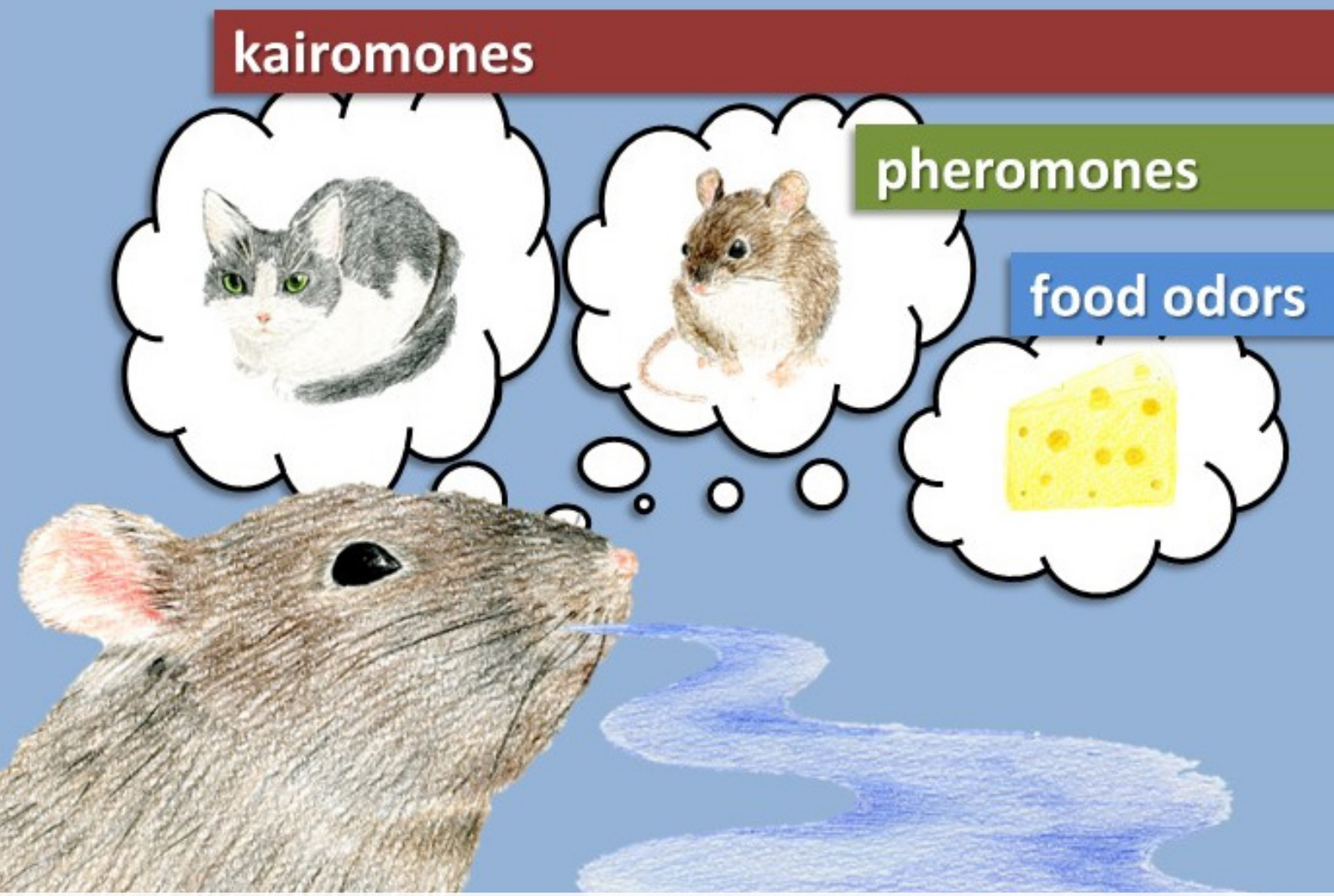
SCENTS THAT MATTER—FROM OLFACTORY STIMULI TO GENES, BEHAVIORS AND BEYOND

EDITED BY: Markus Fendt, Yasushi Kiyokawa and Thomas Endres
PUBLISHED IN: Frontiers in Neuroscience and Frontiers in Behavioral Neuroscience



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88919-813-9
DOI 10.3389/978-2-88919-813-9

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

SCENTS THAT MATTER—FROM OLFACTORY STIMULI TO GENES, BEHAVIORS AND BEYOND

Topic Editors:
Markus Fendt, Institute for Pharmacology and Toxicology, Otto-von-Guericke-University Magdeburg, Germany
Yasushi Kiyokawa, Laboratory of Veterinary Ethology, The University of Tokyo, Japan
Thomas Endres, Institute for Physiology, Otto-von-Guericke-University Magdeburg, Germany

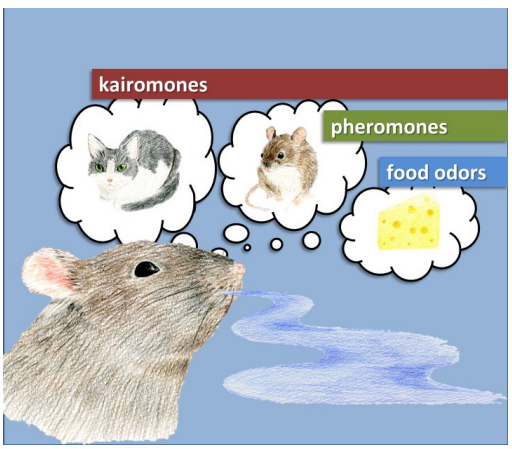


Image by Kaori Kiyokawa and Yasushi Kiyokawa

Scents can carry a lot of important information about the environment, conspecifics and other species. While some of these scents are positively related, as the odor of food, mating partners, or familiar conspecifics, other scents are associated with negative situations and events, e.g. the occurrence of a predator, an aggressive territorial conspecific or spoiled food. The present research topic is focused on such "scents that matter", i.e., scents that are crucial for the survival of an organism. Since many years, the importance of scents always attracts scientists to investigate how scents affect the behavior of mammals, via which mechanisms scents are perceived and how scents modulate neural circuitries responsible for behavior.

We believe that this research topic gives a nice overview on current 'olfactory research.' Many of the contributions are focused on scents with aversive effects, i.e. kairomones or pheromones that warn about potential threats. These studies range from research articles identifying new active odor components of predator odors, describing the induced behavioral changes and the underlying neuroanatomical and neurochemical mechanisms, to review articles summarizing the findings of the last decades on this field. Other articles are focused on the effects of scents in social behaviors or on associative learning. This research topic also represents nicely the current combination of methodological approaches in 'olfactory research': cell biologists, geneticists, behavioral pharmacologists, neuroanatomists, and computational modelers work effectively together to unravel the mechanisms of how scents matters in humans and animals.

Citation: Fendt, M., Kiyokawa, Y., Endres, T., eds. (2016). Scents that Matter — from Olfactory Stimuli to Genes, Behaviors and Beyond. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88919-813-9

Table of Contents

frontiers in Neuroscience

EDITORIAL
published: 09 February 2016
doi: 10.3389/fnins.2016.00029

Editorial: Scents that Matter—from Olfactory Stimuli to Genes, Behaviors and Beyond

Markus Fendt[1,2]*, *Yasushi Kiyokawa*[3] *and Thomas Endres*[4]

[1] Institute for Pharmacology and Toxicology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany, [2] Center of Behavioral Brain Sciences, Otto-von-Guericke University Magdeburg, Magdeburg, Germany, [3] Laboratory of Veterinary Ethology, The University of Tokyo, Tokyo, Japan, [4] Institute for Physiology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany

Keywords: scents, behavior, mammals, pheromones, kairomones, olfactory pathways, defense mechanisms

The Editorial on the Research Topic

Scents that Matter—from Olfactory Stimuli to Genes, Behaviors and Beyond

Mammals can recognize a large variety of scents that give information about the environment, conspecifics, and other species. The present research topic is focused on "scents that matter," i.e., scents that indicate stimuli which are crucial for the survival of an organism. These can be positively related stimuli like the smell of familiar conspecifics, mating partners, or food, but also negatively related stimuli like the scent of potential predators, spoiled food, or territorial and aggressive conspecifics.

A prerequisite for this important role of scents in animals' lives is that they can be well detected and recognized. During the last decades, our understanding of olfactory perception has been largely improved, mainly inspired by the work of Linda Buck and Richard Axel (e.g., Buck and Axel, 1991), which was awarded by the Nobel Prize in 2004. Many of the scents studied in this research topic are processed by the vomeronasal system (e.g., Haga-Yamanaka et al.; Yu), but quite often the main olfactory system is additionally involved (e.g., Rattazzi et al.). A lot of current research addresses the questions about which molecules activate which olfactory receptors and which molecular cascades are modulated by these receptors, or how the different olfactory receptors and the two olfactory systems work together. In the current research topic the articles of Ben-Shaul, Kelliher and Munger, Rattazzi et al. and Yu provide new perspectives in this interesting field of research.

Besides the detection mechanisms of relevant scents, many studies are focusing on the behavioral changes induced by these scents. Most of these studies are analyzing scents signaling potential dangers. One reason for focusing on danger-signaling odors may be that the behavioral effects of these scents are easier to be induced and measured. In addition, it is widely believed that these scents are more critical for fostering the survival of animals. Basically, such danger-signaling scents with aversive-like effects are classified as (a) kairomones, which are emitted by another species such as predators (e.g., Apfelbach et al.; Osada et al.) or (b) pheromones, that are emitted by conspecifics such as alarm pheromones (e.g., Kobayashi et al.; Breitfeld et al.). Both classes of scents warn about a potential threat, which is intended in the case of pheromones, but unintended in the case of kairomones as they lead to a detriment of the emitter (see Nielsen et al.). It is widely believed that predator odors and alarm pheromones are innately recognized, as these stimuli are still effective in laboratory animals that have lived many generations in the absence of predators (Apfelbach et al.; Fendt et al., 2005).

OPEN ACCESS

Edited and reviewed by:
Aldana Maximino, Universidad Nacional Autónoma de México, Mexico

***Correspondence:**
Markus Fendt
markus.fendt@med.ovgu.de

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *03 December 2015*
Accepted: *25 January 2016*
Published: *09 February 2016*

Citation:
Fendt M, Kiyokawa Y and Endres T (2016) Editorial: Scents that Matter—from Olfactory Stimuli to Genes, Behaviors and Beyond. Front. Neurosci. 10:29. doi: 10.3389/fnins.2016.00029

In addition to the general impact of predator odors on the behavior of prey animals, an interesting line of research is the identification of active components in these scents. In the case of predator odors, several molecules have been identified so far: trimethlythiazoline (Taugher et al.; Fortes-Marco et al.; summarized in Rosen et al.), different pyrazines (Osada et al.), and pyridines (Brechbühl et al.), or 2-phenylethylamine (Ferrero et al., 2011). In the present research topic, a number of studies demonstrating that these compounds are able to induce a wide array of defensive responses in laboratory rodents such as avoidance behavior (Wernecke and Fendt; Brechbühl et al.; Fortes-Marco et al.), freezing (Taugher et al.; Fortes-Marco et al.), risk assessment behavior (Breitfeld et al.), or an inhibition of appetitive-like behavior (Kobayashi et al.), as well as physiological changes like a modulation of blood pressure (Brechbühl et al.), or breathing (Taugher et al.). Although these single molecules have the advantage that they can be better controlled in an experimental procedure (e.g., concentration), the natural scents, i.e., blends, are usually more efficient in inducing behavioral changes (summarized in Apfelbach et al.).

The neural mechanisms underlying the behavioral and physiological changes induced by danger-signaling scents are meanwhile partly understood. In the current research topic, studies are focused on brain sites like the bed nucleus of the stria terminalis (Breitfeld et al.; Taugher et al.), the medial amygdala (Carvalho et al.), the periaqueductal gray (Canteras et al.), and different subnuclei of the hypothalamus (Canteras et al.; Kobayashi et al.). Interestingly, these brain sites are of minor or no importance for learned fear whose neural basis is well understood (Fendt and Fanselow, 1999; LeDoux, 2012), suggesting a clear neuronal differentiation between innate and learned fear.

In fear learning, the danger-predicting property of a stimulus is learned by Pavlovian associative learning. Of course, olfactory stimuli can be used for such associative learning, either as unconditioned (Yuan et al.; Fortes-Marco et al.) or conditioned stimuli (Ferry et al.; Yuan et al.). The latter means that a scent without emotional valance can gain danger-predicting, i.e., fear-inducing, properties. Notably, even if a stimulus from another sensory modality is used as a conditioned stimulus in such a fear learning experiment, scents may still play some roles, since they are usually part of the experimental context (e.g., conditioning box, experimenter) and may be associated with the danger simultaneously. In fear learning, the lateral amygdala is important for associating a discrete cue with a danger stimulus, whereas the hippocampus plays an important role in contextual fear learning. Interestingly, novel work of the present research topic demonstrated that different regions of the hippocampus have different roles during contextual fear conditioning with odors (Yuan et al.). In addition to the hippocampus, several cortical areas such as the entorhinal cortex are involved in contextual fear learning (Ferry et al.).

So far, there is little research on the effects of danger signaling scents in humans. However, the defensive behaviors induced by danger-predicting scents and the respective physiological changes observed in animals are connected to anxiety in humans. Therefore, one perspective is that a deeper understanding of the neuroanatomical and neuropharmacological basis of odor-induced fear in animals may also help to find new treatment strategies for anxiety disorders in humans.

As noted above, scents can also serve as positive stimuli. This is of specific interest in the context of social behavior (Wöhr; Noack et al.; Fuzzo et al.) and foraging (Kelliher and Munger). These aspects are also covered by several articles in this special issue. It has been shown that one important function of these scents is to help to recognize social partners (Ben-Shaul; Noack et al.). Thereby, they induce and modulate a variety of behaviors, including ultrasonic calls which are typical for pleasant situations (Wöhr). In the case of social buffering, the scent of a conspecific is able to reduce fear (Fuzzo et al.). These two, quite different effects of social scents are mediated by different subnuclei of the amygdala (Fuzzo et al.; Noack et al.). Notably, there is also potential for translational research with "social scents." For example, a genetic mouse model of autism is less able to modulate ultrasonic vocalization in response to familiar scents (Wöhr).

The present research topic nicely represents the different approaches used in "olfactory research" of relevant scents. These approaches include cell biology, genetics, behavioral pharmacology, neuroanatomy, as well as computational neuroscience. Scientists from all these fields work effectively together to unravel the mechanisms of how scents matter in humans and animals.

We are grateful to all contributors of this research topic. Eighty-five different authors from 10 different countries contributed with research and review articles. Furthermore, we thank the reviewers which helped us and the authors to create an interesting and high-quality research topic.

We hope that you enjoy reading this research topic as much as we have enjoyed editing it.

AUTHOR CONTRIBUTIONS

MF wrote the first draft of the editorial, all authors revised the manuscript and approved the final version of it.

REFERENCES

Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* 65, 175–187. doi: 10.1016/0092-8674(91)90418-X

Fendt, M., Endres, T., Lowry, C. A., Apfelbach, R., and McGregor, I. S. (2005). TMT-induced autonomic and behavioral changes and the neural basis of its processing. *Neurosci. Biobehav. Rev.* 29: 1145–1156. doi: 10.1016/j.neubiorev.2005.04.018

Fendt, M., and Fanselow, M. S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neurosci. Biobehav. Rev.* 23, 743–760. doi: 10.1016/S0149-7634(99)00016-0

Ferrero, D. M., Lemon, J. K., Fluegge, D., Pashkovski, S. L., Korzan, W. J., Datta, S. R., et al. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11235–11240. doi: 10.1073/pnas.1103317108

LeDoux, J. (2012). Rethinking the emotional brain. *Neuron* 73, 653–676. doi: 10.1016/j.neuron.2012.02.004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

PERSPECTIVE
published: 23 June 2015
doi: 10.3389/fnins.2015.00221

TRICK or TRP? What Trpc2$^{-/-}$ mice tell us about vomeronasal organ mediated innate behaviors

C. Ron Yu[1,2]*

[1] Stowers Institute for Medical Research, Kansas City, MO, USA, [2] Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS, USA

The vomeronasal organ (VNO) plays an important role in mediating semiochemical communications and social behaviors in terrestrial species. Genetic knockout of individual components in the signaling pathways has been used to probe vomeronasal functions, and has provided much insights into how the VNO orchestrates innate behaviors. However, all data do not agree. In particular, knocking out Trpc2, a member of the TRP family of non-selective cationic channel thought to be the main transduction channel in the VNO, results in a number of fascinating behavioral phenotypes that have not been observed in other animals whose vomeronasal function is disrupted. Recent studies have identified signaling pathways that operate in parallel of Trpc2, raising the possibility that Trpc2 mutant animals may display neomorphic behaviors. In this article, I provide a critical analysis of emerging evidence to reconcile the discrepancies and discuss their implications.

Keywords: vomeronasal organ, Trpc2, signaling pathways, mating behavior, aggressive behavior, neomorphic behaviors

OPEN ACCESS

Edited by:
Markus Fendt,
Otto-Von-Guericke University
Magdeburg, Germany

Reviewed by:
Pablo Chamero,
University of Saarland, Germany
Marc Spehr,
RWTH-Aachen University, Germany

***Correspondence:**
C. Ron Yu,
Stowers Institute for Medical Research, 1000 E. 50th Street, Kansas City, MO 64110, USA
cry@stowers.org

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *20 April 2015*
Accepted: *02 June 2015*
Published: *23 June 2015*

Citation:
Yu CR (2015) TRICK or TRP? What Trpc2$^{-/-}$ mice tell us about vomeronasal organ mediated innate behaviors. Front. Neurosci. 9:221. doi: 10.3389/fnins.2015.00221

In terrestrial vertebrates, endocrine changes, and stereotypic innate behaviors are often triggered by pheromones. The mammalian vomeronasal organ (VNO) plays an important role in orchestrating pheromone-mediated behaviors (Eisthen and Wyatt, 2006; Tirindelli et al., 2009). In early studies, the functional role of VNO has been derived from ablation experiments in which the VNO is surgically disrupted (VNX) (Bean, 1982; Clancy et al., 1984; Beauchamp et al., 1985; Lepri et al., 1985; Maruniak et al., 1986; Lepri and Wysocki, 1987; Bean and Wysocki, 1989; Labov and Wysocki, 1989; Wysocki and Lepri, 1991; Wysocki et al., 2004). The advent of molecular biology made it possible to genetically manipulate individual components in VNO signaling pathways and provide insights into the mechanisms of VNO mediated behaviors (Del Punta et al., 2002; Leypold et al., 2002; Stowers et al., 2002; Norlin et al., 2003; Kelliher et al., 2006; Kimchi et al., 2007; Chamero et al., 2011; Kim et al., 2012; Leinders-Zufall et al., 2014; Oboti et al., 2014). A consensus that emerges from these studies is that the VNO is essential in triggering territorial aggression. In line with surgical ablation experiments, removing any component of the VNO signaling pathway, including vomeronasal receptors, G proteins, or ion channels, results in diminished aggression in mice (Bean, 1982; Clancy et al., 1984; Maruniak et al., 1986; Bean and Wysocki, 1989; Labov and Wysocki, 1989; Del Punta et al., 2002; Leypold et al., 2002; Stowers et al., 2002; Norlin et al., 2003; Kimchi et al., 2007; Chamero et al., 2011; Kim et al., 2012; Oboti et al., 2014). Genetic mutations that affect VNO function also lead to loss of avoidance to predator or sick animals (Papes et al., 2010; Boillat et al., 2015).

The data on mating behaviors, especially the mounting behaviors displayed by male animals, are less consistent. One of the most interesting behavioral observations comes from mice with knock out mutation of Trpc2, a member of the TRP superfamily of ion channels (Liman et al., 1999). Although several TRP members have been detected in the VNO (Zufall, 2014), Trpc2 appears to be the only one expressed in the vomeronasal sensory neurons (VSNs) as verified by *in situ* hybridization, immunofluorescent staining and electron microscopy (Liman et al., 1999; Menco et al., 2001; Leypold et al., 2002). While Trpc2$^{-/-}$ males display normal mounting behaviors toward female mice, they also indiscriminately mount intruder males (Leypold et al., 2002; Stowers et al., 2002). Most strikingly, female Trpc2$^{-/-}$ mice exhibit hallmarks of male mating behaviors, including solicitation, mounting, and pelvic thrust, toward female and male mice alike (Kimchi et al., 2007). The behavioral phenotypes of Trpc2$^{-/-}$ mice do not recapitulate those observed in VNX rodents (Powers and Winans, 1975; Winans and Powers, 1977; Clancy et al., 1984; Meredith, 1986; Saito and Moltz, 1986; Lepri and Wysocki, 1987; Wysocki and Lepri, 1991; Pfeiffer and Johnston, 1994; Kolunie and Stern, 1995).

In the conventional model of VNO function, male mounting behavior is triggered by pheromone stimulation, through what is considered as the releasing effect of pheromones (Vandenbergh, 1983). Based on the observations from the Trp2$^{-/-}$ mice, Dulac and colleagues proposed an alternative model of VNO function (Stowers et al., 2002). In this new model, mounting is the default behavior triggered by non-VNO sensory input. The function of the VNO is to "ensure gender specific behavior," which inhibits a male mouse from mounting a male (Stowers et al., 2002).

The new interpretation of VNO function is controversial and the discrepancies in behavioral data raise important questions about the functional role of VNO in innate behaviors. At the center of this controversy are two important questions: what is the role played by Trpc2 in pheromone sensing? And is mounting a default behavior that does not require VNO activation? Here I evaluate recent development in the field and attempt to reconcile differences in the experimental results.

Have Trpc2$^{-/-}$ Mice Lost VNO Function Specifically and Completely?

Two groups generated the Trpc2$^{-/-}$ mice independently and reported the loss of territorial aggression and the display of male-male mounting behaviors (Leypold et al., 2002; Stowers et al., 2002). However, they disagreed on whether Trpc2$^{-/-}$ animals completely lost pheromone induced responses. Whereas Stowers and colleagues reported a complete loss of pheromone-triggered activities, residual responses were observed in the studies of Leypold et al. Indeed, Leypold and colleagues cautioned that the residual response might affect how the behavioral data was interpreted.

Since the publication of the initial Trpc2$^{-/-}$ papers, new evidence has emerged from electrophysiological studies challenging the notion that Trpc2 mutation resulted a "null" VNO. Liman first discovered a calcium-activated non-selective (CaNS) cationic channel in hamster VNO neurons (Liman, 2003). A similar conductance was later reported in mouse (Spehr et al., 2009). Although the identity of the channel remains unknown to date, these studies provide the first evidence of Trpc2 independent activation of VNO neurons.

Recently a comprehensive picture of VNO signaling has emerged from the studies by several groups. Delay and colleagues described calcium-activated BK and calcium-activated chloride channel (CACC) in mouse VNO (Zhang et al., 2008; Yang and Delay, 2010). My group later demonstrated that pheromone triggered CACC current was present in VNO neurons of the Trpc2$^{-/-}$ mice (Kim et al., 2011). The CACC now has been identified as TMEM16A/anoctamin1 (Amjad et al., 2015). Delay and colleagues also identified an arachidonic acid dependent signaling pathway in VNO of the Trpc2$^{-/-}$ mouse, with a different knockout line of Trpc2 (Zhang et al., 2010).

In addition, calcium-activated small conductance potassium channel SK3 and G-protein activated inward rectifier potassium channel GIRK were found to act as primary conductance channel in the VSN dendrite and acted in parallel of Trpc2 (Kim et al., 2012). Importantly, the two K channels were depolarizing *in vivo* due to the unusually high K^+ concentrations in the VNO lumen (Kim et al., 2012). Changes in this ionic environment can regulate VNO responses by altering the reversal potential of K^+, and it remains to be determined whether conditions such as strain, age, and hormonal status can influence K^+ homeostasis in the lumen. These discoveries have led to a revised version of the signaling pathways in the VNO that include at least four ion channels directly activated by pheromone stimulation (**Figure 1**). Pheromones can trigger CACC, SK3, and GIRK independent of Trpc2, although Ca^{2+} entry through Trpc2 can augment CACC and SK3 activation. Trpc2 channel accounts for ∼30–40% of the total excitation and Trpc2$^{-/-}$ neurons retain substantial response to pheromones (Kim et al., 2012).

Electrophysiological evidence of Trpc2-independent activation of VNO are supported by histology and behavior analyses. Hasen and Gammie reported that the medial amygdala, which primarily received input from the VNO, was strongly activated in Trp2$^{-/-}$ mice by soiled bedding (Hasen and Gammie, 2009, 2011). Zufall and colleagues found that Bruce effect, pregnancy block induced by strange males, was intact in Trpc2$^{-/-}$ but not VNX mice (Kelliher et al., 2006).

The impact of Trpc2 mutation on pheromone signaling is likely not uniform. An important observation of Trpc2$^{-/-}$ VNO was a significant loss of basal layer neurons (Stowers et al., 2002; Kim et al., 2012), which expressed Gαo and the V2r family of receptors (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Unfortunately, the data were buried in supplemental materials and did not garner the attention they deserved (Stowers et al., 2002; Kim et al., 2012). The study by Hasen and Gammie, on the other hand, clearly showed pronounced reduction of the posterior accessory olfactory bulb in Trpc2$^{-/-}$ mice (Hasen and Gammie, 2009). Thus, it is possible that the activation of basal VSNs is more severely affected than those in the apical layer. This difference may have important implications in behaviors (see below) and

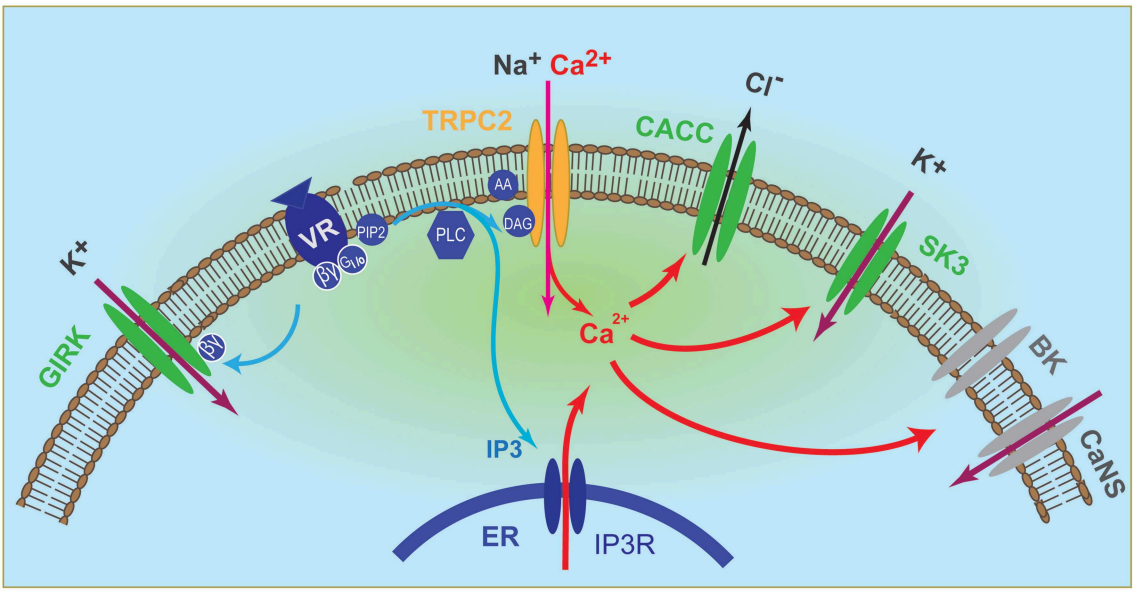


FIGURE 1 | Illustration of vomeronasal neuron signaling pathway. Binding of ligands to their cognate receptors trigger the activation of Gαi2/ Gαo, which in turn activate the phospholipase C (PLC) to produce inositol- 1, 4, 5 - triphosphate (IP3) and diacylglycerol (DAG). DAG activates Trpc2 channel, leading to influx of cationic ions, including Ca^{2+}, whereas IP3 triggers release of Ca^{2+} from intracellular stores. Elevated intracellular Ca^{2+} in turn activates calcium-activated chloride conductance (CACC) and the small conductance calcium-activated potassium channel SK3. Activation of G protein also releases βγ subunits, which activate the G-protein activated inward rectifier channel (GIRK). Both GIRK and SK3 mediate influx of potassium to depolarize the VSN because of a high extracellular [K^+] in the vomeronasal lumenal mucus. Elevated Ca^{2+} can also activates the large conductance calcium-activated potassium channel BK and an unidentified calcium-activated non-selective (CaNS) cationic channel. These two conductance may reside in the dendrite or in the cell body.

explain the apparent loss of VNO activation in the Stowers et al. study. In this study, the major difference in VSN activity between control and Trpc2$^{-/-}$ mice were recorded by laying the sensory epithelium face up on top of an electrode array with the electrodes preferentially made contact with the basal VSNs (Stowers et al., 2002). If Trpc2$^{-/-}$ have a more severe impact on the basal cells, this recording configuration may report diminished activity. Activity in the apical layer, which is less affected, may be occluded from recording by the remaining basal cells.

Does Trpc2 mutation affect the VNO specifically? Trpc2 was initially thought to be exclusively expressed in VNO. Recent evidence suggests that Trpc2 is expressed in a subset of MOE neurons, embryonic brain tissues, and non-neuronal cells, raising the question whether Trpc2$^{-/-}$ affects VNO function specifically (Elg et al., 2007; Boisseau et al., 2009; Hirschler-Laszkiewicz et al., 2012; Omura and Mombaerts, 2014, 2015). In an elegant study, Mombaerts and colleagues knocked in the lacZ gene into the Trpc2 locus and traced the projections of Trpc2-expressing neurons. They discovered that Trpc2 was expressed by two types of MOE neurons projecting to specific glomeruli in ventral side of the main olfactory bulb (Omura and Mombaerts, 2014, 2015). These findings suggest that Trpc2 may carry additional functions in the main olfactory system, as well as other brain areas, and the behavioral phenotypes observed in Trpc2$^{-/-}$ mice are unlikely to be the sole results of VNO disruption.

Is Mounting Behavior Dependent on a Functional VNO?

VNO ablation experiments, performed by a number of labs over several decades, have consistently shown that VNX rodents exhibit diminished mating behaviors (Powers and Winans, 1975; Winans and Powers, 1977; Clancy et al., 1984; Meredith, 1986; Saito and Moltz, 1986; Lepri and Wysocki, 1987; Wysocki and Lepri, 1991; Pfeiffer and Johnston, 1994; Kolunie and Stern, 1995). Trpc2$^{-/-}$ males, on the other hand, show indiscriminate mounting toward intruders (Leypold et al., 2002; Stowers et al., 2002). The most striking observation is that Trpc2$^{-/-}$ females also display mounting behaviors (Kimchi et al., 2007). These behavior phenotypes are rarely observed in wildtype animals. In an attempt to explain the discrepancy in the results, Kimchi and colleagues suggested that VNX surgery could inadvertently cause blood clog in the nasal passage and block odor entry (Kimchi et al., 2007). This scenario is unlikely because mice are obligate nasal breathers. Indeed, several studies have shown that VNX animals display normal approach and investigation of odor sources, indicating that the animals can smell normally [reviewed (Wysocki and Lepri, 1991)]. VNX mice also exhibit investigation of urine source, even though they no longer show preference for urine from the opposite sex (Pankevich et al., 2004, 2006). A careful study also failed to replicate some of the male-typical responses in VNX female mice in the Kimchi study (Martel and Baum, 2009).

In addition to VNX, chemical and genetic ablations of the MOE also lead to diminished investigation of the conspecifics, urine preference and mating behaviors (Thor and Flannelly, 1977; Bean, 1982; Kolunie and Stern, 1995; Keller et al., 2006). CNGA2 knockout mice, which are anosmic because of the loss of an essential component in the olfactory signal transduction pathway, are compromised in mating behaviors (Mandiyan et al., 2005). These observations suggest that attraction by urinary odors can bring the animals to investigate the sources and enable the direct physical contact with non-volatile pheromones by the VNO. Loss of MOE function leads to the loss of odor-evoked investigation and, in turn, could diminish pheromone detection by the VNO. These data should not be construed as definitive evidence that the MOE, but not the VNO, is required to trigger mounting.

Along with studies of VNX animals of several species, a number of transgenic mouse lines that have various deficiencies in VNO function have been studied. These lines include mice with deletion of a V1r receptor cluster, knockout mutations of signaling molecules Gαi2 and Gαo, and mutations of ion channels SK3 and GIRK1(Del Punta et al., 2002; Norlin et al., 2003; Chamero et al., 2011; Kim et al., 2012). None of these lines exhibit male-male mounting or male-like sexual behaviors in the females.

Whereas, the loss of function studies suggesting that the VNO is required to trigger mating behaviors, our recent data demonstrate that pheromones components are sufficient to trigger mating behavior (Haga-Yamanaka et al., 2014). We have previously shown that the VNO recognizes cues that signal the sex and reproductive status of the animal (He et al., 2008, 2010). We have recently identified two sets of pheromone cues (Haga-Yamanaka et al., 2014). A urinary fraction purified from female urine, which we call T16, contains sex-specific cues that signal the carrier as females. This fraction is recognized by a subset of the V1re clade of receptors. We also show that sulfated estrogens specifically activate the V1rj clade of receptors and signal estrus status of the female mice. These cues do not activate the MOE. Although neither sulfated estrogens nor T16 alone alters baseline mating toward ovariectomized females, combining the two cues together elicits strong mounting behaviors (Haga-Yamanaka et al., 2014).

The confluence of data, therefore, suggest that mounting is not a default behavioral output and the VNO is required to trigger this mating behaviors. The notions that non-VNO sensory cues conveying conspecific information to elicit mating as a default behavior is primarily derived from observations of Trpc2$^{-/-}$ mice. This conclusion critically depends on the assumption that Trpc2$^{-/-}$ causes a complete loss of VNO function. As the VNO retains partial function in Trpc2$^{-/-}$mice, it is likely that aberration in VNO signaling in transmitting pheromone information causes aberrant mating behaviors. Indeed, male to male mounting exhibited by Trpc2$^{-/-}$ mice is also observed in double mutant mice that also carry Cnga2$^{-/-}$ or SK3$^{-/-}$ alleles (Kim et al., 2012; Fraser and Shah, 2014).

Neomorphic or Displacement Behaviors in Trpc2$^{-/-}$ Mutants?

What is the nature of the aberrant behaviors observed in Trpc2$^{-/-}$ mice? Classically, the display of behaviors out of context is categorized as displacement activities (Tinbergen, 1989). Animals have a restricted repertoire of innate behaviors preprogrammed in the brain circuitry. Within the same animal, circuit mechanism exists to ensure that antagonistic behavioral patterns are displayed in a mutually exclusive fashion. Displacement reactions arise when there are motivational conflicts, frustration of consummatory acts or physical thwarting of performance (Tinbergen, 1989). Lorenz has described that when fighting drives are obstructed in cranes, they exhibit displacement preening (Lorenz, 1935). Trpc2$^{-/-}$ males have the ability to fight when provoked in a neutral arena, yet they mount instead of attack intruder males (Leypold et al., 2002). Female Trpc2$^{-/-}$ mice show diminished female-specific behaviors such as maternal aggression and lactation, but instead exhibit male-typical sexual behaviors (Leypold et al., 2002; Stowers et al., 2002; Kimchi et al., 2007). It is possible that pheromone signaling in Trpc2$^{-/-}$ mice generate conflicting motivational drive, leading to the replacement of normal responses with an out-of-context substitute. However, no classical case of displacement activities involves a genetic mutation. Therefore, although one could add genetic changes as a cause of displacement activities, it will be more appropriate to characterize behaviors in Trpc2$^{-/-}$ mice as neomorphic. Mating and aggression may be on the same continuum of a behavioral spectrum. The same set of neurons in the ventral medial hypothalamic nucleus drive either mating or aggression depending on the level of activation (Lee et al., 2014). Aberrant input from the VNO is likely to feed into this circuit and induce inappropriate display of mating or aggression.

What may cause neomorphic behaviors in the Trpc2$^{-/-}$ mice? I present two hypotheses to stimulate discussion. The first concerns the development of the vomeronasal circuit, which is linked to gonadotropin releasing hormone (GnRH) neurons in the hypothalamus and preoptic area (Meredith, 1998). GnRH cells migrate along the vomeronasal projection to reach the brain (Schwanzel-Fukuda, 1999). It remains unknown how the development of vomeronasal neurons may affect this migration and the establishment of GnRH neuron connections. A substantial loss of the basal neurons may cause a mis-wiring of the mating/aggression circuit. In addition, physiological changes in Trpc2$^{-/-}$ mice may impact circuit development. Both male and female Trpc2$^{-/-}$ mice have higher testosterone levels than wildtypes (Leypold et al., 2002; Kimchi et al., 2007). As masculinization of the brain could result from elevated testosterone or estrogen levels in adults, as well as from estrogen treatment in neonatal pups (Paup et al., 1972; Baum, 2009; Martel and Baum, 2009; Wu et al., 2009), it is possible that deficiency in pheromone detection during development could lead to brain masculinization in females.

Second, Trp2$^{-/-}$ may directly influence how pheromones are perceived. The basal layer, V2r-expressing VSNs that are lost in Trpc2$^{-/-}$ mice detect polypeptide pheromones, some of which

have been shown to elicit aggression (Chamero et al., 2007). ESP22, a peptide pheromone secreted by juvenile mice, has a powerful effect in inhibiting male mating behaviors (Ferrero et al., 2013). Loss of either Trpc2 or ESP22 leads to mounting of juveniles (Ferrero et al., 2013). It is possible that the loss of the basal layer cells in Trpc2$^{-/-}$, compounded by the partial loss of sensitivity in the remaining neurons, weakens signals that inhibit mating and trigger aggression. The net effect could be the misinterpretation of pheromone cues and a switch from aggression to mating. Finally, it remains possible that the loss of Trpc2 outside of VNO could contribute to neomorphic behaviors.

Concluding Remarks

Behaviors displayed by the Trpc2$^{-/-}$ are fascinating. They capture the imagination of the public and the experts alike. It also has become a requirement to use these mice to demonstrate whether an innate behavior is dependent on VNO function. However, the impact of Trpc2$^{-/-}$ on VNO function is more nuanced than previously thought. How Trpc2$^{-/-}$ causes neomorphic behaviors remains largely unknown. As disruption of VNO function may influence both brain development and pheromone-triggered responses, more detailed studies are required to understand the physiological changes of Trpc2$^{-/-}$ mice. It is important to use caution in using these mice to assess innate behaviors.

Acknowledgments

I thank Drs. L. Ma and S. Haga-Yamanaka for helpful discussion and comments on the manuscript. This work is supported by funding from the Stowers Institute and the US National Institutes of Health (National Institute on Deafness and Other Communication Disorders 008003) to C.R.Y.

References

Amjad, A., Hernandez-Clavijo, A., Pifferi, S., Maurya, D. K., Boccaccio, A., Franzot, J., et al. (2015). Conditional knockout of TMEM16A/anoctamin1 abolishes the calcium-activated chloride current in mouse vomeronasal sensory neurons. *J. Gen. Physiol.* 145, 285–301. doi: 10.1085/jgp.201411348

Baum, M. J. (2009). Sexual differentiation of pheromone processing: links to male-typical mating behavior and partner preference. *Horm. Behav.* 55, 579–588. doi: 10.1016/j.yhbeh.2009.02.008

Bean, N. J., and Wysocki, C. J. (1989). Vomeronasal organ removal and female mouse aggression: the role of experience. *Physiol. Behav.* 45, 875–882. doi: 10.1016/0031-9384(89)90209-6

Bean, N. J. (1982). Olfactory and vomeronasal mediation of ultrasonic vocalizations in male mice. *Physiol. Behav.* 28, 31–37. doi: 10.1016/0031-9384(82)90097-X

Beauchamp, G. K., Wysocki, C. J., and Wellington, J. L. (1985). Extinction of response to urine odor as a consequence of vomeronasal organ removal in male guinea pigs. *Behav. Neurosci.* 99, 950–955. doi: 10.1037/0735-7044.99.5.950

Boillat, M., Challet, L., Rossier, D., Kan, C., Carleton, A., and Rodriguez, I. (2015). The vomeronasal system mediates sick conspecific avoidance. *Curr. Biol.* 25, 251–255. doi: 10.1016/j.cub.2014.11.061

Boisseau, S., Kunert-Keil, C., Lucke, S., and Bouron, A. (2009). Heterogeneous distribution of TRPC proteins in the embryonic cortex. *Histochem. Cell Biol.* 131, 355–363. doi: 10.1007/s00418-008-0532-6

Chamero, P., Katsoulidou, V., Hendrix, P., Bufe, B., Roberts, R., Matsunami, H., et al. (2011). G protein G(alpha)o is essential for vomeronasal function and aggressive behavior in mice. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12898–12903. doi: 10.1073/pnas.1107770108

Chamero, P., Marton, T. F., Logan, D. W., Flanagan, K., Cruz, J. R., Saghatelian, A., et al. (2007). Identification of protein pheromones that promote aggressive behaviour. *Nature* 450, 899–902. doi: 10.1038/nature05997

Clancy, A. N., Coquelin, A., Macrides, F., Gorski, R. A., and Noble, E. P. (1984). Sexual behavior and aggression in male mice: involvement of the vomeronasal system. *J. Neurosci.* 4, 2222–2229.

Del Punta, K., Leinders-Zufall, T., Rodriguez, I., Jukam, D., Wysocki, C. J., Ogawa, S., et al. (2002). Deficient pheromone responses in mice lacking a cluster of vomeronasal receptor genes. *Nature* 419, 70–74. doi: 10.1038/nature00955

Eisthen, H. L., and Wyatt, T. D. (2006). The vomeronasal system and pheromones. *Curr. Biol.* 16, R73–R74. doi: 10.1016/j.cub.2006.01.038

Elg, S., Marmigere, F., Mattsson, J. P., and Ernfors, P. (2007). Cellular subtype distribution and developmental regulation of TRPC channel members in the mouse dorsal root ganglion. *J. Comp. Neurol.* 503, 35–46. doi: 10.1002/cne.21351

Ferrero, D. M., Moeller, L. M., Osakada, T., Horio, N., Li, Q., Roy, D. S., et al. (2013). A juvenile mouse pheromone inhibits sexual behaviour through the vomeronasal system. *Nature* 502, 368–371. doi: 10.1038/nature12579

Fraser, E. J., and Shah, N. M. (2014). Complex chemosensory control of female reproductive behaviors. *PLoS ONE* 9:e90368. doi: 10.1371/journal.pone.0090368

Haga-Yamanaka, S., Ma, L., He, J., Qiu, Q., Lavis, L. D., Looger, L. L., et al. (2014). Integrated action of pheromone signals in promoting courtship behavior in male mice. *eLife* 3:e03025. doi: 10.7554/eLife.03025

Hasen, N. S., and Gammie, S. C. (2009). Trpc2 gene impacts on maternal aggression, accessory olfactory bulb anatomy and brain activity. *Genes Brain Behav.* 8, 639–649. doi: 10.1111/j.1601-183X.2009.00511.x

Hasen, N. S., and Gammie, S. C. (2011). Trpc2-deficient lactating mice exhibit altered brain and behavioral responses to bedding stimuli. *Behav. Brain Res.* 217, 347–353. doi: 10.1016/j.bbr.2010.11.002

He, J., Ma, L., Kim, S., Nakai, J., and Yu, C. R. (2008). Encoding gender and individual information in the mouse vomeronasal organ. *Science* 320, 535–538. doi: 10.1126/science.1154476

He, J., Ma, L., Kim, S., Schwartz, J., Santilli, M., Wood, C., et al. (2010). Distinct signals conveyed by pheromone concentrations to the mouse vomeronasal organ. *J. Neurosci.* 30, 7473–7483. doi: 10.1523/JNEUROSCI.0825-10.2010

Herrada, G., and Dulac, C. (1997). A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. *Cell* 90, 763–773. doi: 10.1016/S0092-8674(00)80536-X

Hirschler-Laszkiewicz, I., Zhang, W., Keefer, K., Conrad, K., Tong, Q., Chen, S. J., et al. (2012). Trpc2 depletion protects red blood cells from oxidative stress-induced hemolysis. *Exp. Hematol.* 40, 71–83. doi: 10.1016/j.exphem.2011.09.006

Keller, M., Douhard, Q., Baum, M. J., and Bakker, J. (2006). Destruction of the main olfactory epithelium reduces female sexual behavior and olfactory investigation in female mice. *Chem. Senses* 31, 315–323. doi: 10.1093/chemse/bjj035

Kelliher, K. R., Spehr, M., Li, X. H., Zufall, F., and Leinders-Zufall, T. (2006). Pheromonal recognition memory induced by TRPC2-independent vomeronasal sensing. *Eur. J. Neurosci.* 23, 3385–3390. doi: 10.1111/j.1460-9568.2006.04866.x

Kim, S., Ma, L., Jensen, K. L., Kim, M. M., Bond, C. T., Adelman, J. P., et al. (2012). Paradoxical contribution of SK3 and GIRK channels to the activation of mouse vomeronasal organ. *Nat. Neurosci.* 15, 1236–1244. doi: 10.1038/nn.3173

Kim, S., Ma, L., and Yu, C. R. (2011). Requirement of calcium-activated chloride channels in the activation of mouse vomeronasal neurons. *Nat. Commun.* 2:365. doi: 10.1038/ncomms1368

Kimchi, T., Xu, J., and Dulac, C. (2007). A functional circuit underlying male sexual behaviour in the female mouse brain. *Nature* 448, 1009–1014. doi: 10.1038/nature06089

Kolunie, J. M., and Stern, J. M. (1995). Maternal aggression in rats: effects of olfactory bulbectomy, ZnSO4-induced anosmia, and vomeronasal organ removal. *Horm. Behav.* 29, 492–518. doi: 10.1006/hbeh.1995.1285

Labov, J. B., and Wysocki, C. J. (1989). Vomeronasal organ and social factors affect urine marking by male mice. *Physiol. Behav.* 45, 443–447. doi: 10.1016/0031-9384(89)90153-4

Lee, H., Kim, D. W., Remedios, R., Anthony, T. E., Chang, A., Madisen, L., et al. (2014). Scalable control of mounting and attack by Esr1+ neurons in the ventromedial hypothalamus. *Nature* 509, 627–632. doi: 10.1038/nature13169

Leinders-Zufall, T., Ishii, T., Chamero, P., Hendrix, P., Oboti, L., Schmid, A., et al. (2014). A family of nonclassical class I MHC genes contributes to ultrasensitive chemodetection by mouse vomeronasal sensory neurons. *J. Neurosci.* 34, 5121–5133. doi: 10.1523/JNEUROSCI.0186-14.2014

Lepri, J. J., and Wysocki, C. J. (1987). Removal of the vomeronasal organ disrupts the activation of reproduction in female voles. *Physiol. Behav.* 40, 349–355. doi: 10.1016/0031-9384(87)90058-8

Lepri, J. J., Wysocki, C. J., and Vandenbergh, J. G. (1985). Mouse vomeronasal organ: effects on chemosignal production and maternal behavior. *Physiol. Behav.* 35, 809–814. doi: 10.1016/0031-9384(85)90416-0

Leypold, B. G., Yu, C. R., Leinders-Zufall, T., Kim, M. M., Zufall, F., and Axel, R. (2002). Altered sexual and social behaviors in trp2 mutant mice. *Proc. Natl. Acad. Sci. U.S.A.* 99, 6376–6381. doi: 10.1073/pnas.082127599

Liman, E. R., Corey, D. P., and Dulac, C. (1999). TRP2: a candidate transduction channel for mammalian pheromone sensory signaling. *Proc. Natl. Acad. Sci. U.S.A.* 96, 5791–5796. doi: 10.1073/pnas.96.10.5791

Liman, E. R. (2003). Regulation by voltage and adenine nucleotides of a Ca2+-activated cation channel from hamster vomeronasal sensory neurons. *J. Physiol.* 548, 777–787. doi: 10.1113/jphysiol.2002.037119

Lorenz, K. (1935). Der Kumpan in der Umwelt des Vogels. *J. Ornithol.* 83, 289–413. doi: 10.1007/BF01905572

Mandiyan, V. S., Coats, J. K., and Shah, N. M. (2005). Deficits in sexual and aggressive behaviors in Cnga2 mutant mice. *Nat. Neurosci.* 8, 1660–1662. doi: 10.1038/nn1589

Martel, K. L., and Baum, M. J. (2009). Adult testosterone treatment but not surgical disruption of vomeronasal function augments male-typical sexual behavior in female mice. *J. Neurosci.* 29, 7658–7666. doi: 10.1523/JNEUROSCI.1311-09.2009

Maruniak, J. A., Wysocki, C. J., and Taylor, J. A. (1986). Mediation of male mouse urine marking and aggression by the vomeronasal organ. *Physiol. Behav.* 37, 655–657. doi: 10.1016/0031-9384(86)90300-8

Matsunami, H., and Buck, L. B. (1997). A multigene family encoding a diverse array of putative pheromone receptors in mammals. *Cell* 90, 775–784. doi: 10.1016/S0092-8674(00)80537-1

Menco, B. P., Carr, V. M., Ezeh, P. I., Liman, E. R., and Yankova, M. P. (2001). Ultrastructural localization of G-proteins and the channel protein TRP2 to microvilli of rat vomeronasal receptor cells. *J. Comp. Neurol.* 438, 468–489. doi: 10.1002/cne.1329

Meredith, M. (1986). Vomeronasal organ removal before sexual experience impairs male hamster mating behavior. *Physiol. Behav.* 36, 737–743. doi: 10.1016/0031-9384(86)90362-8

Meredith, M. (1998). Vomeronasal, olfactory, hormonal convergence in the brain. Cooperation or coincidence? *Ann. N.Y. Acad. Sci.* 855, 349–361. doi: 10.1111/j.1749-6632.1998.tb10593.x

Norlin, E. M., Gussing, F., and Berghard, A. (2003). Vomeronasal phenotype and behavioral alterations in G alpha i2 mutant mice. *Curr. Biol.* 13, 1214–1219. doi: 10.1016/S0960-9822(03)00452-4

Oboti, L., Perez-Gomez, A., Keller, M., Jacobi, E., Birnbaumer, L., Leinders-Zufall, T., et al. (2014). A wide range of pheromone-stimulated sexual and reproductive behaviors in female mice depend on G protein Galphao. *BMC Biol.* 12:31. doi: 10.1186/1741-7007-12-31

Omura, M., and Mombaerts, P. (2014). Trpc2-expressing sensory neurons in the main olfactory epithelium of the mouse. *Cell Rep.* 8, 583–595. doi: 10.1016/j.celrep.2014.06.010

Omura, M., and Mombaerts, P. (2015). Trpc2-expressing sensory neurons in the mouse main olfactory epithelium of type B express the soluble guanylate cyclase Gucy1b2. *Mol. Cell. Neurosci.* 65, 114–124. doi: 10.1016/j.mcn.2015.02.012

Pankevich, D. E., Baum, M. J., and Cherry, J. A. (2004). Olfactory sex discrimination persists, whereas the preference for urinary odorants from estrous females disappears in male mice after vomeronasal organ removal. *J. Neurosci.* 24, 9451–9457. doi: 10.1523/JNEUROSCI.2376-04.2004

Pankevich, D. E., Cherry, J. A., and Baum, M. J. (2006). Effect of vomeronasal organ removal from male mice on their preference for and neural Fos responses to female urinary odors. *Behav. Neurosci.* 120, 925–936. doi: 10.1037/0735-7044.120.4.925

Papes, F., Logan, D. W., and Stowers, L. (2010). The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. *Cell* 141, 692–703. doi: 10.1016/j.cell.2010.03.037

Paup, D. C., Coniglio, L. P., and Clemens, L. G. (1972). Masculinization of the female golden hamster by neonatal treatment with androgen or estrogen. *Horm. Behav.* 3, 123–131. doi: 10.1016/0018-506X(72)90014-1

Pfeiffer, C. A., and Johnston, R. E. (1994). Hormonal and behavioral responses of male hamsters to females and female odors: roles of olfaction, the vomeronasal system, and sexual experience. *Physiol. Behav.* 55, 129–138. doi: 10.1016/0031-9384(94)90020-5

Powers, J. B., and Winans, S. S. (1975). Vomeronasal organ: critical role in mediating sexual behavior of the male hamster. *Science* 187, 961–963. doi: 10.1126/science.1145182

Ryba, N. J., and Tirindelli, R. (1997). A new multigene family of putative pheromone receptors. *Neuron* 19, 371–379. doi: 10.1016/S0896-6273(00)80946-0

Saito, T. R., and Moltz, H. (1986). Copulatory behavior of sexually naive and sexually experienced male rats following removal of the vomeronasal organ. *Physiol. Behav.* 37, 507–510. doi: 10.1016/0031-9384(86)90215-5

Schwanzel-Fukuda, M. (1999). Origin and migration of luteinizing hormone-releasing hormone neurons in mammals. *Microsc. Res. Tech.* 44, 2–10.

Spehr, J., Hagendorf, S., Weiss, J., Spehr, M., Leinders-Zufall, T., and Zufall, F. (2009). Ca2+ -calmodulin feedback mediates sensory adaptation and inhibits pheromone-sensitive ion channels in the vomeronasal organ. *J. Neurosci.* 29, 2125–2135. doi: 10.1523/JNEUROSCI.5416-08.2009

Stowers, L., Holy, T. E., Meister, M., Dulac, C., and Koentges, G. (2002). Loss of sex discrimination and male-male aggression in mice deficient for TRP2. *Science* 295, 1493–1500. doi: 10.1126/science.1069259

Thor, D. H., and Flannelly, K. J. (1977). Peripheral anosmia and social investigatory behavior of the male rat. *Behav. Biol.* 20, 128–134. doi: 10.1016/S0091-6773(77)90682-4

Tinbergen, N. (1989). *The Study of Instinct* (Oxford; New York: Clarendon Press; Oxford University Press).

Tirindelli, R., Dibattista, M., Pifferi, S., and Menini, A. (2009). From pheromones to behavior. *Physiol. Rev.* 89, 921–956. doi: 10.1152/physrev.00037.2008

Vandenbergh, J. G. (1983). *Pheromones and Reproduction in Mammals* (New York, NY: Academic Press).

Winans, S. S., and Powers, J. B. (1977). Olfactory and vomeronasal deafferentation of male hamsters: histological and behavioral analyses. *Brain Res.* 126, 325–344. doi: 10.1016/0006-8993(77)90729-6

Wu, M. V., Manoli, D. S., Fraser, E. J., Coats, J. K., Tollkuhn, J., Honda, S., et al. (2009). Estrogen masculinizes neural pathways and sex-specific behaviors. *Cell* 139, 61–72. doi: 10.1016/j.cell.2009.07.036

Wysocki, C. J., and Lepri, J. J. (1991). Consequences of removing the vomeronasal organ. *J. Steroid Biochem. Mol. Biol.* 39, 661–669. doi: 10.1016/0960-0760(91)90265-7

Wysocki, C. J., Yamazaki, K., Curran, M., Wysocki, L. M., and Beauchamp, G. K. (2004). Mice (*Mus musculus*) lacking a vomeronasal organ can discriminate MHC-determined odortypes. *Horm. Behav.* 46, 241–246. doi: 10.1016/j.yhbeh.2004.02.010

Yang, C., and Delay, R. J. (2010). Calcium-activated chloride current amplifies the response to urine in mouse vomeronasal sensory neurons. *J. Gen. Physiol.* 135, 3–13. doi: 10.1085/jgp.200910265

Zhang, P., Yang, C., and Delay, R. J. (2008). Urine stimulation activates BK channels in mouse vomeronasal neurons. *J. Neurophysiol.* 100, 1824–1834. doi: 10.1152/jn.90555.2008

Zhang, P., Yang, C., and Delay, R. J. (2010). Odors activate dual pathways, a TRPC2 and a AA-dependent pathway, in mouse vomeronasal neurons. *Am. J. Physiol. Cell Physiol.* 298, C1253–C1264. doi: 10.1152/ajpcell.00271.2009

Zufall, F. (2014). TRPs in olfaction. *Handb. Exp. Pharmacol.* 223, 917–933. doi: 10.1007/978-3-319-05161-1_8

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

HYPOTHESIS AND THEORY
published: 20 November 2015
doi: 10.3389/fnins.2015.00439

Extracting Social Information from Chemosensory Cues: Consideration of Several Scenarios and Their Functional Implications

Yoram Ben-Shaul *

Department of Medical Neurobiology, Hebrew University Medical School, Jerusalem, Israel

Across all sensory modalities, stimuli can vary along multiple dimensions. Efficient extraction of information requires sensitivity to those stimulus dimensions that provide behaviorally relevant information. To derive social information from chemosensory cues, sensory systems must embed information about the relationships between behaviorally relevant traits of individuals and the distributions of the chemical cues that are informative about these traits. In simple cases, the mere presence of one particular compound is sufficient to guide appropriate behavior. However, more generally, chemosensory information is conveyed via relative levels of multiple chemical cues, in non-trivial ways. The computations and networks needed to derive information from multi-molecule stimuli are distinct from those required by single molecule cues. Our current knowledge about how socially relevant information is encoded by chemical blends, and how it is extracted by chemosensory systems is very limited. This manuscript explores several scenarios and the neuronal computations required to identify them.

Keywords: chemosensory cue, social communication, olfactory circuitry, traits, neuronal computation, pheromones

OPEN ACCESS

Edited by:
Markus Fendt,
Otto-von-Guericke University
Magdeburg, Germany

Reviewed by:
Radwa Khalil,
Otto-von-Guericke University
Magdeburg, Germany
Yasushi Kiyokawa,
The University of Tokyo, Japan

***Correspondence:**
Yoram Ben-Shaul
yoramb@ekmd.huji.ac.il

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *31 August 2015*
Accepted: *30 October 2015*
Published: *20 November 2015*

Citation:
Ben-Shaul Y (2015) Extracting Social Information from Chemosensory Cues: Consideration of Several Scenarios and Their Functional Implications. Front. Neurosci. 9:439. doi: 10.3389/fnins.2015.00439

INTRODUCTION

In many species, chemosensory cues are crucial for obtaining information about the environment and particularly for interactions with other individuals (Wyatt, 2014). At one end of the spectrum are tasks involving single compounds, such as detection of females by male moths (Sakurai et al., 2014). Here, a communication system involving emission of one specific compound has likely co-evolved with a dedicated sensory processing channel. A related situation in vision is phototaxis: attraction to high photon levels, also occurring in moths and other insects (Yamaguchi and Heisenberg, 2011). At the other extreme is a task like face recognition which requires a complex computation involving sampling and comparison of relational information across multiple detectors. This article is motivated by the view that analyses of chemosensory scenes involve non-trivial comparison of information across compounds and receptors, a task far more challenging than detection of levels of single-compounds.

The chemical profile associated with any animal, and mammals in particular, is a highly complex mix of various molecules that can convey information about the emitting organism (Albone and Shirley, 1984). One important distinction is between pheromones, which elicit some response but are anonymous with respect to the sender, and signature mixtures, which provide information about individuality or colony/family identity (Wyatt, 2010). In addition, social information can

involve detection of specific *traits*. Throughout this manuscript, the term *trait* refers to a particular property of an individual, which may be relevant for guiding behavior toward it. A trait may be permanent (e.g., *species*, *sex*) or temporary (e.g., *age*, *health status*). Such information is conveyed by chemical cues that cannot be strictly defined as either pheromones or as signature mixes. Yet, they are clearly important for guiding behavior.

Sensory systems evolved to extract those statistical features that are relevant for identifying information important to the organism (Rieke et al., 1995; Barlow, 2001; Bradbury and Vehrencamp, 2011). Understanding a sensory system therefore requires identification of the problems that it must solve. For example, detection of light and identification of specific faces involve distinct statistical features and therefore, distinct neural networks (Purves, 2012). Likewise, chemosensation can serve in various contexts involving distinct computations. The problems solved by the olfactory system are varied and include stimulus identification (Chapuis and Wilson, 2012; Rokni et al., 2014), discrimination among stimuli (Kepecs et al., 2007), or source tracking (Thesen et al., 1993; Cardé and Willis, 2008). The chemosensory task considered here is the recognition of specific *traits* using chemical cues. Specifically, this manuscript focuses on the links between specific distributions of chemical cues and potential neuronal solutions to detect them. Our approach is to examine various scenarios of chemosensory cue distributions and to spell out the steps required to extract the relevant information under each of them. Evidence for some scenarios is well established, while others are more speculative. The hope is that an explicit description of the required computations, even if abstract, will help to eventually identify of the actual circuit elements that realize these computations.

METHODS

All plots illustrating specific networks and decision rules were created using MATLAB code. The purpose of the networks is to demonstrate the logical steps involved, and not to implement any realistic neuronal modeling. In other words, the purpose is to show *what* must be calculated, but not *how*. Therefore, the code realizes the networks in a very literal manner. For example, linear input units such as appear in many of the networks (e.g., **Figure 2A**) were implemented by the following code: $R = max(0,(C\text{-}T)\cdot G)$, where R is the response of a unit, with a threshold T, and a gain G, to a stimulus concentration C (Supplementary code file: thresh_unit). The *max* condition ensures that a response will only occur if the concentration C is larger than the threshold, T. The output unit in **Figure 2A** was realized by subtracting the responses of two such linear input units with different gains and thresholds and applying a threshold to the (normalized) response (Supplementary code file: soft_range_unit). The plots in **Figures 2B,C** were created using the script simulate_one_compound_scenarios which itself calls the soft_range_unit function with a set of concentrations that was randomly sampled from a uniform random distribution in the range [0, 10]. All manuscript figures showing outputs of other networks were generated in an analogous manner using specific MATLAB scripts. The code is extensively documented to explain each of the calculations and a *readme* file lists which scripts were used for each figure (Supplementary Material)

RESULTS

Communication and detection of social information via chemosensation involves several stages which are illustrated in **Figure 1**. The source of the signals are individuals with specific *traits* (**Figure 1A**). To be detectable, these traits must be associated with particular distributions of molecules, which are determined by trait-specific metabolic pathways or gene expression patterns (**Figures 1B,C**). Finally, specific neuronal networks (**Figure 1D**) detect particular traits from profiles of chemosensory cues. **Figure 1C** depicts a three dimensional space (i.e., defined by three compounds), of which only two are relevant for detection (**Figure 1C**, right). In this hypothetical example, the relevant parameter is a specific *ratio* between the levels of two compounds, c1 and c2. The nervous system must therefore elicit a well-defined change in neuronal activity (**Figure 1E**)

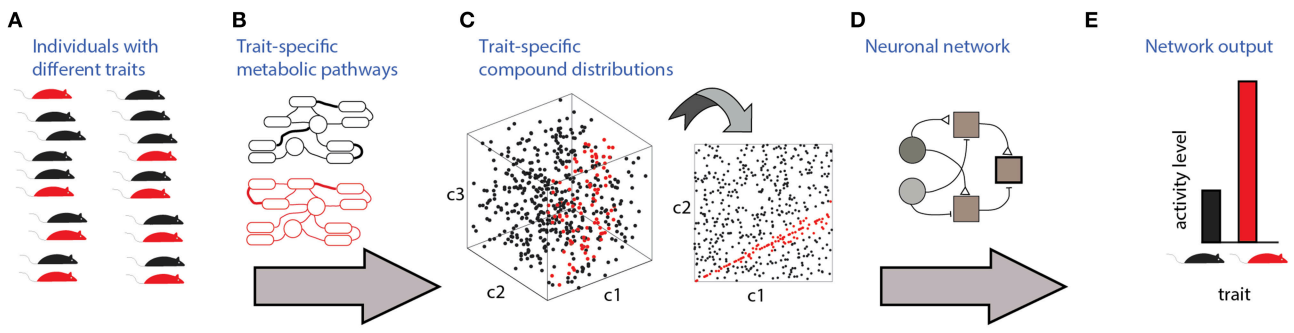


FIGURE 1 | Stages involved in obtaining social information from chemical cues. Different traits (red or black in **A**) are associated with distinct metabolic pathways/expression patterns **(B)**, which lead to distinct profiles of emitted signals **(C)**. In this example, chemical space is defined by levels of 3 components, c1, c2, and c3. Distinguishing among the traits requires neuronal networks **(D)** that can identify the relevant features in chemical space. Here, the feature is a specific ratio between levels of compounds c2 and c1. Ultimately, these networks elicit neuronal activity **(E)** that can distinguish among chemosensory profiles associated with different traits.

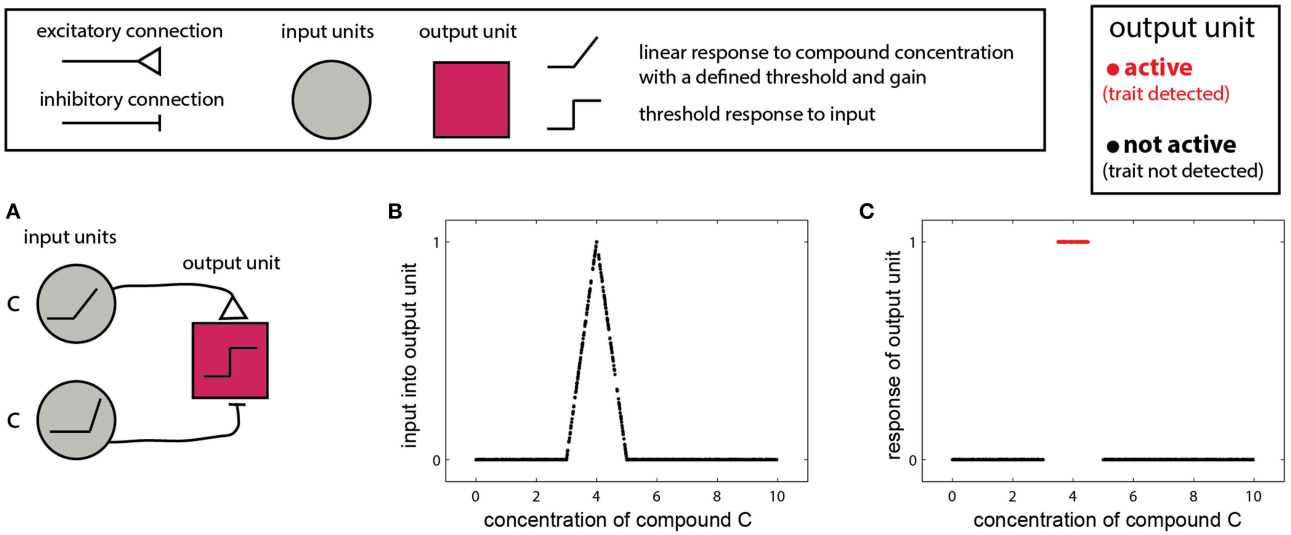


FIGURE 2 | Detection of a single compound. (A) Simple network that detects the presence of a compound c in a specified range. The network comprises two linearly responding units. Note that the threshold of the inhibitory unit is higher and its response function is steeper (higher gain). A threshold response to the difference between the inputs from the two first-layer units results in a selective response in the output unit (red) only when c is within a specific level range. The range can be set by altering the response functions of the first-layer units and by the threshold of the output unit. **(B)** Input to the network in **(A)** as a function of the level of c. **(C)** Output of the network. Legend at top explains network elements.

following the detection of a particular ratio. For simplicity, the traits considered here are binary, assuming one of two values. The networks are composed of units responding to excitatory or inhibitory inputs. The input units represent chemically selective receptor neurons, and thus respond monotonically, though not necessarily linearly, to concentrations of individual molecules. Each network includes one output unit, whose activity reflects the network's decision regarding the trait's value. The proposed networks are *not* intended to be biophysically realistic and are agnostic about particular neuronal codes (e.g., rate vs. temporal coding) and about the actual neuronal hardware used to implement the computations (e.g., dendritic vs. somatic integration). Instead, they serve to illustrate the essential computations required for each type of classification. In the Discussion, an attempt is made to map these model networks to specific elements of the olfactory system.

Single Compound Codes and Their Limitations

In the simplest scenario, a trait is reflected by the presence or absence of one molecule. In this case, the detecting organism needs to detect whether the concentration of the molecule is above a certain threshold or within a given range. The single-compound scenario is related to the classic definition of a pheromone—namely, that the mere presence of one specific compound can elicit a particular behavioral outcome (Wyatt, 2010). Examples include the male silkworm moth's (*Bombyx mori*) response to bombykol (Butenandt et al., 1961), or suppression of mating in mice, due to detection of a peptide indicative of a juvenile state (Ferrero et al., 2013). In such cases, the stimulus space is one dimensional, and the corresponding networks are simple. One version involves two units: one excitatory and one inhibitory both of which respond linearly to the stimulus, above some threshold (**Figure 2A**). The excitatory unit has a lower threshold and gain, and is thus active at lower concentrations, while at higher concentrations the inhibitory unit is recruited, suppressing the output unit. The output unit implements a threshold on the integrated inputs (**Figure 2B**), so that its output represents a binary decision of whether the compound is, or is not, within a certain range (**Figure 2C**). Removal of the threshold operation in the last unit will lead to a continuous measure of similarity to some optimal cue level, as shown in **Figure 2B**. The slope of the response function in **Figure 2B** is a direct function of the slopes of the input units.

Taken to the extreme, under the one compound scenario, each biologically relevant trait is independently represented by levels of one specific compound. For example, levels of one compound would convey an individual's sex, another its age, a third its reproductive status and so on. While such a scheme would simplify *decoding*, it presents a very inefficient code since it requires dedicated metabolic pathways to generate compounds for each trait. Unique single-compound signatures of individuality, are particularly unfeasible. Another critical shortcoming of single compound codes is the likelihood of not being specific for a given species. For species that do not interact, this does not present a problem (Kelly, 1996). However, in some cases, the simplicity of the code facilitates mimicry and therefore allows a predatory species to bait a prey organism (Gemeno et al., 2000). An even more fundamental problem with single compound codes is their failure to provide invariant information. For example, if a particular trait is associated with a certain level of some compound, stimulus source dilution would present on

obvious confounding factor. Likewise, vital information about the state of metabolic pathways is often manifest by the *relative* levels of several compounds, rather than absolute levels of individual compounds.

Multi Compound Codes

Transmission of chemical information by combinations of multiple compounds is widespread (Wyatt, 2014). In the nematode *C. elegans*., different combinations of modular components can promote avoidance, reproduction, long range attraction, and developmental diapause (Srinivasan et al., 2012). Likewise, even though a single component may suffice to elicit a behavioral response, female moth signals are much more effective when present as a combination of components (Linn et al., 1987), thus providing specificity and minimizing interference among related moth species (Linn et al., 1988). A similar mechanism for species-specific mating, using multicomponent blends, is present in goldfish (Levesque et al., 2011). In social insects such as bees, communication is also based on complex codes involving combinations of multiple components (Slessor et al., 2005). Similarly, naked mole-rats, which are social mammals, use a unique odor signature, composed of multiple components, to identity colony members (Oriain and Jarvis, 1997). In mice, some volatiles act synergistically (Novotny et al., 1985) to elicit aggression, while particular combinations of major urinary proteins convey information about individuality (Cheetham et al., 2007; Kaur et al., 2014).

What mechanism is suitable for detecting a combination of individual compounds? An obvious solution involves summation of inputs from multiple neurons, each of which is responsive to one component. **Figure 3A** shows a network that detects the presence of two components and its performance on simulated data (**Figures 3B,C**). Note that the weights of the input units and the threshold of the output units must be tuned to ensure that the output unit will be active *only* under the presence of *both* compounds. Specifically, the influence of the inputs must be capped to ensure that neither could activate the output unit on its own. For linear summation of n components, each with a maximal input of 1, setting the threshold in the range [n-1, 1] will satisfy the condition. However, as the number of components is increased, the ratio $(n-1)/n$ approaches unity, and small random fluctuations can lead to activation of the output unit even without the presence of all components. Non-linear, synergistic, input summation (Silver, 2010), as has been demonstrated for olfactory cortex neurons (Davison and Ehlers, 2011), can at least partially resolve the problem of accidental activation by only a subset of the inputs. In a related scenario, a given biological trait could involve the presence of some compounds, combined with the *absence* of others. An example is the inhibitory effect of heterospecific cues on flight in moths (Lelito et al., 2008). This computation can be realized by an output unit that receives both excitatory and inhibitory inputs that reflect the levels of each of these compounds (**Figures 3D–F**). To enforce the requirement for the absence of a specific compound, its inhibitory effect must be large enough to "veto" activation of the output unit. As in the previous example, this is more difficult when the output unit integrates many excitatory inputs. More generally, it may be required to detect whether levels of each of multiple compounds fall within particular ranges. This can be achieved by a network (**Figures 3G–I**) that includes an output unit receiving inputs from two range-detection networks such as those shown in **Figure 2**. Note that because the decision rules embodied by the networks in **Figures 3A–G** refer to the *sum* of compounds, they do not impose a specific condition about the level of the individual compounds. Graphically, this results in decision boundaries with diagonal lines in the 2D decision space (**Figures 3C,F,I**). This is distinct from a situation in which *each* of the compounds is above a certain value, or within a given range. The latter condition can be achieved if the modules associated with each individual compound impose a threshold (or range), as shown in **Figures 3J–L** for the case of detection of two components.

Multi-compound Codes Involving Relationships

The scenarios above involved traits that were associated with levels of multiple compounds, but not by explicit relationships among them. Yet, in both vertebrates and invertebrates, specific *relationships* among compounds can be highly informative and often constitute the important message. In ants, for instance, the relative *proportions* of multiple cuticular hydrocarbons provide the basis for colony recognition (Martin et al., 2008), and in mice, ratios of distinct major urinary proteins provide information about the stimulus donor (Kaur et al., 2014). Although such analog codes present challenges for readout, they allow enhanced coding capacity as compared to codes defined by the presence or absence of individual components. An example for a postulated binary code is the use of major urinary proteins to convey individuality (Cheetham et al., 2007). More generally, analyses of mouse (Zhang et al., 2007), and rat (Zhang and Zhang, 2011) urinary profiles have shown that the relative abundance (rather than the presence or absence) of particular components is the best indicator of relatedness among different strains. Another fundamental reason for the importance of relationships involves the temporal and/or spatial aspects of the stimulus source. The concentration of a single volatile component decays with the distance from its source. Likewise, the concentration will change with time at any distance. For a soluble component, evaporation of either the solvent or the compound will lead to concentration changes. In a multiple molecule mix, if rates of diffusion/dispersion and evaporation are similar for all compounds, then evaluation of their ratios (at any point in time and space) provides a better estimate of stimulus identity than any component in isolation. Indeed, it has been shown that rats can discriminate binary odor mixtures based on the ratios of the components (Uchida and Mainen, 2007). Furthermore, the rats can generalize their decision rules to other mixtures with identical component ratios, but different total concentrations. On the other hand, if two or more compounds have different (and known) dispersal or evaporation rates, as well as known concentrations at the original stimulus source, then their concentration ratios can provide information about the distance of the stimulus source or the time since its deposition. Various species can discriminate fresh urine from old urine,

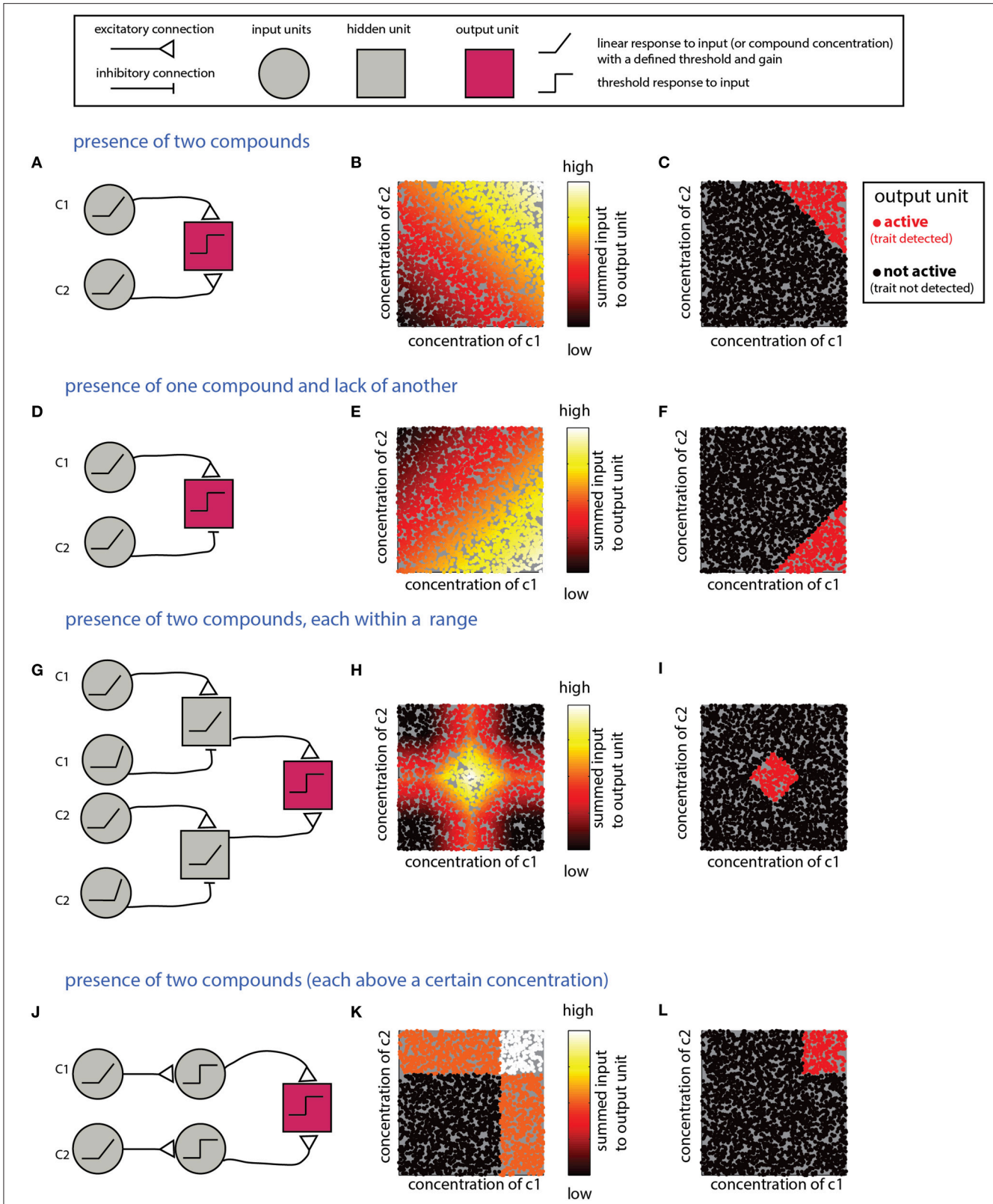


FIGURE 3 | Detection of multiple (two) compounds. (A–C) Detection of the sum of two compounds. **(A)** A simple network that detects the presence of two compounds c1 and c2. The network comprises two linearly responding units that feed into a third integrating unit. **(B)** Inputs into the output unit in the network in **(A)**

(Continued)

FIGURE 3 | Continued
as a function of the levels of c1 and c2. The magnitude of the inputs is indicated by the color of the dots (arbitrary units). **(C)** Activity of the output unit after thresholding the input shown in **(B)**. **(D–F)** Detection of a difference between two compounds. Here, the output unit receives an excitatory input from a unit that detects c1 and an inhibitory input from a unit that detects c2. Assuming that the input units' response functions and efferent connections are similar, the output unit be active when c1 is larger than c2. **(G–I)** Detection that each of two compounds is within a specific range. **(G)** The output unit in this network receives inputs from two range-detecting units as shown in **Figure 2A** (with each network sensitive to the range of one of the compounds). **(H)** Input to the output unit in **(G)**. **(I)** Output of the output unit in **(G)** after thresholding. **(J–L)** A network that detects that *each* of two compounds is above some threshold. Similar modifications can be applied to all the other networks in this section, to impose conditions on each of the compounds. Legend at top explains network elements.

and this could be achieved by comparing levels of multiple components with different volatility (Rich and Hurst, 1999).

The scenarios described in this section require computations that detect relationships between multiple compounds. Integration of excitatory and inhibitory inputs from units reflecting levels of individual compounds can be used to derive information about differences or the ratios between them. Linear input units are suitable for calculating differences, whereas logarithmic responses (or their approximations) are naturally suitable for calculating ratios (Uchida and Mainen, 2007). The networks in **Figure 4** implement detection of difference- (**Figures 4A–C**) or ratio-ranges (**Figures 4D–F**) between two compounds. The two networks differ only in the response function of the input units. The more general situation, involving relationships among more than two compounds, can be addressed by combining several networks such as those in **Figures 4A,D**. One possible network, and its respective decision boundary for a specific ratio range among four compounds, is shown in **Figures 4G,H**. Note that this network utilizes one "anchor" component (c1) to which all the others are referenced. An elegant solution to a similar pattern recognition problem, using temporal coding, has been raised by Hopfield (1995) and subsequently elaborated by Brody and Hopfield (2003). Logically, the networks shown in **Figure 4G** and that suggested by Brody and Hopfield (2003) are similar, but whereas the Hopfield network detects a pattern defined by a fixed ratio, the network in **Figure 4D** allows each compound to vary within a certain ratio *range*.

Context Dependent Trait-compound Relationships

In the preceding sections, only individual traits were considered, but in fact, the levels of any chemical compound may depend on *several* traits. Consequently, the relationship between any one trait and chemical profiles will be context dependent. This notion is supported by experimental evidence. For instance, comparative analysis of urinary components across sex and strain (Zhang et al., 2007; Zhang and Zhang, 2011) or across strains and reproductive states (Schwende et al., 1984), revealed that many of the individual components are modulated by both of these traits. Consistent with this observation, a search for chemical markers of diet, maturation, stress and diurnal rhythm (Schaefer et al., 2010) revealed a large overlap between reliable markers for each of these factors, indicating that many individual markers are modulated by multiple factors (or traits). This idea is illustrated graphically in **Figure 5AI** for a trait reflected by levels of a single compound (as in **Figure 2**). The presence of the "red" trait, and its absence (black) are associated with distinct probability distributions of the corresponding compound. If the distributions do not overlap, then perfect discrimination can be achieved by a simple network such as shown in **Figure 2A**. Overlapping distributions present a different complication which is not considered here.

If a compound's level depends on multiple traits, then identification of any particular trait from that compound, requires consideration of all others. **Figure 5A** illustrates this idea with two hypothetical scenarios. In the first (**Figure 5AII**), there is a general shift of compound level distributions as compared to the reference condition (**Figure 5AI**). Here, a different decision threshold is required for correctly detecting the "red" trait. In the more exotic case, shown in **Figure 5AIII**, the *direction* of change as a function of the trait also depends on other traits. Importantly, these particular scenarios are (qualitatively) evident from actual measurements of urinary volatiles for different strain and reproductive-state combinations (Schwende et al., 1984). Consequently, a network such as shown in **Figure 2A** simply cannot reliably detect the "red" trait across all the conditions shown in **Figure 5A**. To illustrate the effect of multiple traits more formally, consider a particular compound i, whose concentration levels C_i are determined by a linear combination of two traits, T_1 and T_2, i.e., $C_i(T_1,T_2) = g_1 \bullet T_1 + g_2 \bullet T_2$. The gain factors g_1 and g_2 determine the influence of each trait on the compound's concentration. The traits are not binary, but can assume one of several numerical values. In **Figure 5BI**, g_1 and g_2 are equal, so both traits exert the same influence. In **Figure 5BII**, the second trait dominates ($g_1 < g_2$), while in **Figure 5BIII**, the first trait is dominant. In **Figure 5BI**, neither trait can be determined without knowing the other. In **Figures 5BII,III**, the dominant trait can be determined without knowledge of the other, but not vice-versa.

Sometimes, the context can be set by physical, rather than physiological factors. One example is stimulus source dilution. **Figure 5C** shows the effects of 2X and 0.5X dilutions of the original stimulus source on compound levels. As in the other examples above, it is not possible to discriminate among trait values without knowledge of the stimulus dilution. Here too, the confounding effect of stimulus dilution depends on how strongly different trait values affect compound distributions. For example, if distinct trait values exert a 100-fold change on a given compound, then the confounding effect of a 2-fold dilution will be minor. On the other hand, if different trait values induce a 2-fold change, then the confounding effect of the same dilution will be critical. Another circumstance where context plays a role is during social investigation, where animals typically sample multiple body regions to obtain information about each other (Johnston, 2003; Luo et al., 2003; Kimoto et al.,

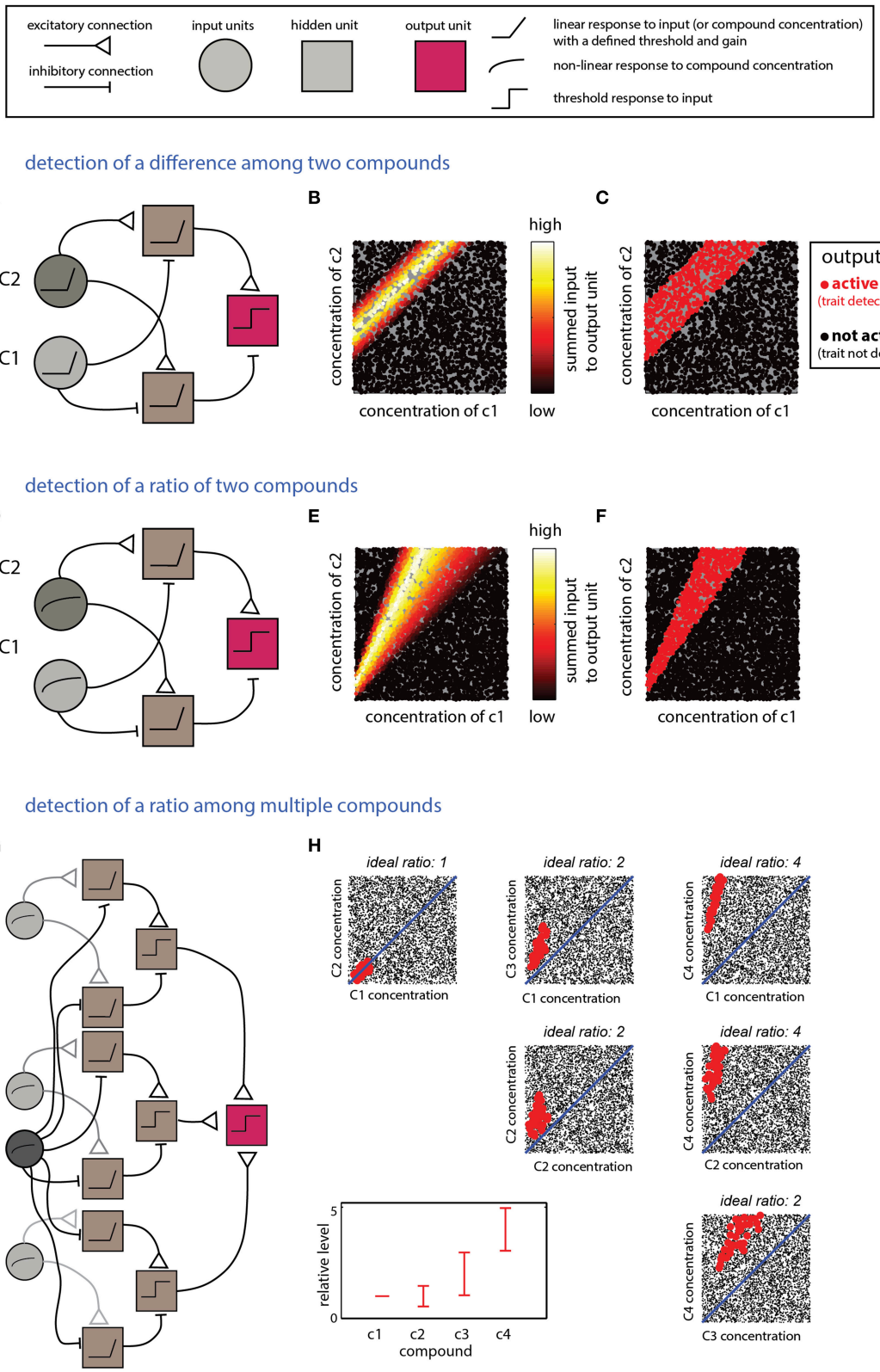


FIGURE 4 | Detection of explicit relationships between compounds. (A–C) Detection of a difference range. The output unit will respond if the difference between two compounds is within a specific range. **(A)** The network comprises two input units which respond linearly (with a threshold) to the two components. Each

(Continued)

FIGURE 4 | Continued
of the two second-layer units respond to the difference between the two compounds but with a different offset. The output unit responds to the difference between the second layer units. **(B)** Inputs into the output unit as a function of the levels of c1 and c2. **(C)** Activity in the output unit after thresholding. The threshold sets the width of the difference range around the ideal difference. **(D–F)** Detection of a ratio range. **(D)** The network is very similar to that shown in **(A)** for detection of a difference, except that the input units respond linearly to the log of the compound concentration. **(E)** Inputs to the output unit in **(D)** as a function of c1 and c2. **(F)** Output of the unit in **(D)** after thresholding the inputs in **(E)**. **(F,G)** Detection of mixtures defined by specific proportions among components. **(G)** A network that detects a mixture of four components (c1–c4) with specific ratio ranges. The network comprises three modules with the same layout as that in **(D)**. Each of the modules compares one of the compounds (c2–c4) to c1. The outputs of these modules are integrated by the output unit that performs a threshold operation on the summed outputs of the three modules. **(H)** Representation of the output of the unit. Each mixture is defined within a 4D space which is shown here by all pairwise projections. The relevant proportions relative to c1 (i.e., those associated with a trait) are indicated in the bottom left panel. Mixtures classified by the network as associated with the trait are shown in red (others are shown in blacks). Thus, each mixture is represented in each of the panels. Legend at top explains network elements.

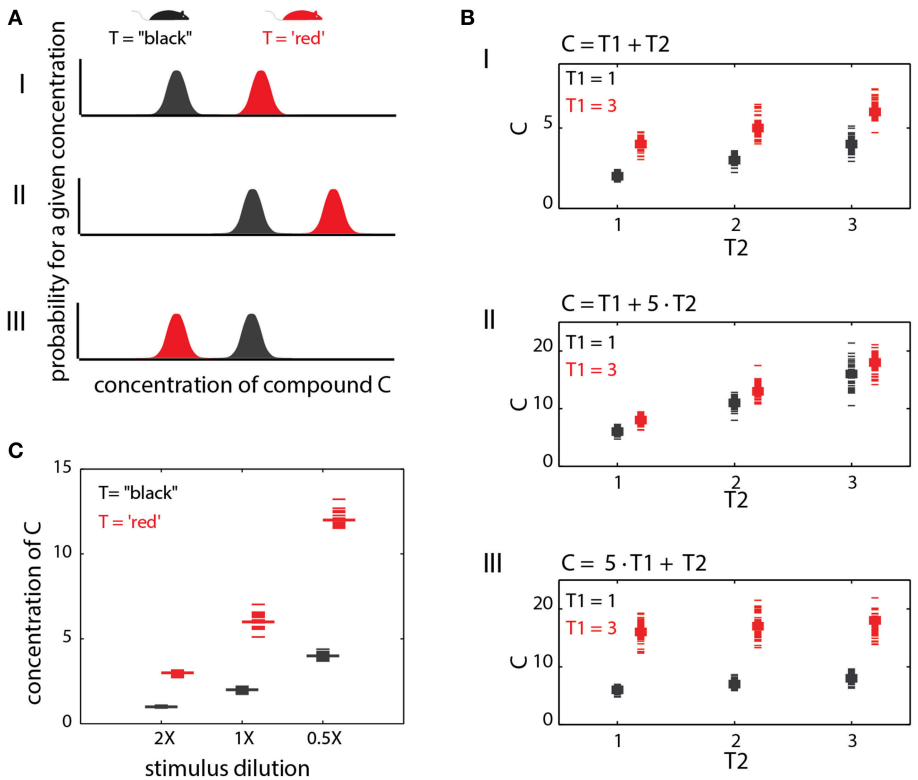


FIGURE 5 | Context effects. (A) Context dependent changes in compound distributions. This example illustrates a single cue with a different distribution under two trait (T) values. **(AI)** shows a baseline condition where the two distributions are non-overlapping. A simple threshold can be set to distinguish the trait values (T = "black" vs. T = "red") based on the level of **(C)**. In **(AII)**, both distributions are shifted. The threshold used in **(AI)** will no longer yield reliable discrimination among the two traits. **(AIII)** the relationships between compound levels and trait values are reversed. Here, the red trait is associated with lower values of **(C)**. **(B)**. Interaction between two different traits in determining compound levels. The first trait (T1) can take one of two values (1 or 3), while the second can take the values 1, 2, or 3. In **(BI)**, both traits exert an equal influence on the levels of **(C)**, which is simply their sum. In **(BII)**, the influence of T2 is dominant, while in **(BIII)**, T1 dominates. **(C)** Effect of dilution on specific compound levels. The example illustrates how different dilutions can confound trait identification.

2005; delBarco-Trillo et al., 2009; Liberles, 2014). For example, determining an individual's sex from urinary cues could involve very different rules as compared to salivary cues. Finally, note that context dependence is not limited to single compound codes as even the *relationships* among compounds may change under different physiological contexts.

Accounting for Context Dependence Using Simple Networks

The networks described above are designed to detect a certain *type* of relationship between compound compositions and a trait. The specific decision criteria for each network are determined by the properties of individual units and their connections. Adjustment of these parameters allows learning of novel distributions and accounting for contextual effects. For example, detecting different ranges as a function of context, as required by the scenarios in **Figure 5B**, calls for a simple modification of a range-detecting network. Such a network is shown in **Figure 6A** where the context is taken into account by introducing an offset to the input units, thereby altering the decision-range (**Figure 6B**). Another example for flexible decision rules is shown in **Figure 6C**, where the offset determines the difference-range for which the network is sensitive.

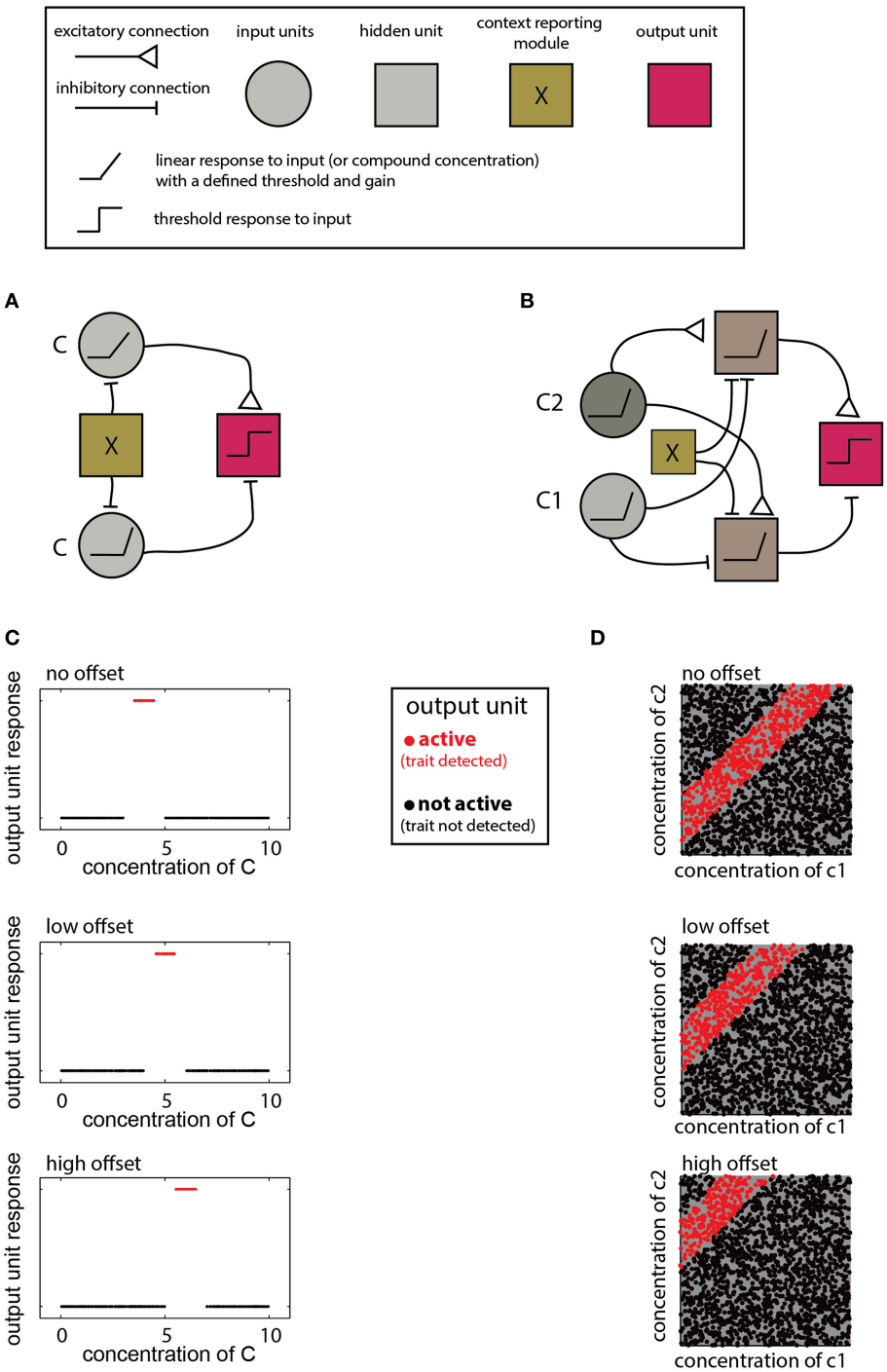


FIGURE 6 | Implementation of context dependent rules. (A) Introduction of a simple bias term (via the context detecting module X) can shift the range detected by a single-compound range-detecting network. **(B)** Similarly, a bias term can change the range associated with a two-component-difference detecting network. **(C)** Different offsets into the network in **(A)** change the detection range of **(C)**. **(D)** Different offsets into the network shown in **(B)** alter the detected difference range. In both **(A,B)**, insertion of different offsets into each of the two units that feed into the output unit can change the upper and lower bounds of the range independently. Legend at top explains network elements.

The contexts accommodated by the networks in **Figure 6** are simple and applicable under very limited conditions. Namely, the context must be defined by a trait that can be coded numerically and which modifies the compound distribution in a simple (e.g., monotonic), manner. In other cases, the context may call for altering the lower and/or the upper limit of a

decision range, or even reverse its direction (e.g., **Figure 5AIII**). For multi-component distributions, the context may influence the distribution of only a subset of components. This can be accounted for by modifying a subset of elements or connections within the network. For example, in the ratio detecting network shown in **Figure 4G**, specific elements can be tuned to change the required proportion of particular compounds. Note that although the context may be reflected by the level of some other compound, context detection itself may be complicated and could involve complex chemical features, or even sensory information from other modalities. In such cases, it may be easiest to simply integrate information from distinct networks, each of which is designed for a particular context.

DISCUSSION

To efficiently map external information to appropriate behavioral outcomes (Purves, 2010) sensory systems must be tuned to the informative statistical features of the environment (Barlow, 2001). Like early vision processing stages that detect regions with high contrast, or lines with specific orientations (Hubel, 1988), early chemosensory processing is likely designed to extract predictable statistical motifs associated with natural stimuli. This implies that understanding chemosensory circuits requires understanding the statistical structure of chemosensory signals. The complexity of chemosensory scenes implies a corresponding complexity of the neuronal circuits detecting them. Below, I discuss whether chemosensory stimuli must really be complex and attempt to map the abstract elements of the networks shown above to actual components of the olfactory system.

Do Chemosensory Signals Really have to be so Complex?

Throughout this manuscript, it was assumed that chemosensory information is conveyed by relationships among multiple compounds. It may be argued that this is an unjustified complication, and that chemical communication only requires detection of individual components. Analyses of natural stimuli reveal that some components are uniquely associated with one sex (Lin et al., 2005; Chamero et al., 2007; Zhang et al., 2007; Haga et al., 2010; Roberts et al., 2010) but such studies compare sex-specific expression patterns across a limited number of genetic backgrounds. Indeed, even when a small number of strains is considered, at least some cues show strain dependence. For example, some murine signals may be associated exclusively with one sex, but only for some strains (Kimoto et al., 2007; Ferrero et al., 2013). Although these findings are based on inbred strains, similar, though perhaps smaller effects, are also likely across individuals in wild populations.

Physiologically, responses to social stimuli have been studied mainly in the context of the vomeronasal system (VNS). Often, neuronal responses at the first brain relay of the VNS, the accessory olfactory bulb (AOB), are sex and strain-specific (Luo et al., 2003; Ben-Shaul et al., 2010; Tolokh et al., 2013). While some neurons, both in the vomeronasal organ (He et al., 2008), and the AOB (Ben-Shaul et al., 2010) do reveal consistent sex-specific responses across multiple strains, the number of tested strains is limited. Basing chemical communication on individual compounds, especially for traits that are subtler than sex, does not exploit the immense combinatorial coding capacity of the olfactory system.

Extraction of traits, rather than identifying specific individuals, is important because they are key to guiding specific behaviors. Because in chemosensation, the relevant quantities are compound levels, and because compound levels are generally influenced by multiple factors, decoding any one trait must take into account knowledge of others. Comparison of levels of various urinary cues in juvenile and sexually mature female mice, across three strains of mice (Schwende et al., 1984), revealed that relative levels of virtually all reported compounds were affected by both the strain *and* the reproductive state of the female. These scenarios closely resemble those shown in **Figure 5A**, and thus provide a direct illustration that decoding a female's reproductive status from chemical cues requires prior knowledge about her genetic background. In addition to these physiological considerations, physical aspects such as stimulus source dilution will also modulate (and complicate) chemosensory scenes. Much of the variability induced by such physical factors can be resolved by considering relationships among multiple components. While much remains unknown about the statistical nature of chemosensory scenes, these lines of evidence suggest that extraction of information from them, requires consideration of multiple components and their relationships.

Mapping Computations to Actual Elements of the Olfactory System

The computational steps described above used the metaphor of networks, but they are not intended to reflect real neuronal elements and synaptic interactions. Implementing some of the processing ascribed here to single units could require multiple neurons (Linster and Cleland, 2009) while on the other hand, some computations assigned to distinct units may be implemented by single neurons. Indeed, individual complex neurons, such as mitral/tufted cells (MTCs), can implement multiple processing stages using distinct cellular compartments (Silver, 2010). Despite these caveats, assuming that the networks reflect real computations, it should be possible to map them to specific elements within the olfactory system. Although there are many similarities between invertebrates and vertebrates (Kaupp, 2010), the discussion below focuses on vertebrate chemosensory systems.

A given odorant can activate multiple receptor types (with different affinity), and likewise, a single receptor type can be activated by multiple odorants (Malnic et al., 1999). This is true for both single molecules and multi-component mixtures. As a consequence, at increasing concentrations, any given odorant will activate a larger set of glomeruli (e.g., Spors and Grinvald, 2002) confounding stimulus intensity and quality. Various solutions have been suggested for maintaining responses to a given odor-stimulus similar across a range of concentrations (Cleland et al., 2011). Here, I simplify and assume that each compound in a multi-compound mixture activates one type of

sensory neuron (defined by its receptor), corresponding to a single input unit in the networks shown above.

The input units in all networks exhibit a linear or a logarithmic response to individual compounds. Obtaining such response profiles by single sensory neurons is not trivial and integration across several receptors with different dynamic ranges is likely required to achieve a consistent response across a broad range (Cleland et al., 2011). In the current context, note that different computations call for different stimulus response relationships. For instance, detection of differences is facilitated by responses that scale linearly with the concentration (e.g., **Figure 4A**), whereas ratio detection is better accomplished by responses that are linear with the logarithm of the concentration (e.g., **Figure 4D**). Other networks involved units that are sensitive to compound levels within a certain range (**Figure 2**). While more complicated, these non-monotonic responses still involve only a single compound, and do not require comparison of activity across distinct channels. Accordingly, all computations involving a single compound could in principle be implemented by intra-glomerular circuits. The considerable complexity of olfactory bulb (OB) circuits suggests several potential ways to implement these computations (Shepherd et al., 2004; Nagayama et al., 2014). For example, intra-glomerular inhibition onto MTCs via periglomerular cells (Nagayama et al., 2014) could form a basis for shaping the slope and range of the relationship between odor concentration and response magnitude. Indeed, glomerular circuits are implicated in various processes including response linearization and decorrelation (Cleland et al., 2011; Banerjee et al., 2015) and contrast enhancement (Cleland and Sethupathy, 2006). For a thorough discussion of glomerular interactions, see (Cleland, 2014). Overall, then, it seems reasonable to map activity related to specific individual components to apical dendrites of MTCs, which reflect the result of glomerular level computations. Indeed, response functions of MTCs show various patterns of dependency on stimulus concentration, including non-monotonic responses that are consistent with detection of a certain compound range (Harrison and Scott, 1986; Meredith, 1986; Wellis et al., 1989). Furthermore, at least in the AOB, it was shown that MTCs can display responses that scale linearly with the logarithm of stimulus concentration (Arnson and Holy, 2013).

The next computational step requires integration of information from multiple channels. While inter-glomerular connections exist, they are generally believed to relay global information (Cleland, 2014; Banerjee et al., 2015), and are thus less suitable to mediate specific interactions among individual channels. The same is likely true for paravalbumin-positive interneurons in the external plexiform layer (Kato et al., 2013; Miyamichi et al., 2013). In contrast, interactions within the external plexiform layer allow specific interactions between MTCs and granule cells (Nagayama et al., 2014). Granule cells receive excitatory inputs from MTCs, and provide inhibition unto the same, or to other MTCs via dendro-dendritic synapses (Shepherd et al., 2004), whose efficacy depends on the distance of the synapse from the MTC soma (Gilra and Bhalla, 2015). This type of connectivity provides an enormous combinatorial capacity for specific interactions. The existence of multiple sister MTCs, (i.e., with apical dendrites sampling from the same individual glomerulus), could allow each to interact with a unique set of non-sister MTCs (Gilra and Bhalla, 2015). Despite similarity in firing rates in response to single odors, sister MTs show distinct timing with respect to the breathing cycle phase (Dhawale et al., 2010). This difference between sister MTCs could be due to distinct inputs that each receives and which are likely to shape the timing with respect to the sniff cycle (Soucy et al., 2009). Thus, sister MTCs are expected to reveal even more dramatic differences in their responses to *combinations* of multiple components than to differences in responses to individual compounds.

A serious problem with assigning across-channel integrative function to MTC-granule cell circuits is that many of the computations require *summation* across distinct channels, and it is not obvious how this can be achieved with the inhibitory MTC-granule cell network. Therefore, it is tempting to discount a central role for OB circuits in coding combinations and attribute all such integrative processing to the cortex, where there is extensive potential for both summative and subtractive processing (Poo and Isaacson, 2011; Bekkers and Suzuki, 2013). This fits with the notion that OB circuits implement basic and preliminary computations like concentration invariance, stimulus decorrelation, or linearization (Miyamichi et al., 2013; Adam et al., 2014; Cleland, 2014; Uchida et al., 2014), while representations of complete patterns and objects are generated in the olfactory cortex (Sosulski et al., 2011; Chapuis and Wilson, 2012). However, the OB may nevertheless play a role in integrative and summative processing. For example, if a MTC is constantly suppressed by some granule cells, reduced activity in those granule cells will lead to MTC disinhibition. Thus, MTC responses might effectively summate by mutually disinhibiting each other. In this context, one study concluded that responses of individual MTCs to combined stimuli are linear sums of responses to individual components (Gupta et al., 2015), while another study found a few cases where the responses to stimulus combinations were qualitatively different from those of the elemental stimuli (Giraudet et al., 2002). Explicitly testing summation by MTCs requires measurement of responses across a fuller extent of the high dimensional stimulus spaces considered here (e.g., 2D space shown in **Figures 3B,E,H,K** and **Figures 4B,E**).

The seemingly limited magnitude of cross-talk (inhibitory, and particularly excitatory) between MTCs, at least when measured as firing rate changes, raises the possibility that temporal coding could play a role in these interactions. The exact timing of MTC responses, particularly within the sniff cycle, carries important information about odor identity (Cury and Uchida, 2010; Shusterman et al., 2011; Smear et al., 2011). As noted above, sister MTCs show distinct timing with respect to the breathing cycle phase (Dhawale et al., 2010) possibly due to selective lateral inputs from specific glomerular channels (Soucy et al., 2009). Interestingly, MTC phase-responses are approximated by negative and positive contributions from distinct glomerular channels with opposing effects on the response within the sniff cycle (Soucy et al., 2009). Olfactory cortex neurons are tuned to particular stimulus combinations

(Lei et al., 2006; Davison and Ehlers, 2011), in a particular temporal order (Haddad et al., 2013; Sanders et al., 2014). Thus, cortical integration of MTC responses could depend on the temporal *alignment* of MTC responses within the sniff cycle, to allow efficient summation within a constrained time window (Uchida et al., 2014). Consistent with this idea, in MTCs, mixtures of two components that elicit distinct phase responses, elicit intermediate phase responses (Khan et al., 2008). Experimentally testing this hypothesis requires evaluation not only of the magnitude, but also of the temporal features of responses to multi-component stimuli.

The next brain stage, the olfactory cortex, seems ideal for implementing both summative (Lei et al., 2006; Apicella et al., 2010; Davison and Ehlers, 2011; Poo and Isaacson, 2011) and subtractive (Suzuki and Bekkers, 2012; Sturgill and Isaacson, 2015) processing via feedforward and recurrent connectivity. Due to the complexity of cortical circuits, however, it is even harder to speculate how specific computations can be mapped onto particular cortical elements. This connectivity provides a rich substrate for implementing multiple stages of processing, including those called for by the networks described here. The notion of cortical integration using the fine temporal aspects of MTCs firing lies at the heart of the model of Hopfield and Brody (Brody and Hopfield, 2003). In broad terms, the model can be thought of as a realization of the more abstract networks shown here (i.e., **Figure 4G**). However, while the Hopfield and Brody model defines a pattern as a combination of odorants at some particular relative proportion, the networks here allow for more general patterns defined by certain *ranges* of stimulus levels or ratios among them, as well as the absence of other stimuli.

Distinctions between the Main and Vomeronasal Olfactory System

An important point that was overlooked thus far is the distinction between the main and the vomeronasal olfactory systems. Although the role of both in social behaviors is well established (Keller et al., 2009; Tirindelli et al., 2009; Stowers and Logan, 2010; Korzan et al., 2013; Beny and Kimchi, 2014; Liberles, 2014), a large body of studies specifically implicates the VNS in social behaviors. Some of the functional differences between the two systems are directly relevant here. For example, unlike the main OB, apical dendrites of AOB MTCs can sample information from multiple glomerular channels (Takami and Graziadei, 1991; Wagner et al., 2006; Larriva-Sahd, 2008). This provides an obvious opportunity for summing inputs from distinct channels and raises the idea that the different connectivity between the systems reflects differences in the statistics of the stimuli that they evolved to detect. Presently, it is not known which computations are realized by this connectivity. While there is evidence for synergistic processing across channels (Ben-Shaul et al., 2010), it does not appear to be the only mode of processing in the AOB (Meeks et al., 2010). Another important difference between the main olfactory and vomeronasal systems is that neuronal responses in the latter are not locked to the sniff cycle, but rather to the non-periodic activation of the vomeronasal organ (Meredith, 1994; Luo et al., 2003; Ben-Shaul et al., 2010). This difference may call for distinct modes of integration by downstream neurons of the VNS. Recent studies have provided important insights about the physiology of the medial amygdala (MeA) (Martinez-Marcos, 2009), a key region receiving inputs from AOB MTCs (Bian et al., 2008; Bergan et al., 2014; Hong et al., 2014; Keshavarzi et al., 2014). Further studies of these regions should reveal the computations realized by them.

Summary and Outlook

A comprehensive understanding of how social information is communicated and detected involves several lines of research (**Figure 1**). Logically, the first stage involves identification of traits that can actually be detect by particular organisms. The second involves characterization of the chemical compound distributions associated with each trait. Third is the investigation of the relationships between neuronal activity and particular patterns of chemical cues. This requires measurement of neuronal activity, across multiple populations, to high-dimensional stimulus spaces. Such knowledge, combined with a better understanding of the connectivity should reveal how the computations discussed here are implemented by the nervous system. This last effort will greatly benefit from realistic computational models to focus physiological hypotheses and experiments.

ACKNOWLEDGMENTS

I deeply thank Thomas Cleland, Ian Davison, Nao Uchida, and Tristram Wyatt for critically reading a draft of this manuscript and providing many insightful comments and suggestions. I thank Avinoam Ben-Shaul for helpful editing suggestions. This work was supported by a Marie Curie career integration grant for Yoram Ben-Shaul (# PCIG10-GA-2011- 303785).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2015.00439

REFERENCES

Adam, Y., Livneh, Y., Miyamichi, K., Groysman, M., Luo, L., and Mizrahi, A. (2014). Functional transformations of odor inputs in the mouse olfactory bulb. *Front. Neural Circuits* 8:129. doi: 10.3389/fncir.2014.00129

Albone, E. S., and Shirley, S. G. (1984). *Mammalian Semiochemistry: The Iinvestigation of Chemical Signals Between Mammals.* New York, NY: Wiley.

Apicella, A., Yuan, Q., Scanziani, M., and Isaacson, J. S. (2010). Pyramidal cells in piriform cortex receive convergent input from distinct olfactory bulb glomeruli. *J. Neurosci.* 30, 14255–14260. doi: 10.1523/JNEUROSCI.2747-10.2010

Arnson, H. A., and Holy, T. E. (2013). Robust encoding of stimulus identity and concentration in the accessory olfactory system. *J. Neurosci.* 33, 13388–13397. doi: 10.1523/JNEUROSCI.0967-13.2013

Banerjee, A., Marbach, F., Anselmi, F., Koh, M. S., Davis, M. B., Garcia da Silva, P., et al. (2015). An interglomerular circuit gates glomerular output and implements gain control in the mouse olfactory bulb. *Neuron* 87, 193–207. doi: 10.1016/j.neuron.2015.06.019

Barlow, H. (2001). The exploitation of regularities in the environment by the brain. *Behav. Brain Sci.* 24, 602–607. discussion: 652–671. doi: 10.1017/S0140525X01000024

Bekkers, J. M., and Suzuki, N. (2013). Neurons and circuits for odor processing in the piriform cortex. *Trends Neurosci.* 36, 429–438. doi: 10.1016/j.tins.2013.04.005

Ben-Shaul, Y., Katz, L. C., Mooney, R., and Dulac, C. (2010). *In vivo* vomeronasal stimulation reveals sensory encoding of conspecific and allospecific cues by the mouse accessory olfactory bulb. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5172–5177. doi: 10.1073/pnas.0915147107

Beny, Y., and Kimchi, T. (2014). Innate and learned aspects of pheromone-mediated social behaviours. *Anim. Behav.* 97, 301–311. doi: 10.1016/j.anbehav.2014.09.014

Bergan, J. F., Ben-Shaul, Y., and Dulac, C. (2014). Sex-specific processing of social cues in the medial amygdala. *Elife* 3:e02743. doi: 10.7554/elife.02743

Bian, X., Yanagawa, Y., Chen, W. R., and Luo, M. (2008). Cortical-like functional organization of the pheromone-processing circuits in the medial amygdala. *J. Neurophysiol.* 99, 77–86. doi: 10.1152/jn.00902.2007

Bradbury, J. W., and Vehrencamp, S. L. (2011). *Principles of Animal Communication.* Sunderland, MA: Sinauer Associates.

Brody, C. D., and Hopfield, J. J. (2003). Simple networks for spike-timing-based computation, with application to olfactory processing. *Neuron* 37, 843–852. doi: 10.1016/S0896-6273(03)00120-X

Butenandt, A., Beckmann, R., and Hecker, E. (1961). On the sexattractant of silk-moths. I. the biological test and the isolation of the pure sex-attractant bombykol. *Hoppe Seylers Z. Physiol. Chem.* 324, 71–83. doi: 10.1515/bchm2.1961.324.1.71

Cardé, R. T., and Willis, M. A. (2008). Navigational strategies used by insects to find distant, wind-borne sources of odor. *J. Chem. Ecol.* 34, 854–866. doi: 10.1007/s10886-008-9484-5

Chamero, P., Marton, T. F., Logan, D. W., Flanagan, K., Cruz, J. R., Saghatelian, A., et al. (2007). Identification of protein pheromones that promote aggressive behaviour. *Nature* 450, 899–902. doi: 10.1038/nature05997

Chapuis, J., and Wilson, D. A. (2012). Bidirectional plasticity of cortical pattern recognition and behavioral sensory acuity. *Nat. Neurosci.* 15, 155–161. doi: 10.1038/nn.2966

Cheetham, S. A., Thom, M. D., Jury, F., Ollier, W. E., Beynon, R. J., and Hurst, J. L. (2007). The genetic basis of individual-recognition signals in the mouse. *Curr. Biol.* 17, 1771–1777. doi: 10.1016/j.cub.2007.10.007

Cleland, T. A. (2014). Construction of odor representations by olfactory bulb microcircuits. *Prog. Brain Res.* 208, 177–203. doi: 10.1016/B978-0-444-63350-7.00007-3

Cleland, T. A., Chen, S. Y., Hozer, K. W., Ukatu, H. N., Wong, K. J., and Zheng, F. (2011). Sequential mechanisms underlying concentration invariance in biological olfaction. *Front. Neuroeng.* 4:21. doi: 10.3389/fneng.2011.00021

Cleland, T. A., and Sethupathy, P. (2006). Non-topographical contrast enhancement in the olfactory bulb. *BMC Neurosci.* 7:7. doi: 10.1186/1471-2202-7-7

Cury, K. M., and Uchida, N. (2010). Robust odor coding via inhalation-coupled transient activity in the mammalian olfactory bulb. *Neuron* 68, 570–585. doi: 10.1016/j.neuron.2010.09.040

Davison, I. G., and Ehlers, M. D. (2011). Neural circuit mechanisms for pattern detection and feature combination in olfactory cortex. *Neuron* 70, 82–94. doi: 10.1016/j.neuron.2011.02.047

delBarco-Trillo, J., LaVenture, A. B., and Johnston, R. E. (2009). Male hamsters discriminate estrous state from vaginal secretions and individuals from flank marks. *Behav. Processes* 82, 18–24. doi: 10.1016/j.beproc.2009.03.003

Dhawale, A. K., Hagiwara, A., Bhalla, U. S., Murthy, V. N., and Albeanu, D. F. (2010). Non-redundant odor coding by sister mitral cells revealed by light addressable glomeruli in the mouse. *Nat. Neurosci.* 13, 1404–1412. doi: 10.1038/nn.2673

Ferrero, D. M., Moeller, L. M., Osakada, T., Horio, N., Li, Q., Roy, D. S., et al. (2013). A juvenile mouse pheromone inhibits sexual behaviour through the vomeronasal system. *Nature* 502, 368–371. doi: 10.1038/nature12579

Gemeno, C., Yeargan, K. V., and Haynes, K. F. (2000). Aggressive chemical mimicry by the bolas spider Mastophora hutchinsoni: identification and quantification of a major prey's sex pheromone components in the spider's volatile emissions. *J. Chem. Ecol.* 26, 1235–1243. doi: 10.1023/A:1005488128468

Gilra, A., and Bhalla, U. S. (2015). Bulbar microcircuit model predicts connectivity and roles of interneurons in odor coding. *PLoS ONE* 10:e0098045. doi: 10.1371/journal.pone.0098045

Giraudet, P., Berthommier, F., and Chaput, M. (2002). Mitral cell temporal response patterns evoked by odor mixtures in the rat olfactory bulb. *J. Neurophysiol.* 88, 829–838. doi: 10.1152/jn.00436.2001

Gupta, P., Albeanu, D. F., and Bhalla, U. S. (2015). Olfactory bulb coding of odors, mixtures and sniffs is a linear sum of odor time profiles. *Nat. Neurosci.* 18, 272–281. doi: 10.1038/nn.3913

Haddad, R., Lanjuin, A., Madisen, L., Zeng, H., Murthy, V. N., and Uchida, N. (2013). Olfactory cortical neurons read out a relative time code in the olfactory bulb. *Nat. Neurosci.* 16, 949–957. doi: 10.1038/nn.3407

Haga, S., Hattori, T., Sato, T., Sato, K., Matsuda, S., Kobayakawa, R., et al. (2010). The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. *Nature* 466, 118–122. doi: 10.1038/nature09142

Harrison, T. A., and Scott, J. W. (1986). Olfactory bulb responses to odor stimulation: analysis of response pattern and intensity relationships. *J. Neurophysiol.* 56, 1571–1589.

He, J., Ma, L., Kim, S., Nakai, J., and Yu, C. R. (2008). Encoding gender and individual information in the mouse vomeronasal organ. *Science* 320, 535–538. doi: 10.1126/science.1154476

Hong, W., Kim, D. W., and Anderson, D. J. (2014). Antagonistic control of social versus repetitive self-grooming behaviors by separable amygdala neuronal subsets. *Cell* 158, 1348–1361. doi: 10.1016/j.cell.2014.07.049

Hopfield, J. J. (1995). Pattern recognition computation using action potential timing for stimulus representation. *Nature* 376, 33–36. doi: 10.1038/376033a0

Hubel, D. H. (1988). *Eye, Brain, and Vision.* New York, NY: Scientific American Library.

Johnston, R. E. (2003). Chemical communication in rodents: from pheromones to individual recognition. *J. Mammal.* 84, 1141–1162. doi: 10.1644/BLe-010

Kato, H. K., Gillet, S. N., Peters, A. J., Isaacson, J. S., and Komiyama, T. (2013). Parvalbumin-expressing interneurons linearly control olfactory bulb output. *Neuron* 80, 1218–1231. doi: 10.1016/j.neuron.2013.08.036

Kaupp, U. B. (2010). Olfactory signalling in vertebrates and insects: differences and commonalities. *Nat. Rev. Neurosci.* 11, 188–200. doi: 10.1038/nrn2789

Kaur, A. W., Ackels, T., Kuo, T. H., Cichy, A., Dey, S., Hays, C., et al. (2014). Murine pheromone proteins constitute a context-dependent combinatorial code governing multiple social behaviors. *Cell* 157, 676–688. doi: 10.1016/j.cell.2014.02.025

Keller, M., Baum, M. J., Brock, O., Brennan, P. A., and Bakker, J. (2009). The main and the accessory olfactory systems interact in the control of mate recognition and sexual behavior. *Behav. Brain Res.* 200, 268–276. doi: 10.1016/j.bbr.2009.01.020

Kelly, D. R. (1996). When is a butterfly like an elephant? *Chem. Biol.* 3, 595–602. doi: 10.1016/S1074-5521(96)90125-8

Kepecs, A., Uchida, N., and Mainen, Z. F. (2007). Rapid and precise control of sniffing during olfactory discrimination in rats. *J. Neurophysiol.* 98, 205–213. doi: 10.1152/jn.00071.2007

Keshavarzi, S., Sullivan, R. K., Ianno, D. J., and Sah, P. (2014). Functional properties and projections of neurons in the medial amygdala. *J. Neurosci.* 34, 8699–8715. doi: 10.1523/JNEUROSCI.1176-14.2014

Khan, A. G., Thattai, M., and Bhalla, U. S. (2008). Odor representations in the rat olfactory bulb change smoothly with morphing stimuli. *Neuron* 57, 571–585. doi: 10.1016/j.neuron.2008.01.008

Kimoto, H., Haga, S., Sato, K., and Touhara, K. (2005). Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature* 437, 898–901. doi: 10.1038/nature04033

Kimoto, H., Sato, K., Nodari, F., Haga, S., Holy, T. E., and Touhara, K. (2007). Sex- and strain-specific expression and vomeronasal activity of mouse ESP family peptides. *Curr. Biol.* 17, 1879–1884. doi: 10.1016/j.cub.2007.09.042

Korzan, W. J., Freamat, M., Johnson, A. G., Cherry, J. A., and Baum, M. J. (2013). Either main or accessory olfactory system signaling can mediate the rewarding

effects of estrous female chemosignals in sexually naive male mice. *Behav. Neurosci.* 127, 755–762. doi: 10.1037/a0033945

Larriva-Sahd, J. (2008). The accessory olfactory bulb in the adult rat: a cytological study of its cell types, neuropil, neuronal modules, and interactions with the main olfactory system. *J. Comp. Neurol.* 510, 309–350. doi: 10.1002/cne.21790

Lei, H., Mooney, R., and Katz, L. C. (2006). Synaptic integration of olfactory information in mouse anterior olfactory nucleus. *J. Neurosci.* 26, 12023–12032. doi: 10.1523/JNEUROSCI.2598-06.2006

Lelito, J. P., Myrick, A. J., and Baker, T. C. (2008). Interspecific pheromone plume interference among sympatric heliothine moths: a wind tunnel test using live, calling females. *J. Chem. Ecol.* 34, 725–733. doi: 10.1007/s10886-008-9475-6

Levesque, H. M., Scaffidi, D., Polkinghorne, C. N., and Sorensen, P. W. (2011). A multi-component species identifying pheromone in the goldfish. *J. Chem. Ecol.* 37, 219–227. doi: 10.1007/s10886-011-9907-6

Liberles, S. D. (2014). Mammalian pheromones. *Annu. Rev. Physiol.* 76, 151–175. doi: 10.1146/annurev-physiol-021113-170334

Lin, D. Y., Zhang, S. Z., Block, E., and Katz, L. C. (2005). Encoding social signals in the mouse main olfactory bulb. *Nature* 434, 470–477. doi: 10.1038/nature03414

Linn, C. E. Jr., Campbell, M. G., and Roelofs, W. L. (1987). Pheromone components and active spaces: what do moths smell and where do they smell it? *Science* 237, 650–652. doi: 10.1126/science.237.4815.650

Linn, C. E. Jr., Hammond, A., Du, J., and Roelofs, W. L. (1988). Specificity of male response to multicomponent pheromones in noctuid mothsTrichoplusia ni andPseudoplusia includens. *J. Chem. Ecol.* 14, 47–57. doi: 10.1007/BF01022530

Linster, C., and Cleland, T. A. (2009). Glomerular microcircuits in the olfactory bulb. *Neural Netw.* 22, 1169–1173. doi: 10.1016/j.neunet.2009.07.013

Luo, M., Fee, M. S., and Katz, L. C. (2003). Encoding pheromonal signals in the accessory olfactory bulb of behaving mice. *Science* 299, 1196–1201. doi: 10.1126/science.1082133

Malnic, B., Hirono, J., Sato, T., and Buck, L. B. (1999). Combinatorial receptor codes for odors. *Cell* 96, 713–723. doi: 10.1016/S0092-8674(00)80581-4

Martin, S. J., Helanterä, H., and Drijfhout, F. P. (2008). Colony-specific hydrocarbons identify nest mates in two species of Formica ant. *J. Chem. Ecol.* 34, 1072–1080. doi: 10.1007/s10886-008-9482-7

Martinez-Marcos, A. (2009). On the organization of olfactory and vomeronasal cortices. *Prog. Neurobiol.* 87, 21–30. doi: 10.1016/j.pneurobio.2008.09.010

Meeks, J. P., Arnson, H. A., and Holy, T. E. (2010). Representation and transformation of sensory information in the mouse accessory olfactory system. *Nat. Neurosci.* 13, 723–730. doi: 10.1038/nn.2546

Meredith, M. (1986). Patterned response to odor in mammalian olfactory bulb: the influence of intensity. *J. Neurophysiol.* 56, 572–597.

Meredith, M. (1994). Chronic recording of vomeronasal pump activation in awake behaving hamsters. *Physiol. Behav.* 56, 345–354. doi: 10.1016/0031-9384(94)90205-4

Miyamichi, K., Shlomai-Fuchs, Y., Shu, M., Weissbourd, B. C., Luo, L., and Mizrahi, A. (2013). Dissecting local circuits: parvalbumin interneurons underlie broad feedback control of olfactory bulb output. *Neuron* 80, 1232–1245. doi: 10.1016/j.neuron.2013.08.027

Nagayama, S., Homma, R., and Imamura, F. (2014). Neuronal organization of olfactory bulb circuits. *Front. Neural Circuits* 8:98. doi: 10.3389/fncir.2014.00098

Novotny, M., Harvey, S., Jemiolo, B., and Alberts, J. (1985). Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. U.S.A.* 82, 2059–2061. doi: 10.1073/pnas.82.7.2059

Oriain, M. J., and Jarvis, J. U. M. (1997). Colony member recognition and xenophobia in the naked mole-rat. *Anim. Behav.* 53, 487–498. doi: 10.1006/anbe.1996.0299

Poo, C., and Isaacson, J. S. (2011). A major role for intracortical circuits in the strength and tuning of odor-evoked excitation in olfactory cortex. *Neuron* 72, 41–48. doi: 10.1016/j.neuron.2011.08.015

Purves, D. (2010). *Brains: How They Seem to Work.* Upper Saddle River, NJ: FT Press Science.

Purves, D. (2012). *Neuroscience.* Sunderland: Sinauer Associates.

Rich, T. J., and Hurst, J. L. (1999). The competing countermarks hypothesis: reliable assessment of competitive ability by potential mates. *Anim. Behav.* 58, 1027–1037. doi: 10.1006/anbe.1999.1217

Rieke, F., Bodnar, D. A., and Bialek, W. (1995). Naturalistic stimuli increase the rate and efficiency of information transmission by primary auditory afferents. *Proc. Biol. Sci.* 262, 259–265. doi: 10.1098/rspb.1995.0204

Roberts, S. A., Simpson, D. M., Armstrong, S. D., Davidson, A. J., Robertson, D. H., McLean, L., et al. (2010). Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol.* 8:75. doi: 10.1186/1741-7007-8-75

Rokni, D., Hemmelder, V., Kapoor, V., and Murthy, V. N. (2014). An olfactory cocktail party: figure-ground segregation of odorants in rodents. *Nat. Neurosci.* 17, 1225–1232. doi: 10.1038/nn.3775

Sakurai, T., Namiki, S., and Kanzaki, R. (2014). Molecular and neural mechanisms of sex pheromone reception and processing in the silkmoth *Bombyx mori*. *Front. Physiol.* 5:125. doi: 10.3389/fphys.2014.00125

Sanders, H., Kolterman, B. E., Shusterman, R., Rinberg, D., Koulakov, A., and Lisman, J. (2014). A network that performs brute-force conversion of a temporal sequence to a spatial pattern: relevance to odor recognition. *Front. Comput. Neurosci.* 8:108. doi: 10.3389/fncom.2014.00108

Schaefer, M. L., Wongravee, K., Holmboe, M. E., Heinrich, N. M., Dixon, S. J., Zeskind, J. E., et al. (2010). Mouse urinary biomarkers provide signatures of maturation, diet, stress level, and diurnal rhythm. *Chem. Senses* 35, 459–471. doi: 10.1093/chemse/bjq032

Schwende, F. J., Wiesler, D., and Novotny, M. (1984). Volatile compounds associated with estrus in mouse urine: potential pheromones. *Experientia* 40, 213–215. doi: 10.1007/BF01963606

Shepherd, G. M., Chen, W. R., and Greer, C. A. (2004). "Olfactory bulb," in *The Synaptic Organization of the Brain*, ed G. M. Shepherd (Oxford: Oxford University Press), 165–216.

Shusterman, R., Smear, M. C., Koulakov, A. A., and Rinberg, D. (2011). Precise olfactory responses tile the sniff cycle. *Nat. Neurosci.* 14, 1039–1044. doi: 10.1038/nn.2877

Silver, R. A. (2010). Neuronal arithmetic. *Nat. Rev. Neurosci.* 11, 474–489. doi: 10.1038/nrn2864

Slessor, K. N., Winston, M. L., and Le Conte, Y. (2005). Pheromone communication in the honeybee (Apis mellifera L.). *J. Chem. Ecol.* 31, 2731–2745. doi: 10.1007/s10886-005-7623-9

Smear, M., Shusterman, R., O'Connor, R., Bozza, T., and Rinberg, D. (2011). Perception of sniff phase in mouse olfaction. *Nature* 479, 397–400. doi: 10.1038/nature10521

Sosulski, D. L., Bloom, M. L., Cutforth, T., Axel, R., and Datta, S. R. (2011). Distinct representations of olfactory information in different cortical centres. *Nature* 472, 213–216. doi: 10.1038/nature09868

Soucy, E. R., Albeanu, D. F., Fantana, A. L., Murthy, V. N., and Meister, M. (2009). Precision and diversity in an odor map on the olfactory bulb. *Nat. Neurosci.* 12, 210–220. doi: 10.1038/nn.2262

Spors, H., and Grinvald, A. (2002). Spatio-temporal dynamics of odor representations in the mammalian olfactory bulb. *Neuron* 34, 301–315. doi: 10.1016/S0896-6273(02)00644-X

Srinivasan, J., von Reuss, S. H., Bose, N., Zaslaver, A., Mahanti, P., Ho, M. C., et al. (2012). A modular library of small molecule signals regulates social behaviors in Caenorhabditis elegans. *PLoS Biol.* 10:e1001237. doi: 10.1371/journal.pbio.1001237

Stowers, L., and Logan, D. W. (2010). Olfactory mechanisms of stereotyped behavior: on the scent of specialized circuits. *Curr. Opin. Neurobiol.* 20, 274–280. doi: 10.1016/j.conb.2010.02.013

Sturgill, J. F., and Isaacson, J. S. (2015). Somatostatin cells regulate sensory response fidelity via subtractive inhibition in olfactory cortex. *Nat. Neurosci.* 18, 531–535. doi: 10.1038/nn.3971

Suzuki, N., and Bekkers, J. M. (2012). Microcircuits mediating feedforward and feedback synaptic inhibition in the piriform cortex. *J. Neurosci.* 32, 919–931. doi: 10.1523/JNEUROSCI.4112-11.2012

Takami, S., and Graziadei, P. P. (1991). Light microscopic Golgi study of mitral/tufted cells in the accessory olfactory bulb of the adult rat. *J. Comp. Neurol.* 311, 65–83. doi: 10.1002/cne.903110106

Thesen, A., Steen, J. B., and Døving, K. B. (1993). Behaviour of dogs during olfactory tracking. *J. Exp. Biol.* 180, 247–251.

Tirindelli, R., Dibattista, M., Pifferi, S., and Menini, A. (2009). From pheromones to behavior. *Physiol. Rev.* 89, 921–956. doi: 10.1152/physrev.00037.2008

Tolokh, I. I., Fu, X., and Holy, T. E. (2013). Reliable sex and strain discrimination in the mouse vomeronasal organ and accessory olfactory bulb. *J. Neurosci.* 33, 13903–13913. doi: 10.1523/JNEUROSCI.0037-13.2013

Uchida, N., and Mainen, Z. F. (2007). Odor concentration invariance by chemical ratio coding. *Front. Syst. Neurosci.* 1:3. doi: 10.3389/neuro.06.003

Uchida, N., Poo, C., and Haddad, R. (2014). Coding and transformations in the olfactory system. *Annu. Rev. Neurosci.* 37, 363–385. doi: 10.1146/annurev-neuro-071013-013941

Wagner, S., Gresser, A. L., Torello, A. T., and Dulac, C. (2006). A multireceptor genetic approach uncovers an ordered integration of VNO sensory inputs in the accessory olfactory bulb. *Neuron* 50, 697–709. doi: 10.1016/j.neuron.2006.04.033

Wellis, D. P., Scott, J. W., and Harrison, T. A. (1989). Discrimination among odorants by single neurons of the rat olfactory bulb. *J. Neurophysiol.* 61, 1161–1177.

Wyatt, T. D. (2010). Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 196, 685–700. doi: 10.1007/s00359-010-0564-y

Wyatt, T. D. (2014). *Pheromones and Animal Behavior: Chemical Signals and Signature Mixes*. Cambridge: Cambridge University Press.

Yamaguchi, S., and Heisenberg, M. (2011). Photoreceptors and neural circuitry underlying phototaxis in insects. *Fly (Austin).* 5, 333–336. doi: 10.4161/fly.5.4.16419

Zhang, J. X., Rao, X. P., Sun, L., Zhao, C. H., and Qin, X. W. (2007). Putative chemical signals about sex, individuality, and genetic background in the preputial gland and urine of the house mouse (Mus musculus). *Chem. Senses* 32, 293–303. doi: 10.1093/chemse/bjl058

Zhang, Y. H., and Zhang, J. X. (2011). Urine-derived key volatiles may signal genetic relatedness in male rats. *Chem. Senses* 36, 125–135. doi: 10.1093/chemse/bjq103

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 25 June 2015
doi: 10.3389/fnins.2015.00226

Behavioral responses to odors from other species: introducing a complementary model of allelochemics involving vertebrates

Birte L. Nielsen[1]*, *Olivier Rampin*[1], *Nicolas Meunier*[1,2] and *Vincent Bombail*[1]

[1] Department of Animal Physiology and Livestock Systems, INRA, UR1197 NeuroBiologie de l'Olfaction, Jouy-en-Josas, France, [2] Department of Biology, Université de Versailles Saint-Quentin-en-Yvelines, Versailles, France

It has long been known that the behavior of an animal can be affected by odors from another species. Such interspecific effects of odorous compounds (allelochemics) are usually characterized according to who benefits (emitter, receiver, or both) and the odors categorized accordingly (allomones, kairomones, and synomones, respectively), which has its origin in the definition of pheromones, i.e., intraspecific communication via volatile compounds. When considering vertebrates, however, interspecific odor-based effects exist which do not fit well in this paradigm. Three aspects in particular do not encompass all interspecific semiochemical effects: one relates to the innateness of the behavioral response, another to the origin of the odor, and the third to the intent of the message. In this review we focus on vertebrates, and present examples of behavioral responses of animals to odors from other species with specific reference to these three aspects. Searching for a more useful classification of allelochemical effects we examine the relationship between the valence of odors (attractive through to aversive), and the relative contributions of learned and unconditioned (innate) behavioral responses to odors from other species. We propose that these two factors (odor valence and learning) may offer an alternative way to describe the nature of interspecific olfactory effects involving vertebrates compared to the current focus on who benefits.

Keywords: olfaction, interspecific interactions, semiochemicals, allomones, kairomones, odor valence, innateness, learning

OPEN ACCESS

Edited by:
Markus Fendt,
Otto-von-Guericke University Magdeburg, Germany

Reviewed by:
Stephen Liberles,
Harvard Medical School, USA
Fabio Papes,
University of Campinas, Brazil

***Correspondence:**
Birte L. Nielsen,
Department of Animal Physiology and Livestock Systems, INRA, UR1197 NeuroBiologie de l'Olfaction, Bâtiment 230, Domaine de Vilvert, F-78352 Jouy-en-Josas, France
birte.nielsen@jouy.inra.fr

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *07 May 2015*
Accepted: *11 June 2015*
Published: *25 June 2015*

Citation:
Nielsen BL, Rampin O, Meunier N and Bombail V (2015) Behavioral responses to odors from other species: introducing a complementary model of allelochemics involving vertebrates. Front. Neurosci. 9:226. doi: 10.3389/fnins.2015.00226

Introduction

Odors can influence the behavior of animals. Behind this simple statement is a hidden world of complex links and interactions, and a large body of scientific studies dealing with various aspects from chemo-sensitivity and implicated brain regions, to evolutionary pathways and functionality. One area of olfactory behavior research is the study of odor-based effects between organisms. These odors are referred to as semiochemicals (Law and Regnier, 1971; Regnier, 1971), and consist of two major groups: pheromones [Karlson and Lüscher, 1959a; originally named ectohormones by Bethe (1932)] for intraspecific interactions, and allelochemics (Whittaker, 1970) for interactions between organisms of different species. Allelochemics are thus odors by which members of one species affect the growth, health, or behavior of members of another species (Whittaker and Feeny, 1971).

Allelochemics were initially divided into two groups, consisting of allomones (of adaptive value to the organism emitting them) and kairomones (of adaptive value to the receiving organism, Brown et al., 1970). Subsequently, Nordlund and Lewis (1976) introduced synomone, an allelochemical where both receiver and emitter benefitted. Whittaker and Feeny (1971) stated that classification of these chemical agents was almost impossible due to their roles combining in "*almost all conceivable directions*." They nevertheless tried to list subcategories of allomones (repellents, escape substances, suppressants, venoms, inductants, counteractants, and attractants) and kairomones (attractants, inductants, signals, and stimulants). The sheer quantity of these categories, partially overlapping in places, and with names that are not always self-explanatory, severely questions the value of such subdivisions.

In their original paper introducing the terms, Brown et al. (1970) talked about mutualistic, antagonistic, and defensive allomones, and presented examples of overlaps between pheromones, allomones, kairomones, and hormones. This gave rise to discussions about the usefulness of the terms, and whether they represented distinct chemical signals (e.g., Blum, 1974). Another term, apneumone, was defined as "*a substance emitted by a nonliving material that evokes a behavioral or physiological reaction adaptively favorable to a receiving organism, but detrimental to an organism, of another species, that may be found in or on the nonliving material*" (Nordlund and Lewis, 1976). This term has thankfully disappeared from use.

Table 1 gives an overview of the most commonly used terms used to describe chemical effects between organisms. These compounds have been studied widely in plants, bacteria and insects, and to a much lesser extent in vertebrates. We therefore set out to review interspecific odor-based effects in vertebrates (mainly in mammals, fish, and birds, but also including examples from amphibians and reptiles), with the specific aim of investigating the extent of and consistency in the use of the terms allomone, kairomone, and synomone. Based on this, we identified three problem areas, which led us to introduce a novel conceptual framework for use when studying interspecific odor-based effects in vertebrates.

What is Wrong with the Current View and Terminology?

The words allomone, kairomone, and synomone have been used increasingly since their coinage in the 1970's. However, among the 2644 publications found in a search on topic in Web of Science™ (ver. 5.16.1; Thomson Reuters © 2015), only 184 (7%) included vertebrates (**Figure 1**). Of these, 98 were concerned with kairomones emitted by vertebrates attracting biting or stinging insects (mainly humans attracting mosquitoes). Another 51 were on the subject of the use of odors by daphnia and other zooplankton to detect aquatic predators, mainly fish. The earliest use of these terms in relation to vertebrates were found in Rothschild and Ford (1973; when scientific papers could still be 50 pages long), on an odor found in newborn rabbit urine which acted as a kairomone accelerating reproduction in the rabbit flea. Overall, only 32 publications were found, which reported responses of vertebrate species to interspecific odors when searching on any of these three terms. In reality, much more research exists on this subject in vertebrates, but the three words are not used, either by omission or because the concept of who benefits does not fit the effects observed.

Ruther et al. (2002) noted that chemicals classified as kairomones had completely different biological functions for the receiving organism. Their suggestion to remedy this was to further sub-divide these compounds according to their function for the benefiting organism, thus introducing foraging, enemy-avoidance, sexual, and aggregation kairomones. However, the terms used in allelochemics are based on the assumption that we know all about the relationship between the two species considered, whereas in reality the olfactory effects are often relative, context specific, and not absolute. The designation of an odor as a kairomone or allomone is only as good as our knowledge of the relationship between the involved species.

Unlike Sbarbati and Osculati (2006), who predicted that the terms kairomone, synomone and allomone would become as popular as the terms hormone or pheromone in vertebrate studies, we are more skeptical. As we will demonstrate below, the terms—although providing a practical categorization—may constrain the way in which interspecific olfactory effects are viewed, especially in vertebrates. In the following sections, we highlight three issues relating to inter-specific odor-based effects, where the terms kairomone, synomone, and allomone do not add clarification. The three issues are innateness of the behavioral response, origin of the odor, and intent of the odorous message; these are discussed in turn below.

Innateness of the Behavioral Response

Before deliberating on the innateness of vertebrate allelochemical responses, we would like to remind the reader of the same issue regarding pheromones. Although the word innate is not included in the original definition of a pheromone (**Table 1**), there is implicitness in the wording, where "release a specific reaction" indicates a certain automation and homogeneity of the response to a pheromone. In their original paper, Karlson and Lüscher (1959a) also invoke the principle that pheromones are effective in minute amounts, which again is more likely to be the case if the evoked response does not require any form of learning. Stowers and Marton (2005) question the notion that the response to pheromones is thought to be unalterable, and suggest that it may be context dependent. Similar caution is shown by Wyatt (2010), who describes the innate response to pheromones as conditional on development as well as context, experience, and internal state. Examples of this are perhaps more likely to be found in vertebrates (e.g., cichlids, Keller-Costa et al., 2015), which have greater cerebral development and a longer lifespan than most insects and bacteria.

Because the words used to describe interspecific odor-based effects were coined along the same root as pheromone (Karlson and Lüscher, 1959b), these "*–mones*" have the same inherent pitfalls when it comes to innateness of the responses. The definition of allomones, for example, include adaptive behavioral reactions upon contact (**Table 1**), thus hinting at

TABLE 1 | Hierarchical overview of terminology commonly used to classify chemical effects within and between organisms.

Terminology	Definition
Infochemical	Generic term synonymous with chemical cue (Sbarbati and Osculati, 2006).
A. Hormone from Greek *hormōn*, "to excite, impel, set in motion."	Chemical messengers, which have to be carried frabom the organ where they are produced to the organ which they affect by means of the blood stream (Starling, 1905)
B. Semiochemical from Greek *ēmeion* "sign"	Chemicals that evoke a behavioral or physiological response in individuals of the same or other species (Sbarbati and Osculati, 2006).
1. Pheromone from Greek *pherein* "convey"	Substances, which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process (Karlson and Lüscher, 1959a).
2. Allelochemical, allelomone from Greek *allēl-* "one another"	[Semio]chemical that mediates interaction between two individuals that belong to different species (Dicke and Sabelis, 1988).
a) Allomone from Greek *allos* "other."	Chemical substance produced or acquired‡ by an organism, which, when it contacts an individual of another species in the natural context, evokes in the receiver a behavioral or physiological reaction adaptively favorable to the emitter (Brown et al., 1970).
b) Kairomone from Greek *kairos* "advantage, opportunity, exploitative"	A transspecific chemical messenger the adaptive benefit of which falls on the recipient rather than on the receiver (Brown et al., 1970).
i. Synomone from Greek *syn,* "with or jointly"	Allelochemical, where both receiver and emitter benefit (Nordlund and Lewis, 1976), thus simultaneously an allomone and a kairomone.

‡*Acquired refers here to odors appropriated (intact) from the food (Brown et al., 1970).*

innate responses, as was the case with pheromones. It is therefore unsurprising that it is not always clear from the literature if a given interspecific odor-based behavioral response is innate or a result of some degree of learning.

Some allelochemical responses in vertebrates appear to be innate. Snakes have been found to use chemical cues from prey species when choosing a site to wait for passing prey to ambush, even without prior experience with the prey (Clark, 2004). A good example of an un-conditioned olfactory response in vertebrates is the startle or freezing behavior of prey animals when exposed to the odor of a predator (Apfelbach et al., 2005). Kangaroos persistently avoid an area of highly palatable food containing predator-related odor cues (dingo urine or feces), and no habituation occur even in the absence of a predator (Parsons and Blumstein, 2010). Papes et al. (2010) observed increased ACTH levels and innate avoidance and risk assessment behaviors in mice exposed to odors obtained from natural mice predators [cat (neck swab), snake (shed skin), and rat (urine)], but not when exposed to rabbit urine. Black-tailed deer from a population with no exposure to wolves for a century showed reduced feeding and increased sniffing when confronted with wolf urine, as compared with black bear urine, gasoline, Cologne, and water at a feeding station (Chamaillé-Jammes et al., 2014). One of the most widely known and studied predator odors is TMT (trimethylthiazoline), which was identified in fox feces almost 40 years ago (Vernet-Maury et al., 1977; Vernet-Maury, 1980). Although some authors have expressed doubt about the kairomonal properties of TMT (McGregor et al., 2002; Fortes-Marco et al., 2013), Fendt and Endres (2008) found clear fear-evoking effects of this molecule. Indeed, mice cannot be conditioned to associate TMT with a (positive) reward as the innate avoidance response is too strong (Kobayakawa et al., 2007). Recently, another molecule (2-phenylethylamine) has been shown to trigger hard-wired aversion circuits in the rodent brain and to provoke danger-associated behavioral responses in mice and rats (Ferrero et al., 2011).

However, it is sometimes difficult to ascertain if an observed olfactory response is truly innate. Cichlids exposed to conspecific alarm cues paired with predator odor while embryos (*in ovo*) showed antipredator behavior post-hatch when exposed to predator odor alone (Nelson et al., 2013). This conditioned response happened at an early stage of development, and could easily be confused with an innate reaction to the smell of predators.

Voluntary behavioral responses, that are not innate, are learned. Animals, often dogs, can be trained to detect certain odorants. These compounds can be of non-biological origin, such as explosives and certain drugs, but sometimes the odors are found in nature, and may even arise from different species. However, it is important to distinguish between human-instigated odor-based learning and allelochemical effects. Rats and dogs have been trained to detect estrus odors in cattle (Kiddy et al., 1978; Ladewig and Hart, 1981; Hawk et al., 1984), and to detect the smell of diseases, cadavers, and even bumblebee nests (e.g., Waters et al., 2011; Bijland et al., 2013). These are not allelochemical effects. Nevertheless, many allelochemical effects *are* a direct result of the animal learning to associate a given odor with the likelihood of an event, such as the presence

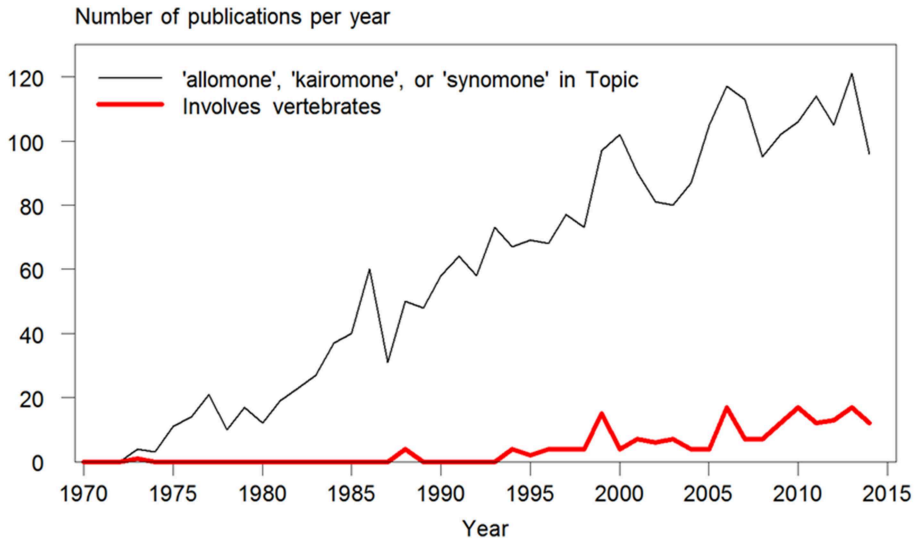


FIGURE 1 | Number of publications per year containing the terms allomone, kairomone, or synomone in their topic. The narrow, black line shows their use overall since their coinage in the 1970's ($N = 2635$ publications) found in a search made in January 2015 in Topic (which includes words in title, keywords, and abstract) in Web of Science™ (ver. 5.16.1; Thomson Reuters © 2015). The bold, red line shows the number of publications among these, which includes vertebrate species ($N = 184$). Of these, 98 were concerned with kairomones emitted by vertebrates (mainly humans, as well as dogs, ruminants, hedgehogs, poultry, snakes, and penguins) attracting biting or stinging insects (mainly mosquitoes, as well as midges, mites, bed bugs, ticks, tsetse flies, and wasps). Only 32 publications concerning odor-based behavioral responses in vertebrate species were found when searching on any of these three terms.

of a predator or a potential sexual encounter. In addition, as was argued with pheromones, seemingly innate responses may improve with training, such as the sexual response of male rats to estrus odors (Nielsen et al., 2013).

Many examples can be found of learned interspecific odor-based responses in vertebrates. Gazdewich and Chivers (2002) found that minnows learned to recognize the odor of a predator fish when it was coupled with a minnow alarm cue, and that this conditioned response improved minnow survival. Based on chemical cues some lizard species are able to discriminate among similar predators that pose different levels of threat (Lloyd et al., 2009). Even humans, whose olfactory capacities are more modest, are able to recognize the smell of their own dog without indicating bias in terms of odor strength or pleasantness (Wells and Hepper, 2000); an ability the authors describe as "*acquired without conscious effort.*"

In conclusion, the use of terms like allomone and kairomone does not make it clear whether a reported allelochemical response is innate or not. Instead, it adds a layer of confusion, as some use the terms to imply innateness, whereas others apply the terms more broadly. From the examples given here it is apparent that although some interspecific odor-based behavioral responses are innate, they may still change with experience. An odor which is initially neutral to a receiver can become an allomone or kairomone through associative learning. Also, a kairomone can become an allomone, as shown in the intriguing example of Toxoplasmosis-infected rats, where the response to cat odors change from antipredator avoidance behavior to attraction and sexual behavior through altered neural activity in limbic brain areas caused by the parasite (House et al., 2011).

Origin of the Odor

In most uses of allelochemic terminology, the odor in question is produced by one of the species involved—often for the specific purpose of allelochemics. In one of the original descriptions of interspecific chemical messengers, the notion that an allomone can be acquired by the emitter refers to the situation where the odor is appropriated (intact) from the food (Brown et al., 1970), and thus is still originating from within the emitter, albeit not produced *de novo*. An example of this is given by Cox et al. (2010), who found that goats and kangaroos showed stronger aversion when they were exposed to the odor from a tiger fed goat or kangaroo, respectively, compared with all other predator–diet combinations. Similarly, salmon display antipredator behavior when exposed to water scented with cues from an otter, but only if the otter has been fed on a salmon diet (Roberts and de Leaniz, 2011). Wyatt (2010) raises a similar issue with pheromones, which he also suggests could be collected from the surroundings, and show individual variations in compound proportions and odor strength.

The role of the microbiota in the production of animal odors also needs to be mentioned. Microbial by-products may contribute a large part of odorants emitted by the animal. Wyatt (2010) distinguishes between pheromones and signature mixture, the latter being an individual's distinctive mix of odorous molecules (odorants), which conspecifics can learn and use for individual recognition. For example, the Indian mongoose uses anal pocket bacteria metabolites as an odorant signature (Ezenwa and Williams, 2014), and we would assign this smell as originating from the individual mongoose. In the case of microbiota by-products arising from an animal cadaver, the

determination of the origin of the odor may be trickier. This is the case in zebrafish displaying a pronounced innate behavioral aversion to cadaverine and putrescine, two diamines emanating from decaying flesh (Hussain et al., 2013). Do those diamines still belong to the semiochemical category as originating from a—now dead—animal? On this basis, Dicke and Sabelis suggested already in 1988 to eliminate the origin criterion from the terminology, and to use the cost-benefit criterion as the sole determinant of infochemical subdivisions.

The message received from an odor emanating from an animal may differ dependent on where the odor is produced. Skin and fur-derived predator odors appear more efficient in evoking fear responses in prey than those derived from urine or feces (Apfelbach et al., 2005; Fendt and Endres, 2008), perhaps because the latter indicates "*a predator was here*" whereas the former could mean "*a predator is here.*" Odors derived from cat collars and cat fur can induce long lasting (up to 4 days) effects in rats following a single exposure (May et al., 2012). Incidentally, male mice can exhibit aggression in response to their own urine when it is presented on the body of a castrated mouse (Stowers and Marton, 2005). This also illustrates how the perception of an odor is highly influenced by the context, as male mice do not usually react aggressively to their own urine patches.

Mammals have also been found to anoint themselves with odors of external origin. This is commonly seen when dogs roll themselves in pungent substances, but the function of this self-scenting is debated. Hyenas show a preference for rolling in animal-based odors, and receive more attention from conspecifics when smelling of carrion (Drea et al., 2002). Ryon et al. (1986) found that wolves preferred to rub themselves in strong-smelling, manufactured odors (perfume and motor oil) above carnivore odors, and that sheep and horse feces did not elicit rubbing in wolves. Among the causal explanations for these observed behaviors is status advertisement, where dominant animals seek to stand out by adding complexity to and increasing the range of their odorous presence (Gosling and McKay, 1990). Some squirrel species anoint themselves with the scent of snakes, by chewing shed snake-skin and subsequently lick their own fur (Clucas et al., 2008). The authors suggest that this is an anti-predatory behavior, and fossil data suggest that such predator scent application in squirrels is ancient in origin (Clucas et al., 2010). Please note that this example would not be included as an allomone in the original definition of the term, as the scent did not originate from within the emitter.

Sometimes the same chemical compounds are found to act as semiochemicals for more than one species, the most famous example being a female sex pheromone shared by the Asian elephant and several species of moth (Rasmussen et al., 1996). The use of the same molecules by different species and even phyla may reflect chemical constraints relating to stability, volatility, or toxicity (Wyatt, 2010). Predator odors can also be hijacked by prey on an evolutionary scale (Papes et al., 2010), and a mouse alarm cue (2-*sec*-butyl-4,5-dihydrothiazole; Novotny et al., 1985) has been found to be similar in structure to sulfur-containing scents from certain predators, such as stoat, fox, and bobcat (Brechbühl et al., 2013). Some animals, such as certain fish species, are able to generalize predator odor recognition across predators within the same family (Ferrari et al., 2007). Rodents are able to detect estrus odors without prior training in a number of non-rodent species (horse and fox: Rampin et al., 2006; cattle: Rameshkumar et al., 2008), indicating a commonality in the estrus odor bouquet of mammals. A molecule (sulcatone) found to be associated with estrus in mammals (Nielsen et al., 2013) is also a human-derived mosquito repellent (Logan et al., 2009, 2010). Thus, the same odor may originate from different sources, some of which are of no biological relevance to the receiver of the odor.

In conclusion, the origins of odors used in a semiochemical context are quite diverse. They may be synthesized by the emitter, but may also arise from microbial by-products, from food items, or from the surroundings. Odors derived from different body areas of an animal may carry different meanings to the receiver, the same odorant can originate from different species, and an odor may be perceived to be of a different origin than is the case. We therefore agree with Dicke and Sabelis (1988) that an odor's origin should not influence whether it can be defined as an allelochemical.

Intent of the Odorous Message

The use of terms like signal, messenger, and communication in the descriptions of allelochemical functions gives an inherent meaning of intent to the odor involved in the interaction. In evolutionary terms, a signal (or sign-stimuli) implies that the function of the stimuli—in this case an emitted odor—has been favored during natural selection to evoke specific behavioral responses in the receiving organism. Indeed, Whittaker and Feeny (1971) saw the evolution of allelochemic agents as a balance between metabolic cost and natural selection. In the definition of allelochemicals, the effect achieved by the emitter (for allomones) and receiver (for kairomones) has to be adaptive (**Table 1**; Dicke and Sabelis, 1988), in order for these effects to have been sustained through the evolutionary development of the species in question. In the case of allomones, this could indicate that an animal emitting an allomone to attract or deter another species do so for this purpose. However, this would imply not only that the odor is being actively released, but also synthesized by the emitter, which we questioned in the previous section. It seems quite unlikely according to the current view of evolutionary processes. For example, specific peptides secreted by a West-African frog species allow it to live in the underground nests of certain ants that otherwise attack and sting intruders (Roedel et al., 2013). These frogs do not secrete such specific substances as a result of contact with the ants or due to particular food items; the skin secretion is not deliberate, but simply the consequence of a serendipitous mutation allowing this frog species to live in a dry environment and protected from intruders by the ants (Roedel et al., 2013). Thus, a more appropriate way to view signaling is proposed by Scarantino (2010), who talks instead about natural information, i.e., information that is grounded in reliable correlations between types of events, such as smoke signaling fire. He encourages us to think of animal signals in a similar manner as "*carriers of natural information,*" as opposed to information being encoded in animal signals.

Viewing interspecific odors in this way also makes it less important from where the odor originates. The snake-odor anointed squirrels mentioned earlier are simply using an odor which carries natural information ("*I am a snake*") to squirrel predators. This may also explain commonalities across species in odor preferences. Mandairon et al. (2009) found that odors rated pleasant by humans were also attractive to mice, suggesting that odor preference may be partially predetermined, based on the physicochemical structure of the odorants (Khan et al., 2007; Secundo et al., 2014). Similarities in odors serving the same purpose could also be included, such as the finding that the odor of male goats (bucks) can be used in the same way as that of male sheep (rams) to promote reproductive receptiveness of ewes (Rosa and Bryant, 2002). In stressed mice, the appeasing effects arising from the smell of conspecifics can be achieved also by exposure to odors from species that are evolutionary close (the Rodentia subclass; Cherng et al., 2012).

Odors as carriers of natural information also make it easier to reconcile divergent messages of the same odor into the concept of allelochemics. The smell of trimethylamine, an odor associated by humans with bad breath and spoiled food, is aversive to rats but attractive to mice (Li et al., 2013). As mouse urine contains high concentrations of trimethylamine, the authors conclude that this may be part of aversive allomones released by mice for defensive behavior against predators, such as rats. On the other hand, not all interspecific odor-based effects can be explained accordingly. The increased play-like behavior seen in cats when exposed to the smell of catnip (Ellis and Wells, 2010) remains enigmatic. The response is genetically determined, but may not develop fully until 3 months of age, and catnip often produces a distinct avoidance response in young kittens (Todd, 1962).

In conclusion, by viewing odors giving rise to interspecific effects as carriers of natural information (Scarantino, 2010), it allows us to include a broader spectrum of olfactory effects in allelochemics. This does not preclude the adaptiveness of the odor-based response, but moves the emphasis from the synthesis of the odor to the information it contains.

A Novel Conceptual Framework for the Study of Interspecific Odor-based Effects

Given the criticisms raised above concerning the usefulness of the terms allomone, kairomone, and synomone when studying vertebrates, could we find another, less constraining way to look at behavioral responses to interspecific odors? Instead of replacing the terms used in allelochemic interactions, we suggest to take a step back and view the concept from a different perspective. Below we propose a simple, yet novel conceptual framework for use when studying interspecific odor-based effects in vertebrates. It is based on two parameters, which are already widely used and referred to in the literature concerning allelochemics: one being the valence of the odor to the recipient and the other the learning involved for the odor-based response to occur.

Odor Valence and Learning

Valence is a useful way to structure the behavioral response to odors (Root et al., 2014), as fast processing of an odor's meaning is important for survival and reproduction (Knaden and Hansson, 2014). Odors with a positive valence are attractive to the recipient, whereas odors with a negative valence are aversive; we thus assign valence to an odor based on the behavioral response observed when exposed to said odor. It is important to bear in mind that valence is not necessarily a fixed quality of the odor, but the motivational significance that a given odor carries to a recipient animal. This may vary between individuals of the same species dependent on sex, physiological state, previous experience, and degree of learning. Nevertheless, when using current animal models, existing behavioral tests can be used to measure relative valence. Understanding the development of aversion and attraction to odors and the mechanisms behind divergent olfactory responses has been highlighted as a model for deciphering sensory systems (Li and Liberles, 2015). Also, Secundo et al. (2014) found the primary dimension of olfactory perception in humans relates to odorant pleasantness on an axis ranging from very unpleasant to very pleasant. Although positive and negative valences are not processed by specific olfactory subsystems in mice, the dorsal olfactory bulb appear to govern avoidance behavior (Kobayakawa et al., 2007; Knaden and Hansson, 2014). A specific valence may still give rise to a specific neuronal pattern, such as the split representation of attractive and repellent odorants found in the antennal lobe of Drosophila (Ai et al., 2010; Knaden et al., 2012; Min et al., 2013). This has also been found in the olfactory tubercle of mice (Gadziola et al., 2015) and rats (Rampin et al., 2012). In the ventral striatum of the rodent brain, encoding of an odor is not fixed, but depends on odor valence; i.e., neurons in this brain region acquire a selective activation to odor exposure through associative learning, and the valence can be reversed for the same odor by the same method (Setlow et al., 2003; Gadziola et al., 2015). In addition, the amygdala has been found to play an essential part in the encoding of stimuli with affective value (Schoenbaum et al., 1999; Armony, 2013; Janak and Tye, 2015). In rodents some amygdala neurons respond to the odor of a predator (Dielenberg and McGregor, 2001; Govic and Paolini, 2015; Pérez-Gómez et al., 2015); and lesioning or inactivation of some amygdala subnuclei can eradicate the fear inducing effect of predator odor (reviewed in Takahashi, 2014). Laterality between stimuli of different valence, with positive stimuli encoded in the left and negative in the right amygdala has also been demonstrated (Young and Williams, 2010, 2013).

As discussed earlier, it is not always clear if an odor-based response is innate when allelochemics are discussed in the literature. It is, however, important for the interpretation of odor-based responses to know the extent to which it is dependent on prior experience and learning, and to take into account the plasticity of seemingly innate responses with time. This has also been highlighted by the search for specific brain regions associated with these two types of responses. For aversive odors, Kobayakawa et al. (2007) found separate sets of glomeruli in the olfactory bulb being dedicated to innate and learned responses, respectively. As was the case for valence, the amygdala is also important for both innate and learned behavioral responses to odors. The posterolateral, medial and cortical amygdala play a critical role in innate odor-driven behaviors (Martinez et al., 2011; Sosulski et al., 2011; Root et al., 2014). This was illustratively

shown in the work by Blanchard and Blanchard (1972), as one of their rats with amygdaloid damage "*climbed onto the [sedated] cat's back and head, and began to nibble on the cat's ear...the cat seized and briefly shook the rat…After the cat released this rat, the rat climbed back onto the cat.*" In contrast, the basolateral amygdala appears to be involved in learned, but not innate fear responses (Ribeiro et al., 2011; Sparta et al., 2014). Also, epigenetic changes in the medial amygdala are behind the change in valence of cat odors mentioned earlier for Toxoplamosis infected rats (Hari Dass and Vyas, 2014). This may be similar to the finding that activation of the lateral amygdala caused by conditioned fear can be suppressed by external events, such as the social buffering caused by the presence of conspecifics (Kiyokawa et al., 2012; Fuzzo et al., 2015).

In conclusion, innateness of the behavioral response to certain odors as well as the learning associated with odor experience are linked to odor valence, both at the processing level and in terms of how an odor obtains a given valence. They are thus important factors involved in many odor-based responses, and therefore lend themselves as obvious candidates for constructing an alternative or complementary conceptual framework to the existing view of allelochemics with which to model interspecific odor-based effects.

The Model

The simplest way of looking at valence and learning is to view them as two binomial parameters: if an odor has a valence (to a recipient) it is either positive (attractive) or negative (aversive), and a given odor response can be either innate or learned. These combine into four groupings, as illustrated in **Figure 2A**. This is, however, a fairly rough division as odors can be more or less aversive, and odor-based behavioral responses may change as a result of learning, even when the initial response is innate, as discussed above. We have therefore, in **Figure 2A**, superimposed the four groupings onto a continual representation of learning and valence, expressing *odor valence* (y-axis) as a function of *learning* (x-axis), where positive and negative parts of the y-axis indicate degree of attraction and aversion, respectively. As described in the previous section, the valence of an odor to an animal is determined by studying the behavioral response of the animal when exposed to said odor. If aversion or attraction is observed at first exposure to the odor, the behavioral response is innate. If no behavioral response is seen upon first exposure, the valence of the odor cannot be determined or is zero (neutral odor). In principle, for a given animal we can place any given odor along the y-axis dependent on the response of the animal at first exposure. However, as soon as the animal has experienced an odor, some degree of learning is taking place, which may or may not change the odor's valence. Thus, valence is a function of learning, where innateness/no learning is at $x = 0$ (**Figure 2A**); in other words odors, which do not elicit an innate behavioral response, have no valence, i.e., are neutral to the animal and thus placed at the origin. It is important to emphasize that innateness refer to the behavioral response, and not the valence (which is a consequence of the former).

The proposed conceptual framework thus consists of the two axes representing odor valence and learning, without necessarily ranking different odors *a priori* or specifying the type of learning involved. It is important to bear in mind that this is just a model, which is a way to simplify reality, in the same way that two

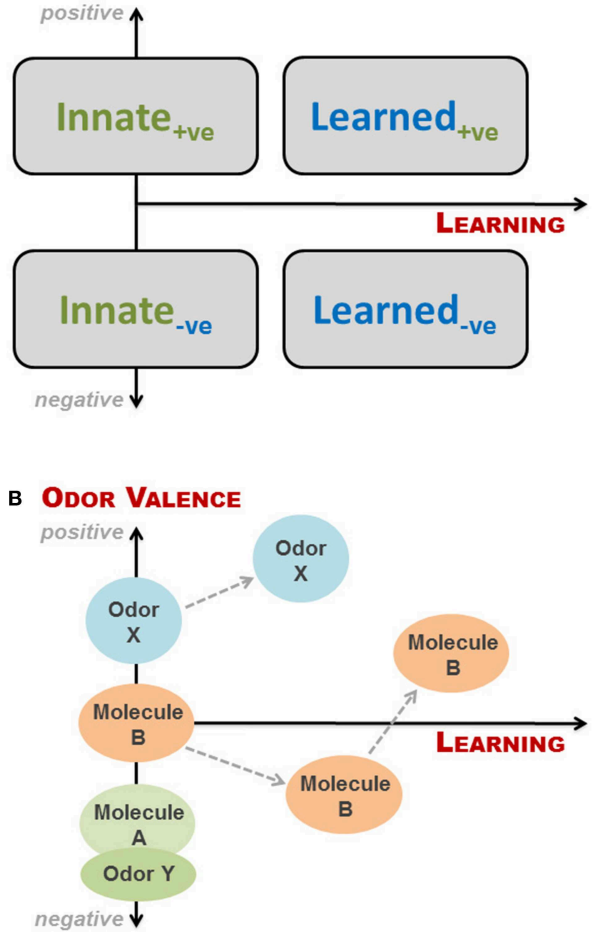


FIGURE 2 | Conceptual framework to describe interspecific olfactory effects for a given animal at a given time. (A) The simplest categorization of odor-based behavioral responses is a binomial split into either innate or learned responses. Likewise, odor valence can be positive (+ve; attractive odors) or negative (–ve; aversive odors). These combine into four groupings, as depicted here by rectangles. However, odors can be more or less aversive, and odor-based behavioral responses may change with learning, even when the initial response is innate. We have therefore superimposed the four groupings onto a continual representation of learning and valence, expressing *odor valence* (y-axis) as a function of *learning* (x-axis), where positive and negative parts of the y-axis indicate degree of attraction and aversion, respectively. It is important to note that odors, which do not elicit an innate behavioral response, have no valence, i.e., are neutral to the animal and thus placed at the origin; Innate responses require no learning, but may be hard to determine and to differentiate from those arising from odors experienced in *utero* via amniotic fluid; **(B)** Examples of how different odors may be categorized relative to each other within the conceptual framework, and how odor valence may change with learning. Odor Y could be the natural smell of a predator, whereas molecule A is TMT or 2-phenylethylamine. Both evoke innate responses, but the natural odor is more fear-inducing. Molecule B is an initially neutral odor, which the animal first learns to find aversive, and subsequently associate with a positive experience. Finally, odor X may be the smell of estrus to a male before and after he gains sexual experience.

Lego® bricks can represent an airplane. Although we may place odors associated with sex or food as being positive, and predators as negative, the relative ranking of odors is highly reliant on context. During rutting season, male ruminants may go for days without eating in search of a female in estrus (Whittle et al., 2000), and her smell is thus likely to be more attractive than food at this point in time; as soon as the breeding season has ended, the relative ranking of food odors will rise again. In a similar way, the smell of a conspecific may be associated with family or an intruder, respectively, depending on the situation. Novel food odors may be attractive to omnivorous species, such as pigs, but initially aversive to more neophobic animals, such as rats. Also, when dealing with complex odors of natural origin, different components of the smell may give rise to quite different responses, as seen when male rats were exposed to feces from vixens in estrus: Rampin et al. (2006) found the behavior of the rats shifting between freezing and penile erections, thus responding to the scent of a predator and a female in estrus, respectively.

Innate behavioral responses require no learning, but they may be hard to determine and to differentiate from those arising from odors experienced in *utero* via amniotic fluid. An example of this, although not interspecific, is the suckling response of newborn mice when presented with a nipple for the first time. Logan et al. (2012) elegantly showed that this response, which appeared innate, was elicited by the presence of signature odors—found in the amniotic fluid—that are learned and recognized prior to first suckling. Learned responses based on imprinting have only a short time window in which to be acquired; these are thus more likely to be intraspecific. The association of odors with positive or negative events has been amply illustrated in the literature, either via classical (e.g., Kvitvik et al., 2010) or operant conditioning (e.g., Rokni et al., 2014), whereas more complex learning paradigms include discrimination of several odors or odorant mixtures, as well as latent and insight learning. In **Figure 2B** are shown examples of how the valence of an odor may change with learning and how one odor may be categorized relative to another within the conceptual framework.

The strength of this model is in its simplicity, yet it still allows us to display the complexity of dynamic odor relationships. It is not meant to replace the terms allomone, kairomone, and synomone, but to offer an alternative viewpoint from which to investigate issues relating to allelochemics, just as the ABO and Rh blood group systems describe different aspects of an individual's blood type. A model can be used not only as a proxy to unravel complex interactions, but also as a media for organizing knowledge integration, as well as a playground for testing assumptions (Martin, 2015). We can envisage the model being expanded to contain additional variables, such as time, odor complexity or odor concentration, depending on the testing paradigm. Indeed, the concepts of who benefits from the interaction could be introduced along the z-axis. The model may be used to construct a diagram of different parts of the brain involved in innate and learned odor-based responses for odors of positive and negative valence, respectively. Finally, it need not be constrained to interspecific odor-based effects, but may help visualize the learning involved in the sculpting of some innate pheromonal responses (Stowers and Marton, 2005; Wyatt, 2010), as well as other odor-based responses.

Conclusion

We have demonstrated the constraining properties and the limits to the usefulness of the terms allomone, kairomone, and synomone when studying interspecific odor-based effects in vertebrates. We do not propose to replace the terms used in allelochemic interactions, but instead to view the concept from a different perspective. We present a simple, yet novel conceptual framework based on two parameters: valence of the odor to the recipient and the learning involved in the observed behavioral response to the odor. This model provides a unifying framework for use when studying interspecific odor-based effects, particularly in vertebrates.

Acknowledgments

This work was supported by WHELP (Welfare–Health in Early Life Programme) as part of the metaprogramme GISA [Gestion Intégrée de la Santé des Animaux (Integrated management of animal health)] funded by INRA, France.

References

Ai, M., Min, S., Grosjean, Y., Leblanc, C., Bell, R., Benton, R., et al. (2010). Acid sensing by the Drosophila olfactory system. *Nature* 468, 691–695. doi: 10.1038/nature09537

Apfelbach, R., Blanchard, C. D., Blanchard, R. J., Hayes, R. A., and McGregor, I. S. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neurosci. Biobehav. Rev.* 29, 1123–1144. doi: 10.1016/j.neubiorev.2005.05.005

Armony, J. L. (2013). Current emotion research in behavioral neuroscience: the role(s) of the amygdala. *Emot. Rev.* 5, 104–115. doi: 10.1177/1754073912457208

Bethe, A. (1932). Vernachlässigte hormone [Neglected hormones]. *Naturwissenschaften* 20, 177–181. doi: 10.1007/BF01504737

Bijland, L. R., Bomers, M. K., and Smulders, Y. M. (2013). Smelling the diagnosis: a review on the use of scent in diagnosing disease. *Neth. J. Med.* 71, 300–307.

Blanchard, D. C., and Blanchard, R. J. (1972). Innate and conditioned reactions to threat in rats with amygdaloid lesions. *J. Comp. Physiol. Psychol.* 81, 281–290. doi: 10.1037/h0033521

Blum, M. S. (1974). Deciphering the communicative Rosetta stone. *Bull. Entomol. Soc. Am.* 20, 30–35. doi: 10.1093/besa/20.1.30

Brechbühl, J., Moine, F., Klaey, M., Nenniger-Tosato, M., Hurni, N., Sporkert, F., et al. (2013). Mouse alarm pheromone shares structural similarity with predator scents. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4762–4767. doi: 10.1073/pnas.1214249110

Brown, W. L. Jr., Eisner, T., and Whittaker, R. H. (1970). Allomones and kairomones: transspecific chemical messengers. *Bioscience* 20, 21. doi: 10.2307/1294753

Chamaillé-Jammes, S., Malcuit, H., Le Saout, S., and Martin, J.-L. (2014). Innate threat-sensitive foraging: black-tailed deer remain more fearful of wolf than of the less dangerous black bear even after 100 years of wolf absence. *Oecologia* 174, 1151–1158. doi: 10.1007/s00442-013-2843-0

Cherng, C. F., Chang, C. P., Su, C. C., Tzeng, W. Y., Chuang, J. Y., Chen, L. H., et al. (2012). Odors from proximal species reverse the stress-decreased neurogenesis via main olfactory processing. *Behav. Brain Res.* 229, 106–112. doi: 10.1016/j.bbr.2011.12.020

Clark, R. W. (2004). Timber rattlesnakes (*Crotalus horridus*) use chemical cues to select ambush sites. *J. Chem. Ecol.* 30, 607–617. doi: 10.1023/B:JOEC.0000018632.27010.1e

Clucas, B., Ord, T. J., and Owings, D. H. (2010). Fossils and phylogeny uncover the evolutionary history of a unique antipredator behaviour. *J. Evol. Biol.* 23, 2197–2211. doi: 10.1111/j.1420-9101.2010.02083.x

Clucas, B., Rowe, M. P., Owings, D. H., and Arrowood, P. C. (2008). Snake scent application in ground squirrels, *Spermophilus spp.*: a novel form of antipredator behaviour? *Anim. Behav.* 75, 299–307. doi: 10.1016/j.anbehav.2007.05.024

Cox, T. E., Murray, P. J., Hall, G. P., and Li, X. (2010). Pest responses to odors from predators fed a diet of target species conspecifics and heterospecifics. *J. Wildl. Manage.* 74, 1737–1744. doi: 10.2193/2009-410

Dicke, M., and Sabelis, M. W. (1988). Infochemical terminology: based on cost-benefit analysis rather than origin of compounds? *Funct. Ecol.* 2, 131–139. doi: 10.2307/2389687

Dielenberg, R. A., and McGregor, I. S. (2001). Defensive behaviour in rats towards predatory odors: a review. *Neurosci. Biobehav. Rev.* 25, 597–609. doi: 10.1016/S0149-7634(01)00044-6

Drea, C. M., Vignieri, S. N., Cunningham, S. B., and Glickman, S. E. (2002). Responses to olfactory stimuli in spotted hyenas (*Crocuta crocuta*): I. Investigation of environmental odors and the function of rolling. *J. Comp. Psychol.* 116, 331–341. doi: 10.1037/0735-7036.116.4.331

Ellis, S. L. H., and Wells, D. L. (2010). The influence of olfactory stimulation on the behaviour of cats housed in a rescue shelter. *Appl. Anim. Behav. Sci.* 123, 56–62. doi: 10.1016/j.applanim.2009.12.011

Ezenwa, V. O., and Williams, A. E. (2014). Microbes and animal olfactory communication: where do we go from here? *Bioessays* 36, 847–854. doi: 10.1002/bies.201400016

Fendt, M., and Endres, T. (2008). 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox odor – just repugnant or really fear-inducing? *Neurosci. Biobehav. Rev.* 32, 1259–1266. doi: 10.1016/j.neubiorev.2008.05.010

Ferrari, M. C., Gonzalo, A., Messier, F., and Chivers, D. P. (2007). Generalization of learned predator recognition: an experimental test and framework for future studies. *Proc. Biol. Sci.* 274, 1853–1859. doi: 10.1098/rspb.2007.0297

Ferrero, D. M., Lemon, J. K., Fluegge, D., Pashkovski, S. L., Korzan, W. J., Datta, S. R., et al. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11235–11240. doi: 10.1073/pnas.1103317108

Fortes-Marco, L., Lanuza, E., and Martinez-Garcia, F. (2013). Of pheromones and kairomones: what receptors mediate innate emotional responses? *Anat. Rec.* 296, 1346–1363. doi: 10.1002/ar.22745

Fuzzo, F., Matsumoto, J., Kiyokawa, Y., Takeuchi, Y., Ono, T., and Nishijo, H. (2015). Social buffering suppresses fear-associated activation of the lateral amygdala in male rats: behavioral and neurophysiological evidence. *Front. Neurosci.* 9:99. doi: 10.3389/fnins.2015.00099

Gadziola, M. A., Tylicki, K. A., Christian, D. L., and Wesson, D. W. (2015). The olfactory tubercle encodes odor valence in behaving mice. *J. Neurosci.* 35, 4515–4527. doi: 10.1523/JNEUROSCI.4750-14.2015

Gazdewich, K. J., and Chivers, D. P. (2002). Acquired predator recognition by fathead minnows: influence of habitat characteristics on survival. *J. Chem. Ecol.* 28, 439–445. doi: 10.1023/A:1017902712355

Gosling, L. M., and McKay, H. V. (1990). Scent-rubbing and status signalling by male mammals. *Chemoecology* 1, 92–95. doi: 10.1007/BF01241649

Govic, A., and Paolini, A. G. (2015). *In vivo* electrophysiological recordings in amygdala subnuclei reveal selective and distinct responses to a behaviorally identified predator odor. *J. Neurophysiol.* 113, 1423–1436. doi: 10.1152/jn.00373.2014

Hari Dass, S. A., and Vyas, A. (2014). *Toxoplasma gondii* infection reduces predator aversion in rats through epigenetic modulation in the host medial amygdala. *Mol. Ecol.* 23, 6114–6122. doi: 10.1111/mec.12888

Hawk, H. W., Conley, H. H., and Kiddy, C. A. (1984). Estrus-related odors in milk detected by trained dogs. *J. Dairy Sci.* 67, 392–397. doi: 10.3168/jds.S0022-0302(84)81314-4

House, P. K., Vyas, A., and Sapolsky, R. (2011). Predator cat odors activate sexual arousal pathways in brains of *Toxoplasma gondii* infected rats. *PLoS ONE* 6:e23277. doi: 10.1371/journal.pone.0023277

Hussain, A., Saraiva, L. R., Ferrero, D. M., Ahuja, G., Krishna, V. S., Liberles, S. D., et al. (2013). High-affinity olfactory receptor for the death-associated odor cadaverine. *Proc. Natl. Acad. Sci. U.S.A.* 110, 19579–19584. doi: 10.1073/pnas.1318596110

Janak, P. H., and Tye, K. M. (2015). From circuits to behaviour in the amygdala. *Nature* 517, 284–292. doi: 10.1038/nature14188

Karlson, P., and Lüscher, M. (1959a). 'Pheromones': a new term for a class of biologically active substances. *Nature* 183, 55–56. doi: 10.1038/183055a0

Karlson, P., and Lüscher, M. (1959b). The proposed biological term 'pheromone'. *Nature* 183, 1835. doi: 10.1038/1831835b0

Keller-Costa, T., Canário, A. V. M., and Hubbard, P. C. (2015). Chemical communication in cichlids: a mini-review. *Gen. Comp. Endocrinol.* doi: 10.1016/j.ygcen.2015.01.001. [Epub ahead of print].

Khan, R. M., Luk, C.-H., Flinker, A., Aggarwal, A., Lapid, H., Haddad, R., et al. (2007). Predicting odor pleasantness from odorant structure: pleasantness as a reflection of the physical world. *J. Neurosci.* 27, 10015–10023. doi: 10.1523/JNEUROSCI.1158-07.2007

Kiddy, C. A., Mitchell, D. S., Bolt, D. J., and Hawk, H. W. (1978). Detection of estrus-related odors in cows by trained dogs. *Biol. Reprod.* 19, 389–395. doi: 10.1095/biolreprod19.2.389

Kiyokawa, Y., Wakabayashi, Y., Takeuchi, Y., and Mori, Y. (2012). The neural pathway underlying social buffering of conditioned fear responses in male rats. *Eur. J. Neurosci.* 36, 3429–3437. doi: 10.1111/j.1460-9568.2012.08257.x

Knaden, M., and Hansson, B. S. (2014). Mapping odor valence in the brain of flies and mice. *Curr. Opin. Neurobiol.* 24, 34–38. doi: 10.1016/j.conb.2013.08.010

Knaden, M., Strutz, A., Ahsan, J., Sachse, S., and Hansson, B. S. (2012). Spatial representation of odorant valence in an insect brain. *Cell Rep.* 1, 392–399. doi: 10.1016/j.celrep.2012.03.002

Kobayakawa, K., Kobayakawa, R., Matsumoto, H., Oka, Y., Imai, T., Ikawa, M., et al. (2007). Innate versus learned odour processing in the mouse olfactory bulb. *Nature* 450, 503–508. doi: 10.1038/nature06281

Kvitvik, I.-L., Berg, K. M., and Ågmo, A. (2010). A neutral odor may become a sexual incentive through classical conditioning in male rats. *Learn. Motiv.* 4, 11–21. doi: 10.1016/j.lmot.2009.06.003

Ladewig, J., and Hart, B. L. (1981). Demonstration of estrus-related odors in cow urine by operant conditioning of rats. *Biol. Reprod.* 24, 1165–1169.

Law, J. H., and Regnier, F. E. (1971). Pheromones. *Annu. Rev. Biochem.* 40, 533–548. doi: 10.1146/annurev.bi.40.070171.002533

Li, Q., Korzan, W. J., Ferrero, D. M., Chang, R. B., Roy, D. S., Buchi, M., et al. (2013). Synchronous evolution of an odor biosynthesis pathway and behavioral response. *Curr. Biol.* 23, 11–20. doi: 10.1016/j.cub.2012.10.047

Li, Q., and Liberles, S. D. (2015). Aversion and attraction through olfaction. *Curr. Biol.* 25, R120–R129. doi: 10.1016/j.cub.2014.11.044

Lloyd, R., Alford, R. A., and Schwarzkopf, L. (2009). Chemical discrimination among predators by lizards: responses of three skink species to the odours of high- and low-threat varanid predators. *Aust. Ecol.* 34, 50–54. doi: 10.1111/j.1442-9993.2008.01881.x

Logan, D. W., Brunet, L. J., Webb, W. R., Cutforth, T., Ngai, J., and Stowers, L. (2012). Learned recognition of maternal signature odors mediates the first suckling episode in mice. *Curr. Biol.* 22, 1–10. doi: 10.1016/j.cub.2012.08.041

Logan, J. G., Seal, N. J., Cook, J. I., Stanczyk, N. M., Birkett, M. A., Clark, S. J., et al. (2009). Identification of human-derived volatile chemicals that interfere with attraction of the Scottish biting midge and their potential use as repellents. *J. Med. Entomol.* 46, 208–219. doi: 10.1603/033.046.0205

Logan, J. G., Stanczyk, N. M., Hassanali, A., Kemei, J., Santana, A. E. G., Ribeiro, K. A. L., et al. (2010). Arm-in-cage testing of natural human-derived mosquito repellents. *Malaria J.* 9:239. doi: 10.1186/1475-2875-9-239

Mandairon, N., Poncelet, J., Bensafi, M., and Didier, A. (2009). Humans and mice express similar olfactory preferences. *PLoS ONE* 4:e4209. doi: 10.1371/journal.pone.0004209

Martin, O. (2015). "Un exemple de modèle systémique pour structurer et interroger nos connaissances [An example of a systemic model to structure and query our knowledge]," *Paper presented at Conseil scientifique, Comment s'affranchir de l'expérimentation animale tout en publiant plus et mieux* (Jouy-en-Josas). (Accessed March 26, 2015).

Martinez, R. C., Carvalho-Netto, E. F., Ribeiro-Barbosa, E. R., Baldo, M. V. C., and Canteras, N. S. (2011). Amygdalar roles during exposure to a live predator and to a predator-associated context. *Neuroscience* 172, 314–328. doi: 10.1016/j.neuroscience.2010.10.033

May, M. D., Bowen, M. T., McGregor, I. S., and Timberlake, W. (2012). Rubbings deposited by cats elicit defensive behavior in rats. *Physiol. Behav.* 107, 711–718. doi: 10.1016/j.physbeh.2012.03.007

McGregor, I. S., Schrama, L., Ambermoon, P., and Dielenberg, R. A. (2002). Not all 'predator odours' are equal: cat odour but not 2,4,5-trimethylthiazoline (TMT; fox odour) elicits specific defensive behaviours in rats. *Behav. Brain Res.* 129, 1–16. doi: 10.1016/S0166-4328(01)00324-2

Min, S., Ai, M., Shin, S. A., and Suh, G. S. B. (2013). Dedicated olfactory neurons mediating attraction behavior to ammonia and amines in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 110, E1321–E1329. doi: 10.1073/pnas.1215680110

Nelson, A. B., Alemadi, S. D., and Wisenden, B. D. (2013). Learned recognition of novel predator odour by convict cichlid embryos. *Behav. Ecol. Sociobiol.* 67, 1269–1273. doi: 10.1007/s00265-013-1554-1

Nielsen, B. L., Jerôme, N., Saint-Albin, A., Rampin, O., and Maurin, Y. (2013). Behavioural response of sexually naïve and experienced male rats to the smell of 6-methyl-5-hepten-2-one and female rat faeces. *Physiol. Behav.* 120, 150–155. doi: 10.1016/j.physbeh.2013.07.012

Nordlund, D. A., and Lewis, W. J. (1976). Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. *J. Chem. Ecol.* 2, 211–220. doi: 10.1007/BF00987744

Novotny, M., Harvey, S., Jemiolo, B., and Alberts, J. (1985). Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. U.S.A.* 82, 2059–2061. doi: 10.1073/pnas.82.7.2059

Papes, F., Logan, D. W., and Stowers, L. (2010). The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. *Cell* 141, 692–703. doi: 10.1016/j.cell.2010.03.037

Parsons, M. H., and Blumstein, D. T. (2010). Familiarity breeds contempt: kangaroos persistently avoid areas with experimentally deployed dingo scents. *PLoS ONE* 5:e10403. doi: 10.1371/journal.pone.0010403

Pérez-Gómez, A., Bleymehl, K., Stein, B., Pyrski, M., Birnbaumer, L., Munger, S. D., et al. (2015). Innate predator odor aversion driven by parallel olfactory subsystems that converge in the ventromedial hypothalamus. *Curr. Biol.* 25, 1340–1346. doi: 10.1016/j.cub.2015.03.026

Rameshkumar, K., Achiraman, S., Karthikeyan, K., and Archunan, G. (2008). Ability of mice to detect estrous odor in bovine urine: roles of hormones and behavior in odor discrimination. *Zoolog. Sci.* 25, 349–354. doi: 10.2108/zsj.25.349

Rampin, O., Bellier, C., and Maurin, Y. (2012). Electrophysiological responses of rat olfactory tubercle neurons to biologically relevant odours. *Eur. J. Neurosci.* 35, 97–105. doi: 10.1111/j.1460-9568.2011.07940.x

Rampin, O., Jérôme, N., Briant, C., Boué, F., and Maurin, Y. (2006). Are oestrus odours species specific? *Behav. Brain Res.* 72, 169–72. doi: 10.1016/j.bbr.2006.04.005

Rasmussen, L. E., Lee, T. D., Roelofs, W. L., Zhang, A., and Daves, G. D. (1996). Insect pheromone in elephants. *Nature* 379:684. doi: 10.1038/379684a0

Regnier, F. E. (1971). Semiochemicals - structure and function. *Biol. Reprod.* 4, 309–326.

Ribeiro, A. M., Barbosa, F. F., Munguba, H., Costa, M. S. M. O., Cavalcante, J. S., and Silva, R. H. (2011). Basolateral amygdala inactivation impairs learned (but not innate) fear response in rats. *Neurobiol. Learn. Mem.* 95, 433–440. doi: 10.1016/j.nlm.2011.02.004

Roberts, L. J., and de Leaniz, C. G. (2011). Something smells fishy: predator-naive salmon use diet cues, not kairomones, to recognize a sympatric mammalian predator. *Anim. Behav.* 82, 619–625. doi: 10.1016/j.anbehav.2011.06.019

Roedel, M.-O., Brede, C., Hirschfeld, M., Schmitt, T., Favreau, P., Stoecklin, R., et al. (2013). Chemical camouflage - a frog's strategy to co-exist with aggressive ants. *PLoS ONE* 8:e81950. doi: 10.1371/journal.pone.0081950

Rokni, D., Hemmelder, V., Kapoor, V., and Murthy, V. N. (2014). An olfactory cocktail party: figure-ground segregation of odorants in rodents. *Nat. Neurosci.* 17, 1225–1232. doi: 10.1038/nn.3775

Root, C. M., Denny, C. A., Hen, R., and Axel, R. (2014). The participation of cortical amygdala in innate, odour-driven behaviour. *Nature* 515, 269–284. doi: 10.1038/nature13897

Rosa, H. J. D., and Bryant, M. J. (2002). The 'ram effect' as a way of modifying the reproductive activity in the ewe. *Small Ruminant Res.* 45, 1–16. doi: 10.1016/S0921-4488(02)00107-4

Rothschild, M., and Ford, B. (1973). Factors influencing the breeding of the rabbit flea (*Spilopsyllus cuniculi*): a spring-time accelerator and a kairomone in nestling rabbit urine with notes on *Cediopsylla simplex*, another "hormone bound" species. *J. Zool.* 170, 87–137. doi: 10.1111/j.1469-7998.1973.tb05046.x

Ruther, J., Meiners, T., and Steidle, J. L. M. (2002). Rich in phenomena-lacking in terms. A classification of kairomones. *Chemoecology* 12, 161–167. doi: 10.1007/PL00012664

Ryon, J., Fentress, J. C., Harrington, F. H., and Bragdon, S. (1986). Scent rubbing in wolves (*Canis lupus*): the effect of novelty. *Can. J. Zool.* 64, 573–577. doi: 10.1139/z86-084

Sbarbati, A., and Osculati, F. (2006). Allelochemical communication in vertebrates: kairomones, allomones and synomones. *Cells Tissues Organs* 183, 206–219. doi: 10.1159/000096511

Scarantino, A. (2010). Animal communication between information and influence. *Anim. Behav.* 79, e1–e5. doi: 10.1016/j.anbehav.2010.03.005

Schoenbaum, G., Chiba, A. A., and Gallagher, M. (1999). Neural encoding in orbitofrontal cortex and basolateral amygdala during olfactory discrimination learning. *J. Neurosci.* 19, 1876–1884.

Secundo, L., Snitz, K., and Sobel, N. (2014). The perceptual logic of smell. *Curr. Opin. Neurobiol.* 25, 107–115. doi: 10.1016/j.conb.2013.12.010

Setlow, B., Schoenbaum, G., and Gallagher, M. (2003). Neural encoding in ventral striatum during olfactory discrimination learning. *Neuron* 38, 625–636. doi: 10.1016/S0896-6273(03)00264-2

Sosulski, D. L., Bloom, M. L., Cutforth, T., Axel, R., and Datta, S. R. (2011). Distinct representations of olfactory information in different cortical centres. *Nature* 472, 213–216. doi: 10.1038/nature09868

Sparta, D. R., Smithuis, J., Stamatakis, A. M., Jennings, J. H., Kantak, P. A., Ung, R. L., et al. (2014). Inhibition of projections from the basolateral amygdala to the entorhinal cortex disrupts the acquisition of contextual fear. *Front. Behav. Neurosci.* 8:129. doi: 10.3389/fnbeh.2014.00129

Starling, E. H. (1905). The chemical control of the functions of the body. Lecture I of the croonian lectures on the chemical correlation of the functions of the body. *Lancet* 166, 339–341. doi: 10.1016/S0140-6736(01)11877-5

Stowers, L., and Marton, T. F. (2005). What is a pheromone? Mammalian pheromones reconsidered. *Neuron* 46, 699–702. doi: 10.1016/j.neuron.2005.04.032

Takahashi, L. K. (2014). Olfactory systems and neural circuits that modulate predator odor fear. *Front. Behav. Neurosci.* 8:72. doi: 10.3389/fnbeh.2014.00072

Todd, N. B. (1962). Inheritance of the catnip response in domestic cats. *J. Hered.* 53, 54–56.

Vernet-Maury, E. (1980). "Trimethyl-thiazoline in fox feces: a natural alarming substance for the rat," in *Olfaction and Taste VII,* ed H. van der Starre (London: IRL Press), 407.

Vernet-Maury, E., Dubois, P., and Chanel, J. (1977). An attempt to isolate and synthesize fox odor. *Chemorecept. Abstr.* 5, 55–56.

Waters, J., O'Connor, S., Park, K. J., and Goulson, D. (2011). Testing a detection dog to locate bumblebee colonies and estimate nest density. *Apidologie* 42, 200–205. doi: 10.1051/apido/2010056

Wells, D. L., and Hepper, P. G. (2000). The discrimination of dog odours by humans. *Perception* 29, 111–115. doi: 10.1068/p2938

Whittaker, R. H. (1970). "Biochemical ecology of higher plants," in *Chemical Ecology,* eds, E. Sondheimer and J. B. Simeone (New York, NY: Academic Press), 43–70.

Whittaker, R. H., and Feeny, P. P. (1971). Allelochemics: chemical interactions between species. *Science* 171, 757–770. doi: 10.1126/science.171.3973.757

Whittle, C. L., Bowyer, R. T., Clausen, T. P., and Duffy, L. K. (2000). Putative pheromones in urine of rutting male moose (*Alces alces*): evolution of honest advertisement? *J. Chem. Ecol.* 26, 2747–2762. doi: 10.1023/A:1026485725805

Wyatt, T. D. (2010). Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 196, 685–700. doi: 10.1007/s00359-010-0564-y

Young, E. J., and Williams, C. L. (2010). Valence dependent asymmetric release of norepinephrine in the basolateral amygdala. *Behav. Neurosci.* 124, 633–644. doi: 10.1037/a0020885

Young, E. J., and Williams, C. L. (2013). Differential activation of amygdala Arc expression by positive and negatively valenced emotional learning conditions. *Front. Behav. Neurosci.* 7:191. doi: 10.3389/fnbeh.2013.00191

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 29 July 2015
doi: 10.3389/fnins.2015.00263

Are single odorous components of a predator sufficient to elicit defensive behaviors in prey species?

Raimund Apfelbach[1]*, *Michael H. Parsons*[2], *Helena A. Soini*[3] *and Milos V. Novotny*[3]

[1] *Animal Physiology, Institute for Neurobiology, University of Tübingen, Tübingen, Germany,* [2] *Department of Biology, Hofstra University, Hempstead, NY, USA,* [3] *Department of Chemistry, Institute for Pheromone Research, Indiana University, Bloomington, IN, USA*

OPEN ACCESS

Edited by:
Thomas Endres, Otto-von-Guericke University, Germany

Reviewed by:
Micheal Baum, Boston University, USA
Lorey K. Takahashi, University of Hawaii at Manoa, USA

***Correspondence:**
Raimund Apfelbach, Animal Physiology, Institute for Neurobiology, University of Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany
raimund.apfelbach@uni-tuebingen.de

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *30 May 2015*
Accepted: *13 July 2015*
Published: *29 July 2015*

Citation:
Apfelbach R, Parsons MH, Soini HA and Novotny MV (2015) Are single odorous components of a predator sufficient to elicit defensive behaviors in prey species? Front. Neurosci. 9:263. doi: 10.3389/fnins.2015.00263

When exposed to the odor of a sympatric predator, prey animals typically display escape or defensive responses. These phenomena have been well-documented, especially in rodents, when exposed to the odor of a cat, ferret, or fox. As a result of these experiments new discussions center on the following questions: (1) is a single volatile compound such as a major or a minor mixture constituent in urine or feces, emitted by the predator sufficient to cause defensive reactions in a potential prey species or (2) is a whole array of odors required to elicit a response and (3) will the relative size or escapability of the prey as compared to the predator influence responsiveness. Most predator-prey studies on this topic have been performed in the laboratory or under semi-natural conditions. Field studies could help to find answers to these questions. Australian mammals are completely naïve toward the introduced placental carnivores. That offers ideal opportunities to analyze in the field the responses of potential prey species to unknown predator odors. During the last decades researchers have accumulated an enormous amount of data exploring the effects of eutherian predator odors on native marsupial mammals. In this review, we will give a survey about the development of olfactory research, chemical signals and their influence on the behavior and—in some cases—physiology of prey species. In addition, we report on the effects of predator odor experiments performed under natural conditions in Australia. When studying all these literature we learned that data gained under controlled laboratory conditions elucidate the role of individual odors on brain structures and ultimately on a comparatively narrow range behaviors. In contrast to single odors odor arrays mimic much more the situation prey animals are confronted to in nature. Therefore, a broad range of methodology—from chemistry to ecology including anatomy, physiology, and behavior—is needed to understand all the different (relevant) stimuli that govern and guide the interactions between a predator and its potential prey.

Keywords: predator odors, aging of odors, predator naive prey, odor avoidance, field studies in Australia

Development of Olfactory Research

Chemical ecology is one of the most fascinating themes in modern biology. Chemical compounds are essential in intraspecific communication as well as in information exchange between different species. Chemical signals are involved in the defense of prey species against predators, competitors, parasites, microbes, and other potentially harmful organisms (Derby and Aggio, 2011). In short, the challenge in chemical ecology is to demonstrate how chemically mediated interactions steer ecology and evolutionary processes at all levels of ecological organization (Vet, 1999).

Among the first scientific reports on chemicals secreted by a carnivore was the paper by Albone and Perry (1975). The authors analyzed the anal sac secretion of the red fox (*Vulpes vulpes*) and of the lion (*Panthera leo*). The major components of red fox urine deposited in their tracks on snow during the mating and breeding season were later structurally identified (Jorgenson et al., 1978). It was noted that the characteristic "skunk-like odor" of these fox tracks is likely due to sulfur-containing constituents of urine. The synthetic blends of the major urinary constituents were made and deposited in the fox natural habitat to test their ecological significance (Whitten et al., 1980; Wilson et al., 1980). Seasonal variations in the excretion of captive fox urinary volatiles were further investigated (Bailey et al., 1980). During the 1970s and early 1980s, chemical constituents of the defensive secretions of the striped skunk (*Mephitis mephitis*) were also positively identified as mainly sulfur-containing compounds (Andersen and Bernstein, 1975; Andersen et al., 1982).

During roughly the same time period, the chemist Schildknecht and his coworkers analyzed the anal gland secretions of several mustelid species including the mink (*Mustela vison*), the polecat (*Mustela putorius*) and the badger (*Meles meles*) (Schildknecht et al., 1976, 1981; Schildknecht and Birkner, 1983; Schildknecht and Hiller, 1984). Considering the limited analytical capabilities at that time, these studies are still among the most detailed reports on semiochemicals in carnivores. The authors reported 3,3-dimethyl-1,2-dithiolane, 2,2-dimethyl-, cis- and trans-2,3-dimethyl-, 2-propyl-, and 2-pentylthietane characteristic to the polecat (*Mustela putorius* L.) and the ferret (*Mustela putorius furo*). In the odorous secretion of the stoat (*Mustela ermine*) they found 2-methyl-, 2-opyl-, and 2-pentylthietan. Similar sulfur-containing components were found in the secretion of the anal glands of weasel *(Mustela nivalis*). On the contrary, no sulfur-containing compounds were observed in the beech-marten (*Martes foina*) and the pine-marten (*Martes martes*) odoriferous secretions (Schildknecht and Birkner, 1983).

As mustelids became more popular in scientific research, more research groups focused on the anal secretions of mustelid species (Crump, 1980; Brinck et al., 1983; Crump and Moors, 1985). The general ideas about the importance of chemical communication in mammals were significantly advanced by the first reports of chemical structures of primer pheromones in rodents (Jemiolo et al., 1986; Novotny et al., 1986, 1990) starting in the 1980s. Importantly, the analytical methodologies developed for this research became a major stimulus for broad studies of ecological significance. All these studies have opened avenues for biologists to investigate chemical communication within individual species and the relationship between predators and prey.

The chemical compounds emitted by a species and the effects they might cause in another species became hot spots of scientific interest. Among the first to launch this research line were Nolte et al. (1994). They sparked a field of highest scientific interest when they published their paper "why are predator odors aversive to prey?" This question is still investigated and debated by biologists, chemists, and ecologists. Until now, no one has introduced an adaptive framework to organize the data and speculations for the broad range of varying results. We will propose a novel adaptive framework to compare the overall risk that a prey animal might accept against the degree of danger represented by one or more predator cues. The most obvious indicator of risk relates to how likely a single encounter of predator-prey would result in foregone feeding or mating opportunities, serious injury, or demise e.g., the ability of the prey to defend, suppress, or survive the encounter. For this reason we propose a likelihood of risk category by the relative size or elusiveness of the prey as compared to the predator (supplement, **Table 1**).

Implicit in our model is an understanding that smaller, more vulnerable, prey may respond aversively to single-molecule odors while larger, or most elusive, prey may attenuate their response according to composite molecules that convey additional information about the degree of risk inherent in the scent. For instance small kangaroos and wallabies may respond aversively to domestic dog urine, predators they have had no evolutionary contact with—or possibly even aged, chemically degraded scents. However, larger macropods such as red kangaroos, may not respond to a single compound. They likely require complex scents that include additional information on the size and recentness of void from the predator, before foregoing mating opportunities or leaving a food patch. It would after all, be evolutionarily disadvantageous for a 50+ kg herbivore to respond to wastes secreted by a 700 g, predator.

Much of the present research concentrates on three basic topics: (1) How effective are carnivorous chemical compounds as repellents to prey species of different size, (2) do such chemical compounds suppress breeding success in prey species and (3) which brain structures of the prey species are involved in the avoidance/fear responses. A comprehensive review of field and laboratory studies about the positive and negative effects of predator odors on mammalian prey species has been reported in detail (Apfelbach et al., 2005). The involvement of brain structures in the avoidance/fear responses has been recently very well-addressed by Takahashi (2014).

The effects of predator odors or individual compounds in a complex odor source can be evaluated in the laboratory and in the field. Besides natural predator odors, synthetic predator semiochemicals (Lindgren et al., 1995) and—in another study—a range of seven predator odors and, in addition diesel oil, as repellents for wildlife have been used in field studies (Engelhart and Müller-Schwarze, 1995). In that study, coyote (*Canis latrans*), lynx (*Lynx canadensis*) and river otter (*Lutra canadensis*) odors had the strongest effects, while diesel oil was effective too, but the effects were weaker. So far, no field data are available about the

TABLE 1 | Literature review of chemical based predator–prey studies from Australia with foci on the source cue, integrity of signal and outcome measured.

Time Period	Literature referenced	Predator species	Prey species	Variations of chemical	Response measured	Risk category
2000–2005	Head et al., 2002	White-lipped snake odors (*Drysdalia coronoides*) from damp paper towel in snake enclosure	Mountain log skink (*Pseudemoia entrecasteauxii*) paper-towel and rocks from cage	Not stated	Shift in habitat use	4
	Blumstein et al., 2002	Feces from red fox (*Vulpes vulpes*), kodiak bear (*Ursus arctos*) and dingo (*Canis L. dingo*) placed beneath feeding tray	Tammar wallaby (*Macropus eugenii*) and rednecked pademelon (*Thylogale thetis*)	Frozen at −20°C	No changes in foraging behavior	2
	Downes, 2002	Yellow-faced whip snake (*Demensaina psammophis*) paper towel and rocks from cage	Common garden skink (*Lampropholis delicata*)	Not stated	20% reduction in mobility of prey	4
	Banks et al., 2003	Domestic dog feces (*Canis domesticus*)	Bush rat (*Rattus fuscipes*)	Fresh	No influence on trapping success	2
	Powell and Banks, 2004	Fox feces (*Vulpes vulpes*)	House mouse (*Mus musculus*)	Fresh	No change in food removed (GUD*)	4
	Ramp et al., 2005	Synthetic dog (*Canis domesticus*) urine	Parma wallaby *(Macropus parma*) and red-necked pademelon (*Thylogale thetis*)	Not stated (synthetic used)	*T. thetis* investigated scent more, *M. Parma* showed aversive response	4
	Russell and Banks, 2005	Red fox (*Vulpes vulpes*), tiger quoll (*Dasyurus maculatus*)	Northern brown bandicoot (*Isoodon macrourus*) and brushtail possum (*Trichosurus vulpecula*)	Stated fresh or frozen	Captured significantly more often in traps scented with tiger quoll odor	4, 3
2006–2010	Hayes et al., 2006	Varied: carpet python, dingo, quoll, red fox (*Vulpes vulpes*)	Fawn-footed melomys (*Melomys cervinipes*), bush rat, (*Rattus fuscipes*), giant white-tailed rat (*Uromys caudimaculatus*)	Frozen at −20°C in airtight vials with Teflon-lined lids	During dry season all species avoided all predator odor stations	4
	Murray et al., 2006	Tiger feces *(Panthera tigris*)	Goat (*Capra hircus*)	Feces mixed in bentonite (>dispersal)	Reduced feeding	2
	Parsons et al., 2007	Coyote (*Canis latrans*), dingo (*Canis L. dingo*)	Western gray kangaroo (*Macropus fuligonosus*)	Pooled urine (12–16 adult males) replenished each trial	Increased GUD and flight/startles for dingo as compared to coyote	2, 3
	Russell and Banks, 2007	Tiger quoll (*Dasyurus maculatus*) and the introduced placental red fox (*Vulpes vulpes*)	Bush rat (*Rattus fuscipes*), swamp rat (*Rattus lutreolus*), eastern chestnut mouse (*Pseudomys gracilicaudatus*), brown antechinus (*Antechinus stuartii*)	Stated fresh and/or frozen	Native rodents more likely trapped in control than treatment. *Antechinus* showed no trapping differential	4, 2
	Lloyd et al., 2009	Goanna (*Varanus tristis*) a species that consumes skinks as a major proportion of its diet, and *V. varius*, which does not	Tropical skink (*Carlia rostralis*), (*C. rubrigularus*) and (*C. storri*)	Filter paper dampened water	Two of the three species avoided *V. tristis*. None avoided *V. varius*	4, 2
	Cox et al., 2010	Feces from tiger (*Panthera tigris*) and tasmanian devil (*Sarcophilus harrisii*)	Goat (*Capra hircus*) and eastern gray kangaroo (*Macropus giganteus*)	Feces mixed in a bentonite carrier (aids in dispersal)	Both prey species avoided odors from predators that had fed on these species prior to trials (diet specific response)	2, 2
	Parsons and Blumstein, 2010a	Dingo (*Canis l. dingo*) urine; feces	Western gray kangaroo (*Macropus fuliginosus*)	Pooled urine/feces (12–16 adult males) repolished trial	Flight; =GUD	3

(Continued)

TABLE 1 | Continued

Time Period	Literature referenced	Predator species	Prey species	Variations of chemical	Response measured	Risk category
	Parsons and Blumstein, 2010b	Dingo urine (*Canis l. dingo*)	Wallabies (Macropus rufogrisius, pademelon (*Thylogale billardierii*), brush-tailed possum (*Trichosurus vulpecula*)	Maintained fresh	Flight; =GUD	2,2
2011–2015	Nersesian et al., 2012	Fox (*Vulpes vulpes*), owl	Brush-tailed possums (*Trichosurus vulpecula*)		< feeding time, <vigilance varied with indirect cues	4
	Anson and Dickman, 2013	Red fox (*Vulpes vulpes*) in areas where fox impacts had been greatest, and to cues of the native lace monitor (*Varanus varius*)	Common ringtail possum (*Pseudocheirus peregrinu*	Collected fresh, 1 part feces mixed with 5 parts water	Flight alarm calling to both odors	4, 3
	Bytheway et al., 2013	Dog/dingo hybrid integument odor collected on towels	Black rat (*Rattus rattus*)	Fresh	Increased GUD, visitation/investigation	4
	Descovich et al., 2012	Dingo feces	Southern hairy nosed wombat (*Lasiorhinus latifrons*)	Frozen at −20°C	When feces were present, the wombats used concealed locations more often than other periods	4
	Cremona et al., 2014	Dingo (*Canis l. dingo*) and the northern quoll (*Dasyurus hallucatus*) feces	Rock rat (*Zyzomys* spp.)	Not stated	Rock rats demonstrated a stronger avoidance to quoll odor than to dingo odor	3
	Mella et al., 2014a	Fox (*Vulpes vulpes*) and dingo (*Canis L. dingo*) feces	Western gray kangaroo (*Macropus fuliginosus*)	Fresh	Modifying space use by rapidly escaping from both odors	3, 3
	Mella et al., 2014b	Domestic dog (*Canis domesticus*) urine, owl pellets	Brushtail possum (*Trichosurus vulpecula*)	Urine used within 24 h after collection	Possums reacted more strongly to indirect cues (no change to direct)	3, 3
	Spencer et al., 2014	Fox (*Vulpes vulpes*) and cat (*Felis catus*) urine	*Notomys alexis*, a terrestrial native rodent	Dowels were soaked overnight in predator urine stored at 1°C	No effect, prey relies on escape	4

*Outcomes based on presumed risk category of species. Risk category: 1, lowest risk; 2, significant size differential, but uncommon predation (non-historic); 3, lethal predator, but comparatively large prey; 4, highest risk of lethality with direct predation common. *GUD = the weight of food that animals leave behind next to treatments, that they otherwise would have consumed in the control tray. Search string = "predator prey scent odor Australia"; search range 2000–2005, 2006–2010, 2011–2015. Inclusive of all experimental journal articles with terrestrial vertebrate field studies involving predator wastes as a source cue including: urine, feces, dander, or integumentary.*

effectiveness of a predator odor to suppress or at least to reduce the reproduction success of potential prey species.

In our review the seminal discussion centers mainly on two questions: (1) is just one single volatile compound in, for example, urine or feces emitted by the predator enough to release defensive reactions in a potential prey species—or must there be a whole profile of odors? (2) Do prey animals respond innately to olfactory predator cues? Our focus is set mainly on mammals, but other vertebrates and lower taxa will be included on some occasions.

Composition and Aging of Olfactory Signals

An odorant is a chemical compound that gives a particular smell to a source. An odor is typically viewed as a volatile molecule, or a set of molecules, that convey some information about the sender to a receiver; generally, the molecules meeting this specification have molecular weights lower than 300 Da.

In mammals, there are different bodily odor sources. Besides urinary odors, other odors emitted from other sources by an animal may serve in the behavioral context to convey information such as degree of hunger or satiety, single, or multiple predators (from over-marking) and importantly, the size, and specificity of the predator- especially important to prey that are otherwise large enough or elusive enough to live comfortably among predators that convey little risk. Among those are anal gland secretions, fecal odors, and vaginal secretions, just to mention the most obvious ones. Fur, dander, sebum, saliva, and tears can also transmit "infochemicals." Some species possess additional glands like the preorbital glands and tarsal organs in many hoofed animals or the supplementary sacculi (located at the opening of the cheek pouches) and the midventral gland of dwarf hamsters (*Phodopus* spec.) secreting substances of relevance for intraspecific communication.

In invertebrates, just one molecular entity is often sufficient to transmit an important message from one individual to another; the best known example is the pheromone bombykol produced by the silkworm *Bombyx mori* (Schneider et al., 1968; Kaissling, 2014). Among insects, pheromones (intraspecific messengers) are typically composed of one or only very few molecular types such as hydrocarbons in insect cuticles (Blomquist and Bagnères, 2010), while most odorous substances secreted by mammals are typically composed of numerous different volatile compounds. Depending on the species, up to 70 or more volatile compounds have been found. For example, in ferret (*Mustela putorius furo*) urine, 31 volatile urinary compounds have been identified and compared with 26 anal gland compounds of the same species. Only 10 compounds were found common to both sources (Zhang et al., 2005). The two marking sources likely convey different messages to conspecifics. This possibility is backed by our observations on wild living ferrets and polecats (*Mustela putorius*). Males and females urinate all over their territories, but defecate only on specific spots. While urine and fecal odors have putative functions in intraspecific communication, the secretions of the anal glands (containing high amounts of sulfur compounds) seem to serve a dual purpose for defense: when cornered or threatened, and as "alarm pheromones" to warn conspecifics of imminent danger. Two simple compounds from the peri-anal gland, 4-methylpentanal and hexenal, function together as the alarm pheromone in Norway rats (*Rattus norvegicus*) (Inagaki et al., 2014). These two molecules lose their function, when acting alone.

The composition of the urine odors and possibly also of the anal gland odors may change during the seasons in accordance with the endocrine status and also the diet consumed by a predator. This was seen in the elevated seasonal levels of isopentyl methyl sulfide in the red fox urine (Bailey et al., 1980). Several volatile compounds showed peak levels also in the wolf urine (*Canis lupus*) depending on the season. These included isopentyl methyl sulfide and several carbonyl compounds (Raymer et al., 1984). Hormone treatment experiments later confirmed that testosterone increased wolf urinary volatile compound levels (Raymer et al., 1986).

Volatile compounds in urine or in a secretion differ in molecular weights and physicochemical properties, such as boiling points, vapor pressure, and solubility properties in water. For instance, in the ferret urine (Zhang et al., 2005) the molecular weight of the individual molecules ranged from 60.05 Da (acetic acid; boiling point: 118°C; vapor pressure: 1290 Pa) to 256.42 Da (hexadecanoic acid; boiling point: 215°C; vapor pressure: 0.5×10^{-4} Pa). In the urine of another carnivore (Osada et al., 2013), the wolf (*Canis lupus*), the molecular weight range extends from 62.13 Da (dimethyl sulfide; boiling point: 35°C; vapor pressure 53,700 Pa) to 122.12 Da (benzoic acid; boiling point: 249°C; vapor pressure 0.1 Pa). In general, compounds with low molecular weights, low polarity, and high vapor pressure evaporate faster than the "heavier," more polar compounds with low vapor pressure properties. These differences might explain why over time the composition of a secretion changes. We will call it "aging of a signal." In this context, aging can mean decreased concentrations or a loss of some components and/or changing ratios between the compounds. This process would be particularly prominent in the arid conditions of Australia, where all compounds, regardless of molecular weight, evaporate more quickly due to increasing of vapor pressures at elevated environmental temperatures. Another consideration is the water solubility of the compound in environmental conditions. Compounds with low water solubility will be less affected by rain and therefore might resist environmental stress much longer than compounds with high water solubility. The much less volatile components of urinary marks and secretions, such as lipids and proteins, may also retain and slowly release volatile chemosignals into the environment.

The numbers of research papers on aging of urine or gland secretion odor compounds are very limited. In contrast to studies on chemical signals in animals, aging has been intensively investigated in food chemistry and interesting results have been reported. For instance, the flavor characteristics of beer appear to deteriorate greatly with time (Gijs et al., 2002). Similarly, changes in some volatile constituents of brandy (Onishi et al., 1977) and sherry wines have been reported (Munoz et al., 2007). Among the first to report on behavioral effects of aged urine were

Coppola and Vandenbergh (1985). These authors determined how long the puberty delay causing chemosignal emitted from an adult female mouse urine remains active to suppress puberty in young females. According to their data, within 7–10 days after collecting urine, the sample will lose its pheromonal potency to delay puberty in recipient females. The authors suspected that the puberty delay causing signal would lose its potency more rapidly in nature than under laboratory conditions due to the destructive influences of the natural elements.

Neuroendocrine and behavioral responses of mice to urine samples from conspecific males and females, which had aged for different time periods, revealed that the quality and intensity of signaling molecules in urine changed over time (Kwak et al., 2013a). In another study, volatile organic compounds in fresh and aged human urine samples were analyzed and compared (Kwak et al., 2013b).

Due to such unpredictable environmentally-induced changes, researchers run experiments with either fresh secretions or with secretions which were deep-frozen until use. Yet, in spite of deep freezing, the secretions can still undergo composition or conformational changes. This has been convincingly demonstrated in a very recent study during which cat feces was stored at −70°C (Hegab et al., 2014). The statement of these researches reports best the effects of aged chemicals: "Behavioral and hormonal responses and changes in the level of medial hypothalamic c-fos nRNA were examined in Brandt's voles (*Lasiopodomys brandtii*) exposed to the feces of a domestic cat (*Felis catus*) stored for different periods. One hundred voles were tested in the defensive withdrawal apparatus. The voles showed an aversion to freshly collected cat feces, indicated by high levels of flight-related behaviors, increased freezing behavior, and more vigilant rearing compared to old feces. The serum levels of adrenocorticotropic hormone and corticosterone significantly increased when the voles were exposed to fresh cat feces. The level of c-fos mRNA in the medial hypothalamus region was highest in the individual exposed to fresh cat feces. All these behavioral, endocrine, and c-fos-mRNA responses were lower when voles were subject to older cat feces" (Hegab et al., 2014). In a study working with meerkats (*Suricata suricatta*), similar results were found. The freshness of the presented wolf (*Canis lupus*) urine increased vigilance in the prey animals while the increased quantity of urine sample did not cause the similar effect (Zöttl et al., 2013). Similarly, in comparisons of the effects of fresh and previously frozen female mouse urine on male mice, fresh urine triggered stronger courtship ultrasonic vocalization in males (Hoffmann et al., 2009). Gas chromatographic analyses of dingo urine convincingly demonstrated changes in the male and female urine after an aging period of about 3 months or even less (**Figure 1**).

Data from such studies indicate that very careful experimentation and interpretation of the data is needed when stored biological compounds are used to study behavioral and physiological parameters. It cannot be excluded that in natural environments, loss of response to a predator odor over time is not caused by habituation, but rather aging of the signal.

Single Odors or Odor Arrays?

Sensing the chemical warnings present in the environment is essential for species survival. Brunswik (1956) suggested the concept of a transient fear scent operating in mice. Emitted by both males and females, this fear scent can be elicited by a single stressful event. In the natural setting, a mouse's tendency to withdraw from sites possessing the fear scent of conspecifics may well-protect mice from predators and other dangerous situations. Similarly, Carr et al. (1970, 1971) reported that male mice avoided an olfactory signal emitted by stressed male and female mice. It was also demonstrated that male mice were repelled by the odor from shocked males and attracted to the odor from non-shocked males (Colyer (1971). Although additional odor sources may be involved, it is believed that a fear scent is contained in the urine (Müller-Velten, 1966). These findings marked the beginning of considering urine as a transmitter of danger odor signals.

Nolte et al. (1994) suspected that predator odors are aversive to prey species due to the high concentrations of sulfurous components (metabolites of protein digestion) in their urine. As reported above, urine or fecal odors are composed of numerous different compounds. Subsequently, many studies in behavioral research have used such complex mixtures and reported the results under these conditions. It is not in the interest of a predator to deposit a signal that persists over longer time periods to warn potential prey about predator's presence. While there is a trade-off involved when predators deploy odors in the environment, it is beneficial for conspecifics to be able to detect these "chemical bulletin boards" for as long as possible. However, the longer the scent is viable, the more likely potential prey could intercept the signal and will respond innately to these kairomones (chemical messages of another species) or will learn to recognize and respond accordingly to these signals. Cheetahs may have evolved the ability to secrete an odorless (elemental) sulfur complex (Burger et al., 2006). On the other hand, secretions may inform conspecifics about a territory owner or a sexual partner ready to mate. In this case, it would be advantageous for the sender when the chemical signal remains in place for a longer time period.

Most mammals were long assumed to have two olfactory systems working independently, the primary olfactory epithelial tissue (MOE) and secondary vomeronasal mediated system (VMO; e.g., pheromones), with the molecular weight of volatile constituents determining the appropriate binding site. This model seemed to have been validated in mice (Trinh and Storm, 2003), but has been since updated to consider the shared role of both organs, now seen to complement one another due to common V1R receptors, and shared processing regions in the amygdala (Kendrick, 2014).

It remains unclear whether single molecule constituents are more or less likely to be received by the primary or secondary systems, than composite chemicals. However, we may operate under the reasonable assumption that predator-secreted compounds are more likely to be detected by the VMO, than foul smelling, or pungent, artificial odors such as diesel fuel, ammonia or feces from non-predatory omnivores (e.g., pigs). These foul odors would contribute less biologically meaningful

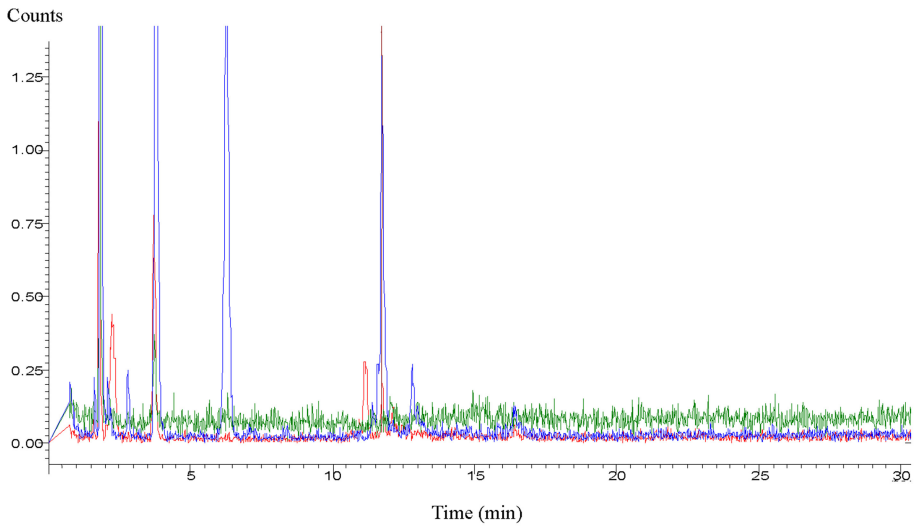


FIGURE 1 | Aged profile of dingo (*Canis lupus dingo*) urine. Blue loop refers to fresh male urine, red loop refers to fresh female urine, and green loop refers to male and female urine aged >3 months (Graph supplied by M.H. Parsons).

information and would thus be more likely to be interpreted by the MOE.

In vertebrates, at least in mammals, generally no single compound is known to convey complex behavioral messages between individuals. Yet, examples that a "bouquet" of odors makes up the message, have been reported with red fox (Whitten et al., 1980) and mice (Jemiolo et al., 1986; Novotny et al., 1986; Ma et al., 1998). However, several papers state that some compounds in urine or fecal secretions are especially effective and will alone, or in combination with other molecules, elicit defensive reactions in prey species and/or suppress breeding success. Some of the four most discussed molecular types include:

(1) **Pyrazines** are known as volatile nitrogen-containing odoriferous compounds present in vertebrates, plants, insects, fungi, and bacteria (Woolfson and Rothschild, 1990). Pyrazines, such as 2,5-dimethylpyrazine (2,5-DMP), seem to have redundant message contents in vertebrates. In the female mouse urine, 2,5-DMP is involved in a puberty inhibition signaling from a female mouse to other female mice together with five other adrenal-mediated urinary metabolites (Andreolini et al., 1987; Ma et al., 1998).
Male wolf (*Canis lupus*) urine samples contained more than 50 compounds in the GC-MS analyses with some significant differences in compound levels (Osada et al., 2013). Such differences may allow, for instance, individual recognition within a wolf pack. However, the urine of all three wolfs contained pyrazine derivatives as the predominant active components capable to induce avoidance and freezing behaviors in mice. Osada further reported that the combination of 2,5-DMP and two other pyrazines, which are present in the wolf urine, induced freezing behavior in mice, which was a similar response as to wolf urine, while each pyrazine compound alone was inactive. In the ferret urine, pyrazines could play a role in the odor-sensing and caution expressed by hamsters to the ferret urine (Apfelbach et al., 2015).

(2) **2-Phenylethylamine (PEA)** (molecular weight, Mw: 121.18) is a component common to many carnivore odors. It also has been found in non-carnivorous species at much lower concentrations (Ferrero et al., 2011). Like pyrazines it is a moderately volatile compound (vapor pressure 35 Pa, Mokbel et al., 2009), therefore, probably not very effective over longer periods of time. In behavioral studies, rodents avoided a PEA odor source similarly as they avoided predator urines. To verify that PEA is the decisive chemical for the avoiding reaction, the researchers experimentally depleted lion urine of this compound. In the subsequent behavioral experiments, rats showed significant avoidance behavior to 10% content in lion urine, but not to 10% PEA-depleted urine specimen. Aversion was fully restored to a 10% PEA-respiked lion urine. The authors interpret from these data that PEA is a key component of a carnivore odor blend detected and avoided by rodents (Ferrero et al., 2011). However, studies under natural conditions have not been reported.

(3) **2,3,5-Trimethyl-3-thiazoline (TMT)** (Mw: 129.22) is found in fox feces (Vernet-Maury, 1980). Over the years, several laboratories reported fear-like alterations in rat behavior due to exposure to synthesized TMT (e.g., Wallace and Rosen, 2000). Physiological data as well as data on brain structures involved in the TMT-elicited defensive responses of rats have been also described (Fendt et al., 2003, 2005; Endres et al., 2005; Dielenberg et al., 2001). The view of TMT as a biologically relevant olfactory stimulus has been challenged. According to Morrow (Morrow et al., 2002), a fear-like biochemical and behavioral response in rats to TMT odor depends on the exposure environment. Others have even failed to observe fearful behaviors (McGregor et al., 2002); since TMT has an acrid, irritating, and powerfully repugnant

odor (at least to humans), its effects are more characteristic of an aversive odor, presumably working through a nociceptive mechanism. According to Staples and McGregor (2006), differences in response to TMT and cat odor could depend on the rat strain.

Blanchard regarded TMT as follows: "These findings suggest that flight/avoidance, although it obviously may occur as one component of a full pattern of defensive and emotional behaviors, is also somewhat separable from the others. When—as appears to be the case with TMT—it is the major, perhaps only consistent defensive behavior elicited, this may reflect a stimulus that is aversive or noxious but with little ability to predict the presence of threat or danger" (Blanchard et al., 2003). To address this criticism, rats were exposed to TMT following either olfactory bulb removal or trigeminal nerve transection (Ayers et al., 2013). The findings indicate that freezing behavior to TMT requires an intact olfactory system, as indicated by the loss of freezing following olfactory bulb removal. Rats with trigeminal nerve transection freeze normally to TMT, suggesting the olfactory system mediates this behavior to TMT. TMT is an ecologically relevant predator odor useful in experiments of unconditioned fear that is mediated via olfaction and not nociception (Ayers et al., 2013).

Very few behavioral field experiments using TMT as a repellent have been reported. To reduce feeding damage by voles (*Microtus spec.*) on apple trees in orchards Sullivan et al. (1988a) applied TMT to traps and quantified the number of voles caught in TMT-free traps and in traps scented with TMT. The animals significantly avoided the TMT scented traps. In a similar field experiment using Northern pocket gophers (*Thomomys talpoides*) animals also avoided the TMT odor (Sullivan et al., 1988b). However, when testing the two synthetic predator odors TMT and DMDIT (3,3-dimethyl-1,2-dithiolane) for their possible repelling effects on roof rats (*Rattus rattus*) in Hawaiian macademia nut orchards no clear results became visible. The authors (Burwash et al., 1998) stated "overall we could not detect significant differences or consistent trends in response of rats to DMDIT or TMT in these field trials."

Without doubt TMT is effective in eliciting fear and escape responses in rats, although they were naive to foxes and fox feces. Interestingly, TMT has not been found in dog feces (Arnould et al., 1998) and also not in anal gland secretions of dog and coyote (Preti et al., 1976), although both carnivore species are closely related to the red fox. These and other results indicate that TMT may be characteristic for the red fox, but possibly not for other predators.

(4) A cat-specific substance is **2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid (L-felinine**) (MW: 207.29). Felinine, a putative pheromone precursor, has been identified in the urine of several members of the felidae family including the domestic cat (*Felis catus*) (Hendriks et al., 1995b). Cat urine contains 3-mercapto-3-methylbutan-1-ol, a degradation product of felinine, and a putative cat pheromone. This compound gives cat urine its typical odor and may have a function in territorial marking (Hendriks et al., 1995a; Miyazaki et al., 2006). However, no experimental proof has been provided up to date for this assumption. Felinine is a non-volatile amino acid that requires a close contact ("close contact signal") for the olfactory perception. To investigate the influence of cat odor on reproductive behavior and physiology in the house mouse, cat urine or 0.05% felinine was directly applied to the bedding of pregnant mice every other day. After having given birth, the total number of offspring was counted as well as the number of pups per female. Exposure of mated females to felinine provoked a pregnancy block in 67.85% female mice, while in the control group a birth reduction of only 17.86% was observed (Voznessenskaya, 2014). Felinine has not been tested outside the laboratory conditions so far.

Rats and mice are averse to the odor of a cat's urine, but after they are infected with the parasite *Toxoplasma gondii*, they are attracted to cat urine. This increases the likelihood of being preyed upon and consequently, infecting the cat (Berdoy et al., 2000). Earlier, a similar decrease in predator avoidance in parasitized mice was reported. Mice infected with the naturally occurring *Eimeria vermiformis* spent a significantly longer time period in the proximity of cat odor, while uninfected mice continued to avoid cat urine. This result indicates that infection with *E. vermiformis* in mice reduces the avoidance of a predator odor through neurochemical systems associated with anxiety involving GABA receptor mechanisms (Kavaliers and Colwell, 1995).

Besides felinine odor, cats emit other chemical signals. Cats mark their territories by rubbing their neck at corners or objects leaving a scent behind ("cat neck odor"). When a rat is exposed to such a scent, it exhibits a strong aversive/flight reaction. When Wistar rats were exposed to a cotton pad wiped on a cat body rubbing location, they showed increased hiding behavior, decreased exploration behavior and reduced stimulus approach and investigation. These defensive responses persisted for up to 4 days following a single stimulus exposure (May et al., 2012). C-fos studies revealed a high activation of the brain structures involved in these fear reactions (Dielenberg et al., 2001). So far no information is published about the compound responsible for the reported behavioral effects.

It may seem unlikely that only one type of a volatile molecule out of the many compounds (e.g., in urine) is sufficient for eliciting escape or defense reactions in a prey. Considering the above findings, one can conclude that the amount of information encoded in one urinary volatile molecule seems limited. The predator odor information system may act more like a "yes—no" (danger—no danger) information system. Much more information can be encoded in a "bouquet" of several volatile compounds. Already in 1994, Nolte and coworkers reported that herbivorous rodents were able to distinguish between urine collected from coyotes fed cantaloupes vs. those fed a meat diet. Similarly, in a paper by Berton et al. (1998), the authors demonstrated that mice were able to distinguish between the fecal odor of cats subjected to either a vegetarian diet or a carnivorous diet. In a recent study, it was demonstrated that the dwarf hamster (*Phodopus campbelli*) was able to readily distinguish

between urine of ferrets fed with chicken, rat, or hamster. While dietary variations are unlikely to result in measurable quantities of additional urinary compounds, the signal-receiving animals may be capable of distinguishing quantitative differences in the urinary volatile compound arrays (shown in **Figure 2**), and subsequently, perceiving different olfactory messages (Apfelbach et al., 2015).

An interesting question is how fast naïve animals are able to learn to respond to odors of a new, previously unknown predator. Anson and Dickman (2013) explored in a field study the ability of the common ringtail possum (*Pseudocheirus peregrinus*), a semi-arboreal Australian marsupial, to recognize and respond to olfactory cues from the introduced fox (*Vulpes vulpes*). Their results show that in the areas with high fox densities, the selection pressure from the fox has been sufficient for ringtails to develop anti-predator behaviors over a few generations since foxes had become established in the area. In contrast to this finding, no such anti-predator behavior patterns were obvious in the areas where foxes either had not been observed or observed only very recently. A recent paper by Dias and Ressler (2014) will likely receive considerable attention among scientists. These authors examined the inheritance of a parental traumatic olfactory exposure. F0 mice were subject to odor fear conditioning before conception, and subsequently in F1 and F2 generations, an increased behavioral sensitivity to the F0-conditioned odor, acetophenone, but not to other odors, was found. Besides behavioral studies, neuroanatomical, and genetic studies including cross-fostering were employed. Taking all their data into account, the authors concluded that their findings provide a framework for addressing how environmental information may be inherited transgenerationally at behavioral, neuroanatomical, and epigenetic levels.

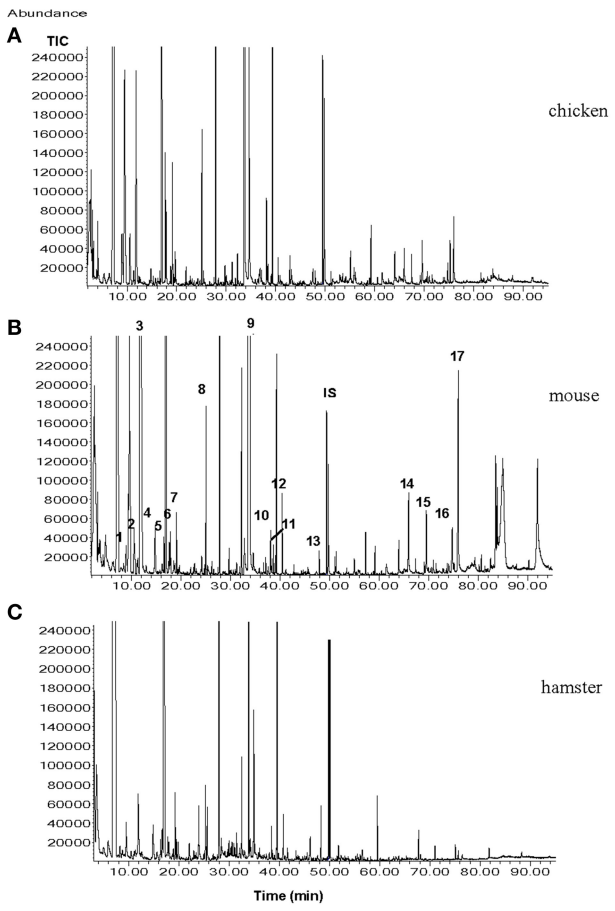


FIGURE 2 | Total ion chromatograms (TICs) from the male ferret urine samples when ferrets were fed with (A), chicken; (B), mouse; (C), hamster. Numbers indicate the following compounds 1, xylene; 2, heptanal; 3, 2,5-dimethylpyrazine; 4, benzaldehyde; 5, 6-methyl-5-hepten-2-one; 6, 2,3,5-trimethylpyrazine; 7, 2-ethenyl-6-methylpyrazine; 8, non-anal; 9, quinoline; 10, o-aminoacetophenone; 11, 2-methylquinoline; 12, 2-methylquinazoline; 13, geranylacetone; 14, tetradecanoic acid; 15, pentadecanoic acid; 16, 9-hexadecanoic acid; 17, hexadecanoic acid; IS internal standard (7-tridecanone) (Graph taken from Apfelbach et al., 2015).

Field Studies in Australia—A Unique Experimental Field

Australia offers a unique opportunity to observe the relationship between predator-naïve native marsupial prey species of various size and introduced placental predators. This situation allows experiments under natural conditions to follow the question whether predator odors will be ignored or investigated or do they elicit aversive reactions. Researchers have accumulated an enormous amount of data exploring the effects of eutherian predator odors on native marsupial mammals. Some of these data will be included in our review.

The introduction of alien predators often has catastrophic effects on populations of native prey species. Australia offers a unique opportunity to observe the relationship between predator-naïve native prey species of various size and introduced predators. It is a challenge to investigate the "evolution" of a balanced relationship between newcomers and endemic species when there is no consensus on how long predator and prey must remain together in order to co-adapt.

For thousands of years, the dingo was the only eutherian predator well-embedded in the Australian food chain. With the arrival of the Europeans in Australia, previously unknown predator species also arrived at this isolated continent. Predators such as the European fox (*Vulpes vulpes*), the feral cat (*Felis catus*), wild dogs (*Canis lupus familiaris*), coyotes (*Canis latrans*), and ferrets (*Mustela putorius furo*) established themselves and started to threaten naïve native species. Correspondingly, quite a high number of field studies have been reported from Australia. In all these studies, complex natural predator odors and no single volatile compounds were employed.

With a few examples, the relationship between predator and prey will be depicted in this section of the review. Special attention is given to the question as to how effective are unknown carnivorous chemicals as repellents to small (<2 Kg), medium sized (>2 Kg) and large prey (>10 Kg) species as compared to their predators. It seems logical that larger prey animals (or those with better defenses or escape ability) respond to a wider variety of chemical signals. Smaller, or more vulnerable, animals

like murids should fear almost any sulfur-rich or nitrogen-containing odor regardless of the type of predator that produced it. There is a question whether a prey (e.g., a rat) should be more discriminating and should respond to single chemical compounds indicating a feline or canine, or whether a kangaroo should respond to canine, but certainly not, feline compounds. Larger prey, or more capable defenders, may also be more discriminating of additional compounds of the odor, such as the concentration ratio (intensity) of meat metabolites, an honest advertisement that a predator has previously consumed prey.

In situations where a prey survives the initial impact of Europe-originated predators, a predator may act as a strong selective agent for prey to develop strategies to manage predation risk. However, Australian studies on the use of unknown predator odor cues by mammalian prey species produced contradictory results. According to Woolhouse and Morgan (1995), some native species avoid the odors of all predators, but these native species were small and fall into the highest risk category for predation (**Table 1**, risk category 4). Data published by Nersesian et al. (2012) and Spencer et al. (2014) supported this finding. Other species appear to respond only to odors of native predators (Dickman, 1993). In some cases, native mammals show no evident avoidance of the odors of native and introduced predators (Blumstein et al., 2002).

Since the establishment of foxes to Australia in the 1870s, these predators have been linked to a local loss and regional extinction of several small to medium-sized mammals and some other vertebrates (Burbidge and McKenie, 1989). Two studies should be particularly emphasized. In 1998, Banks reported on the responses of wild Australian bush rats (*Rattus fuscipes*) to the odor (fresh feces tainted with the urine) of the European fox (*Vulpes vulpes*). Trapping success of rats was compared between clean traps and traps scented with fox odor in winter, spring, and summer. Trapping success was statistically analyzed and no difference between scented and unscented traps was found. Bush rats behaved naïvely toward the predator odor (Banks, 1998). In a similar second study, Banks et al. (2003) analyzed trapping success again using bush rats, with traps scented with dog feces and unscented traps. Bush rats showed no aversion to dog fecal odors and entered unscented and dog-scented traps equally. The researchers concluded that this lack of response may be because rats do not identify fox or dog scats as a cue to predation risk. However, another interpretation for these findings was that animals may have been startled (fled) into traps instead of away from them (a common occurrence that has been corroborated by video evidence), and thus trap-presence should not be a clear indicator for lack of vigilance. This is because animals are often recruited toward a scent in order to investigate additional scent-related information. In another field study (Anson and Dickman, 2013), behavior and in addition glucocorticoid hormone levels were analyzed on the marsupial ringtail possum (*Pseudocheirus peregrinus*). Animals were exposed to fox-suppressed areas and to areas where foxes were abundant. Ringtails showed no physiological or behavioral differences between the two areas. This lack of response to the fox odor may represent complete naivité or strong rapid selection to the invasive predator. Or, based on the relative risks posed by a predator relative to the size of prey, it could reflect the animal's category of risk. For instance, ringtail possums are capable defenders and fit within category 3 (lethal predator but uncommon predation), and would likely require more information such as a composite cue with direct predator presence in the immediate vicinity, or predator cues from a more threatening predator or group of counter-marking predators, before retreating from a preferred food patch. All these findings are somehow surprising, since fox feces is a very powerful odor to elicit escape reactions in European rodents. Mice (highest risk of lethality with predation common; category 4) would be more likely to be wary of any predator scent, regardless of the composite nature of timestamp of delivery. Macropods, the largest of the marsupials, would be the most selective in which predator scents to avoid. This may help explain why macropods can differentiate among risks posed by coyotes, domestic dogs, and dingo—with the primary avoidance response being to urine by dingoes.

Kovacs et al. (2012) reported on two common species of Australian small mammals (bush rat, *Rattus fuscipes,* and brown antechinus, *Antechinus stuartii*) that have persisted for over a century in the presence of the European fox). No difference in prey abundance in sites with high and low fox activity was found. However, survival of the bush rat was almost two-fold higher where fox activity was low. The conclusion of the authors was that populations of both species perform better where the activity of the predator is low. Interestingly, juvenile, but not adult rats, avoided fox odor on traps more strongly where fox activity was high than where it was low, but neither adult *R. fuscipes* nor *A. stuartii* responded differently to different levels of fox activity. Avoidance of fox odor declined over time.

In most areas of Australia, kangaroos enter farming areas and compete with farm animals for food, and are the primary selective agent in shaping forest rehabilitation following fire or anthropogenic disturbance, e.g., the kangaroo palate determines the composition of plant rehabilitation. As a consequence of this behavior, researchers have sought methods to influence food patch selection such that kangaroos would leave food patches with vulnerable, moist seedlings in exchange for mature forage that could compensate following herbivory. They tested the effectiveness of the urine of dingoes and non-native predators like coyotes (*Canis latrans*) to protect farming areas. When they experimentally deployed recent voids of dingo urine, kangaroos (*Macropus* spp.) were highly aroused and fled, some in excess of 50 m from the odor source (Parsons and Blumstein, 2010a). When they presented the novel coyote urine, kangaroos (large prey animals category 3) did not flee, but rather investigated the new smell—possibly to determine whether the scent conveyed enough risk to forgoing a feeding opportunity. This experiment was repeated in Tasmania (Parsons and Blumstein, 2010b) where wallabies (*Macropus rufogrisius*), pademelons (smaller macropods, *Thylagale stigmatica*) and brushtail possums (*Trichosuras vulepcula*) had never been exposed to dingoes (category 2). The outcomes, however, were similar in that all three small species (usually in the high risk categories) avoided the dingo scent (Parsons and Blumstein, 2010b). These outcomes may have been influenced because the dingoes had been regularly fed kangaroo carcasses prior to collection. While attempting to

"synthesize" the urine to assist land rehabilitation, the authors determined that aged urine had completely lost its effectiveness (**Figure 1**) to such a degree that it could actually be used to attract the same animals the fresh urine had once repelled (Parsons et al., 2012). Again, it is assumed this "attraction" is based on attempts by the discriminating animal to obtain further information to warrant a behavioral response.

Kangaroos are both large in comparison to their predators and mobile, and should have a higher level of confidence that a specific chemical represents tangible risks before making a decision to forgo feeding. Therefore, kangaroos should be more discriminating than smaller mammals. This approach was supported when Cox et al. (2010), who learned that kangaroos were not repelled by carnivorous Tasmanian devils (*Sarcophilus harrisii*, a small predator); the authors reversed the outcomes: they fed kangaroo meat to Tasmanian devils, and the kangaroos fled.

Interestingly, Cox et al. (2014) were able to generate a similar response from an exotic non-native species, the tiger (*Pandera tigris*). When fed on kangaroo meat, tiger feces became an effective repellent for kangaroos, sometimes generating an "area-effect." More recently, southern hairy-nosed wombats (*Lasiorhinus latifrons*) have been found to avoid digging in the area where dingo urine or feces had been deployed. Wombats remained in the area, as indicated by fresh tracks, but chose to dig tunnels in areas farther from dingo odors. Similar experiments with dingo urine were performed in Tasmania. Dingoes never entered Tasmania, but it was found that during the trials with natural dingo urine, supplemented with a gel, 78% of wallabies and 80% of possums were repelled (Macey, 2008). The authors again concluded that the smaller prey animals in higher risk categories may have been more sensitive and less discriminating to an unfamiliar predator. Unfortunately, the information about 200 chemical ingredients of dingo urine have not yet been published. Therefore, a comparison with the urine of old world carnivores is not available.

An examination of **Table 1** suggests that the only category 2 interactions where a significant prey response was recorded, occurred from exotic—but large or pack-hunting predators—the tiger and the dingo. Whereas, 14 of the 18 occasions where category 3 or 4 interactions were inferred related in some level of aversion (**Table 1**). The remaining variation may be explained by a combination of single molecule deterrents being trialed such as the domestic dog synthetic used by Ramp et al. (2005), unique response variables such as measuring levels of novel food that has been placed in close proximity to the predator odor—where mixed plumes may conflate the identification of each molecule—or the level of preservation of frozen or partly degraded scents.

At least some prey species do not necessarily escape when a predator odor is encountered. This has been very convincingly shown in an open environment with the spinifex hopping-mouse (*Notomys alexis*), an Australian desert rodent. Spencer et al. (2014) tested the foraging and movement responses of the rodent to non-native predator (fox and cat) urine odor. Urines were collected from a fox just killed prior to urine sampling and stored at ∼1°C until use; cat urine was obtained from euthanized cats. (Unfortunately no information is given in the paper for how long the urines were stored.) Rodents did not respond to these predator odors as one might have expected. Experience with an unknown predator and the stimuli emitted by the predator are for sure decisive for a balanced interaction between predators and prey (Anson and Dickman, 2013). Even in a fish predator, experience, and feeding history determines prey behavior and survival (Lönnstedt et al., 2012).

Conclusions

Based on the lessons we have learned from the recent literature, particularly from field trials in Australia, we now have a heightened awareness of the following three factors that may influence reported outcomes:

(1) Complexity of the molecular signal: Several studies have tried to find universal carnivore signals that, when received by a potential prey species, will be adequately responded to, even when the prey species never had encountered that predator or its odor before. Indeed, there are some volatile compounds found in many carnivores that elicit defensive and/or even fear reactions in a prey. Almost any sulfur-rich or nitrogen-containing compound elicits such behaviors regardless of the predator source. Generally, these single compounds are not effective alone (except TMT), but require other accompanying molecules to gain efficiency. Most often, single volatile substances convey very limited information about the predator. In contrast, arrays of different volatile compounds may convey more relevant information to the receiver, for example, the type of food the predator has consumed.

(2) Chemical stability of the signal: A major consideration in all predator-prey studies is the stability of the chemical compounds and thus stability of the message. As demonstrated, aging of an odor bouquet could result in a modified information or even loss of the message.

(3) Variable risk posed by the predator as compared to the prey: When evaluating the effectiveness of an odor used as a repellent, the size, and defensive abilities of the prey species has to be taken into account. In particular, Cox et al. (2010) showed that the largest macropods, Eastern gray kangaroos, will ignore recently-voided urine from the non-threatening, small Tasmanian devil. However, this wild-type response (e.g., ignoring small predators) was changed when the risk category was reversed by adding kangaroo meat to the predator diet. And this helped support our position that single molecule scents are less likely to be effective in deterring large herbivores, because larger animals may require more biologically relevant information than what a single molecule can provide. The Australian studies also demonstrate that not all potential prey species respond to unknown carnivore odors (not even to fox feces containing TMT) with defensive behaviors. Obviously they do not identify innately predator scents as a cue to predation risk; some even will show odor exploration behaviors when an unknown odor is presented. However, some species originally ignoring unknown predator odors

learn to associate such odors over time, especially after the predator has preyed conspecifics.

Acknowledgments

This review article is the result of a cooperation project between RA (he designed the paper and wrote the first draft), MP (he added the Australian studies), HS, and MN (both worked and expanded the chemical part of the here presented article). We thank the reviewers for their help to improve the article by suggesting aspects we did not think about. We acknowledge support by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tübingen University.

References

Albone, E. S., and Perry, G. C. (1975). Anal sac secretion of the red fox, *Vulpes vulpes*; volatile fatty acids and diamines: implications for a fermentation hypothesis of chemical recognition. *J. Chem. Ecol.* 2, 101–111. doi: 10.1007/BF00988029

Andersen, K. K., and Bernstein, D. T. (1975). Some chemical constituents of the scent of the striped skunk (*Mephitis mephitis*). *J. Chem. Ecol.* 1, 493–499.

Andersen, K. K., Bernstein, D. T., Caret, R. L., and Romanczyk, L. J. Jr. (1982). Chemical constituents of the defensive secretion of the striped skunk (*Mephitis mephitis*). *Tetrahedron* 38, 1965–1970. doi: 10.1016/0040-4020(82)80046-X

Andreolini, F., Jemiolo, B., and Novotny, M. V. (1987). Dynamics of excretion of urinary chemosignals in the house mouse (*Mus musculus*) during the natural estrous cycle. *Experientia* 43, 998–1002. doi: 10.1007/BF01952217

Anson, J. R., and Dickman, C. R. (2013). Behavioral responses of native prey to disparate predators: naivité and predator recognition. *Oecologia* 17, 367–377. doi: 10.1007/s00442-012-2424-7

Apfelbach, R., Blanchard, C. D., Blanchard, R., Hayes, R. A., and McGregor, I. S. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neurosci. Biobehav. Rev.* 29, 1123–1144. doi: 10.1016/j.neubiorev.2005.05.005

Apfelbach, R., Soini, H. A., Vasilieva, N. Y., and Novotny, M. V. (2015). Behavioral responses of predator-naïve dwarf hamsters (*Phodopus campbelli*) to odor cues of the European ferret fed with different prey species. *Physiol. Behav.* 146, 57–66. doi: 10.1016/j.physbeh.2015.04.014

Arnould, C., Malosse, C., Signoret, J. P., and Descoins, C. (1998). Which chemical constituents from dog feces are involved in its food repellent effect in sheep? *Chem. Senses* 24, 559–576.

Ayers, L. W., Asok, A., Heyward, F. D., Jeffrey, B., and Rosen, J. B. (2013). Freezing to the predator odor 2,4,5-dihydro-2,5-trimethylthiazoline (TMT) is disrupted by olfactory bulb removal but not trigeminal deafferentation. *Behav. Brain Res.* 253, 54–59. doi: 10.1016/j.bbr.2013.06.034

Bailey, S., Bunyan, P. J., and Page, M. J. (1980). "Variation in the levels of some components of the volatile fraction of urine from captive red foxes (*Vulpes vulpes*) and its relationship to the state of the animal," in Chemical Signals: Vertebrates and Aquatic Invertebrates, eds D. Müller-Schwartze and R. M. Silverstein (New York, NY: Plenum Press), 391–404.

Banks, P. B. (1998). Responses of Australian bush rats, *Rattus fuscipes*, to the odor of introduced *Vulpes vulpes*. *J. Mammal.* 79, 1260–1264. doi: 10.2307/1383017

Banks, P. B., Nelika, K., Hughes, K. N., and Rose, T. (2003). Do native Australian small mammals avoid faeces of domestic dogs? Responses of Rattus fuscipes and Antechinus stuartii. *Aust. Zool.* 32, 406–409. doi: 10.7882/AZ.2002.018

Berdoy, M., Webster, J. P., and MacDonald, D. W. (2000). Fatal attraction in rats infected with *Toxoplasma gondii*. *Proc. Biol. Sci.* 267, 1591–1594. doi: 10.1098/rspb.2000.1182

Berton, F., Vogel, E., and Belzung, C. (1998). Modulation of mice anxiety in response to cat odor as a consequence of predator's diet. *Physiol. Behav.* 65, 247–254. doi: 10.1016/S0031-9384(98)00126-7

Blanchard, D. C., Griebel, G., and Blanchard, R. J. (2003). Conditioning and residual emotionality effects of predator stimuli: some reflections on stress and emotion. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 1177–1185. doi: 10.1016/j.pnpbp.2003.09.012

Blomquist, G. J., and Bagnères, A.-G. (eds.). (2010). *Insect Hydrocarbons. Biology, Biochemistry and Chemical Ecology*. Cambridge: Cambridge University Press.

Blumstein, D. T., Mari, M., Daniel, J. C., Ardron, J. G., Griffin, A. S., and Evans, C. S. (2002). Olfactory predator recognition: wallabies may have to learn to be wary. *Anim. Conserv.* 5, 87–93. doi: 10.1017/S1367943002002123

Brinck, C., Erlinge, S., and Sandell, M. (1983). Anal sac secretion in mustelids. A comparison. *J. Chem. Ecol.* 9, 727–744. doi: 10.1007/BF00988779

Brunswik, E. (1956). *Perception and Representative Design of Psychological Experiments*. Berkeley, CA: University of California Press.

Burbidge, A. A., and McKenie, N. L. (1989). Patterns in the modern decline of Western Australia vertebrate fauna – causes and conservation implications. *Biol. Conserv.* 50, 143–198. doi: 10.1016/0006-3207(89)90009-8

Burger, B. V., Visser, R., Moses, A., and Le Roux, M. (2006). Elemental sulfur identified in urine of cheetah, *Acinonyx jubatus*. *J. Chem. Ecol.* 32, 1347–1352. doi: 10.1007/s10886-006-9056-5

Burwash, M. D., Tobin, M. E., Woolhouse, A. D., and Sullivan, T. P. (1998). Field testing synthetic predator odors for roof rats (*Rattus rattus*) in Hawaiian Macademia nut orchards. *J. Chem Ecol.* 24, 603–630. doi: 10.1023/A:1022304917652

Bytheway, J. P., Carthey, A. J., and Banks, P. B. (2013). Risk vs. reward: how predators and prey respond to aging olfactory cues. *Behav. Ecol. Sociobiol.* 67, 715–725. doi: 10.1007/s00265-013-1494-9

Carr, W. J., Martorano, R. D., and Krames, L. (1970). Response of mice to odors associated with stress. *J. Comp. Physiol. Psychol.* 71, 223–228. doi: 10.1037/h0029164

Carr, W. J., Roth, P. A., and Amore, M. (1971). Responses of male mice to odors from stressed vs nonstressed males and females. *Psychon. Sci.* 25, 275–276. doi: 10.3758/BF03335874

Colyer, S. W. (1971). *Valence of Stress and Nonstress Index in Odors and the Effectiveness of post Stress Odor Emission in Mice*. Unpublished doctoral dissertation, Temple University.

Coppola, D. M., and Vandenbergh, J. G. (1985). Effect of density, duration of grouping and age of urine stimulus on the puberty delay pheromone in female mice. *J. Reprod. Fertil.* 73, 517–522. doi: 10.1530/jrf.0.0730517

Cox, T. E., Murray, P. J., Bengsen, A. J., Hall, G. P., and Li, X. (2014). Do fecal odors from native and non-native predators cause a habitat shift among macropods? *Wildl. Soc. Bull.* 39, 159–164. doi: 10.1002/wsb.509

Cox, T. E., Murray, P. J., Hall, G. P., and Li, X. (2010). Pest responses to odors from predators fed a diet of target species conspecifics and heterospecifics. *J. Wildl. Manag.* 74, 1737–1744. doi: 10.2193/2009-410

Cremona, T., Crowther, M. S., and Webb, J. K. (2014). Variation of prey responses to cues from a mesopredator and an apex predator. *Aust. Ecol.* 39, 749–754. doi: 10.1111/aec.12138

Crump, D. R. (1980). Anal gland secretion of the ferret. *J. Chem. Ecol.* 6, 837–844. doi: 10.1007/BF00990407

Crump, D. R., and Moors, P. J. (1985). Anal gland secretions of the stoat (*Mustela ermine*) and the ferret (*Mustela putorius* forma *furo*). Some additional thietane components. *J. Chem. Ecol.* 11, 1037–1043. doi: 10.1007/BF01020673

Derby, C. D., and Aggio, J. F. (2011). The neuroecology of chemical defenses. *Int. Comp. Biol.* 51, 771–780. doi: 10.1093/icb/icr063

Descovich, K. A., Lisle, A. T., Johnston, S., Nicolson, V., and Phillips, C. J. (2012). Differential responses of captive southern hairy-nosed wombats (*Lasiorhinus latifrons*) to the presence of faeces from different species and male and female conspecifics. *Appl. Anim. Behav. Sci.* 138, 110–117. doi: 10.1016/j.applanim.2012.01.017

Dias, B. G., and Ressler, K. J. (2014). Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nat. Neurosci.* 17, 89–96. doi: 10.1038/nn.3594

Dickman, C. R. (1993). Raiders of the last ark: cats in island Australia. *Aust. Nat. Hist.* 24, 44–52.

Dielenberg, R. A., Hunt, G. E., and MacGregor, I. S. (2001). "When a rat smells a cat": the distribution of Fos immunoreactivity in rat brain following

exposure to a predator odor. *Neuroscience* 104, 1085–1097. doi: 10.1016/S0306-4522(01)00150-6

Downes, S. J. (2002). Does responsiveness to predator scents affect lizard survivorship? *Behav. Ecol. Sociobiol*. 52, 38–42. doi: 10.1007/s00265-002-0477-z

Endres, T., Apfelbach, R., and Fendt, M. (2005). Behavioral changes induced in rats by exposure to trimethylthiazoline, a component of fox odor. *Behav. Neurosci*. 119, 1004–1010. doi: 10.1037/0735-7044.119.4.1004

Engelhart, A., and Müller-Schwarze, D. (1995). Responses of beaver (*Castor canadensis* Kuhl) to predator chemicals. *J. Chem. Ecol*. 21, 1349–1364. doi: 10.1007/BF02027567

Fendt, M., Endres, T., and Apfelbach, R. (2003). Temporary inactivation of the bed nucleus of the stria terminalis but not of the amygdala blocks freezing induced by trimethylthiazoline, a component of fox feces. *J. Neurosci*. 23, 23–28.

Fendt, M., Endres, T., Lowry, C. A., Apfelbach, R., and McGregor, I. S. (2005).TMT-induced autonomic and behavioral changes and the neural basis of its processing. *Neurosci. Biobehav. Rev*. 29, 1145–1156. doi: 10.1016/j.neubiorev.2005.04.018

Ferrero, D. M., Lemon, J. K., Fluegge, D., Pashkovski, S. L., Korzan, W. J., Datta, S. R., et al. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. U.S.A*. 108, 11235–11240. doi: 10.1073/pnas.1103317108

Gijs, L., Chevance, F., Jerkovic, V., and Collin, S. (2002). How low pH can intensify β-damascenone and dimethyltrisulfide production through beer aging. *J. Agric. Food Chem*. 50, 5612–5616 doi: 10.1021/jf020563p

Hayes, R. A., Nahrung, H. F., and Wilson, J. C. (2006). The response of native Australian rodents to predator odours varies seasonally: a by-product of life history variation? *Anim. Behav*. 71, 1307–1314. doi: 10.1016/j.anbehav.2005.08.017

Head, M. L., Keogh, J. S., and Doughty, P. (2002). Experimental evidence of an age-specific shift in chemical detection of predators in a lizard. *J. Chem. Ecol*. 28, 541–554. doi: 10.1023/A:1014592028693

Hegab, I. M., Jin, Y., Ye, M., Wang, A., Yin, B., Yang, S., et al. (2014). Defensive responses of Brandt's voles (*Lasiopodomys brandtii*) to stored cat feces. *Physiol. Behav*. 123, 193–199. doi: 10.1016/j.physbeh.2013.10.030

Hendriks, W. H., Moughan, P. J., Tarttelin, M. F., and Woolhouse, A. D. (1995b). Felinine: a urinary amino acid of felidae. *Comp. Biochem. Physiol. B Biochem. Mol. Biol*. 112, 581–588. doi: 10.1016/0305-0491(95)00130-1

Hendriks, W. H., Tarttelin, M. E., and Moughan, P. J. (1995a). Twenty-four hour felinine (corrected) excretion patterns in entire and castrated cats. *Physiol. Behav*. 58, 467–469. doi: 10.1016/0031-9384(95)00084-V

Hoffmann, F., Musolf, K., and Penn, D. J. (2009). Freezing urine reduces its efficacy for eliciting ultrasonic vocalizations from male mice. *Physiol. Behav*. 96, 602–605. doi: 10.1016/j.physbeh.2008.12.014

Inagaki, H., Kiyokawa, Y., Tamogami, S., Watanabe, H., Takeuchi, Y., and Mori, Y. (2014). Identification of a pheromone that increases anxiety in rats. *Proc. Nat. Acad. Sci. U.S.A*. 111, 18751–18756. doi: 10.1073/pnas.1414710112

Jemiolo, M., Harvey, S., and Novotny, M. V. (1986). Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents. *Proc. Natl. Acad. Sci. U.S.A*. 83, 4576–4579. doi: 10.1073/pnas.83.12.4576

Jorgenson, J. W., Novotny, M. V., Carmack, M., Copland, G. B., Wilson, S. R., Katona, S., et al. (1978). Chemical scent constituents in the urine of the red fox (*Vulpes vulpes* L.) during the winter season. *Science* 199, 796–798. doi: 10.1126/science.199.4330.796

Kaissling, K. E. (2014). "Pheromone reception in insects: the example of silk moths," in *Neurobiology of Chemical Communication, Chapter 4*, ed C. Mucignat-Caretta (Boca Raton,FL: CRC Press), 99–146.

Kavaliers, M., and Colwell, D. D. (1995). Decreased predator avoidance in parasitized mice: neuromodulatory correlates. *Parasitology* 111, 257–263. doi: 10.1017/S0031182000081816

Kendrick, K. M. (2014). Pheromones: the scent of a male. *Curr. Biol*. 24, R228–R230. doi: 10.1016/j.cub.2014.02.019

Kovacs, E. K., Crowther, M. S., Webb, J. K., and Dickman, C. R. (2012). Population and behavioral responses of native prey to alien predation. *Oecologia* 168, 947–957. doi: 10.1007/s00442-011-2168-9

Kwak, J., Grigsby, C. C., Preti, G., Rizki, M. M., Yamazaki, K., and Beauchamp, G. K. (2013a). Changes in volatile compounds of mouse urine as it ages: their interactions with water and urinary proteins. *Physiol. Behav*. 120, 211–219. doi: 10.1016/j.physbeh.2013.08.011

Kwak, J., Grigsby, C. C., Smith, B. R., Rizki, M. M., and Preti, G. (2013b). Changes in volatile compounds of human urine as it ages: their interaction with water. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci*. 941, 50–53. doi: 10.1016/j.jchromb.2013.09.040

Lindgren, P. M. F., Sullivan, T. P., and Crump, D. R. (1995). "Review of synthetic predator odor semiochemicals as repellents for wildlife management in the Pacific Northwest," in *National Wildlife Research Center Repellents Conference, Paper 24* (Lincoln: University of Nebraska).

Lloyd, R., Alford, R. A., and Schwarzkopf, L. (2009). Chemical discrimination among predators by lizards: responses of three skink species to the odours of high—and low—threat varanid predators. *Aust. Ecol*. 34, 50–54. doi: 10.1111/j.1442-9993.2008.01881.x

Lönnstedt, O. M., McCormick, M. I., Meekan, M. G., Ferrari, M. C. O., and Chivers, D. P. (2012). Learn and live: predator experience and feeding history determines prey behaviour and survival. *Proc. R. Soc. B* 279, 2091–2098. doi: 10.1098/rspb.2011.2516

Ma, W., Miao, Z., and Novotny, M. V. (1998). Role of the adrenal gland and adrenal-mediated chemosignals in suppression of estrus in the house mouse: the Lee-Boot Effect revisited. *Biol. Reprod*. 59, 1317–1320. doi: 10.1095/biolreprod59.6.1317

Macey, R. (2008, June 12). Dingo pee a potent possum 'piss off'. *Environment*.

May, M. D., Bowen, M. T., McGregor, I. S., and Timberlake, W. (2012). Rubbings deposited by cats elicit defensive behavior in rats. *Physiol. Behav*. 107, 711–718. doi: 10.1016/j.physbeh.2012.03.007

McGregor, I. S., Schrama, L., Ambermoon, P., and Dielenberg, R. A. (2002). Not all "predator odours" are equal: cat odor but not 2,4,5-trimethylthiazoline (TMT; fox odour) elicits specific defensive behaviours in rats. *Behav. Brain Res*. 129, 1–16. doi: 10.1016/S0166-4328(01)00324-2

Mella, V. S. A., Banks, P. B., and McArthur, C. (2014b). Negotiating multiple cues of predation risk in a landscape of fear: what scares free—ranging brushtail possums? *J. Zool*. 294, 22–30. doi: 10.1111/jzo.12146

Mella, V. S. A., Cooper, C. E., and Davies, S. J. (2014a). Behavioural responses of free—ranging western grey kangaroos (*Macropus fuliginosus*) to olfactory cues of historical and recently introduced predators. *Aust. Ecol*. 39, 115–121. doi: 10.1111/aec.12050

Miyazaki, M., Yamashita, T., Suzuki, Y., Saito, Y., Soeta, S., Taira, H., et al. (2006). A major urinary protein of the domestic cat regulates the production of felinine, a putative pheromone precursor. *Chem. Biol*. 13, 1071–1079. doi: 10.1016/j.chembiol.2006.08.013

Mokbel, I., Razzouk, A., Sawaya, T., and Jose, J. (2009). Experimental vapor pressures of 2-phenylethylamine, benzylamine, trimethylamine, and cis-2,6-dimethylpiperidine in the range between 0.2 Pa and 75 *Pa. J. Chem. Eng. Data* 54, 919–822. doi: 10.1021/je800603z

Morrow, B. A., Elsworth, J. D., and Roth, R. H. (2002). Fear-like biochemical and behavioral responses in rats to the predator odor, TMT, are dependent on the exposure environment. *Synapse* 46, 11–18. doi: 10.1002/syn.10109

Müller-Velten, H. (1966). Über den angstgeruch bei der Hausmaus (*Mus musculus* L.). *Z. vergl. Physiol*. 52, 401–429. doi: 10.1007/BF00302294

Munoz, D., Peinado, R. A., Medina, M., and Moreno, J. (2007). Biological aging of sherry wines under periodic and controlled microaerations with *Saccharomyces cerevisiae* var. capensis: effect on odorant series. *Food Chem*. 100, 1188–1195. doi: 10.1016/j.foodchem.2005.10.065

Murray, P. J., Burns, A. C., and Davy, J. R. (2006). Development of an animal repellent—selection, efficacy and presentation. *Anim. Product. Sci*. 46, 851–856. doi: 10.1071/EA06004

Nersesian, C. L., Banks, P. B., and McArthur, C. (2012). Behavioural responses to indirect and direct predator cues by a mammalian herbivore, the common brushtail possum. *Behav. Ecol. Sociobiol*. 66, 47–55. doi: 10.1007/s00265-011-1250-y

Nolte, D. L., Mason, J. R., Epple, G., Aranov, E., and Campbell, D. L. (1994). Why are predator odors aversive to prey? *J. Chem. Ecol*. 20, 1505–1516. doi: 10.1007/BF02059876

Novotny, M. V., Harvey, S., and Jemiolo, B. (1990). Chemistry of male dominance in the house mouse, *Mus domesticus*. *Experientia* 46, 109–113. doi: 10.1007/BF01955433

Novotny, M. V., Jemiolo, B., Harvey, S., Wiesler, D., and Marchlewska-Koj, A. (1986). Adrenal-mediated endogenous metabolites inhibit puberty in female mice. *Science* 231, 722–725. doi: 10.1126/science.3945805

Onishi, M., Guymon, J. F., and Crowell, E. A. (1977). Changes in some volatile constituents of brandy during aging. *Am. J. Enol. Vitic.* 28, 152–158.

Osada, K., Kurihara, K., Izumi, H., and Kashiwayanagi, M. (2013). Pyrazine analogues are active components of wolf urine that induce avoidance and freezing behaviours in mice. *PLoS ONE* 8:e61753. doi: 10.1371/journal.pone.0061753

Parsons, M. H., and Blumstein, D. T., (2010a). Familiarity breeds contempt: kangaroos persistently avoid areas with experimentally deployed dingo scents. *PLoS ONE* 5:e10403. doi: 10.1371/journal.pone.0010403

Parsons, M. H., and Blumstein, D. T. (2010b). Feeling vulnerable? Indirect risk cues differently influence how two marsupials respond to novel dingo urine. *Ethology* 116, 972–980. doi: 10.1111/j.1439-0310.2010.01810.x

Parsons, M. H., Blumstein, D. T., and Dods, K. C. (2012). An *in situ* vertebrate bioassay helps identify potential matrices for a predator—based synthetic management tool. *Wildl. Soc. Bull.* 36, 383–388. doi: 10.1002/wsb.144

Parsons, M. H., Lamont, B. B., Kovacs, B. R., and Davies, S. J. (2007). Effects of novel and historic predator urines on semi—wild Western Grey Kangaroos. *J. Wildl. Manag.* 71, 1225–1228. doi: 10.2193/2006-096

Powell, F., and Banks, P. B. (2004). Do house mice modify their foraging behaviour in response to predator odours and habitat? *Anim. Behav.* 67, 753–759. doi: 10.1016/j.anbehav.2003.08.016

Preti, G., Muetterties, E. L., Furman, J. M., Kennelly, J. J., and Johns, B. E. (1976). Volatile constituents of dog (*Canis familiaris*) and coyote (*Canis latrans*) anal sacs. *J. Chem. Ecol.* 2, 177–186. doi: 10.1007/BF00987740

Ramp, D., Russell, B. G., and Croft, D. B. (2005). Predator scent induces differing responses in two sympatric macropodids. *Aust. J. Zool.* 53, 73–78. doi: 10.1071/ZO04053

Raymer, J., Wiesler, D., Novotny, M., Asa, C., Seal, U. S., and Mech, L. D. (1984). Volatile constituents of wolf (*Canis lupus*) urine as related to gender and season. *Experientia* 40, 707–709. doi: 10.1007/BF01949734

Raymer, J., Wiesler, D., Novotny, M., Asa, C., Seal, U. S., and Mech, L. D. (1986). Chemical scent constituents in urine of wolf (*Canis lupus*) and their dependence on reproductive hormones. *J. Chem. Ecol.* 12, 297–314. doi: 10.1007/BF01045612

Russell, B. G., and Banks, P. B. (2005). Responses of four Critical Weight Range (CWR) marsupials to the odours of native and introduced predators. *Aust. Zool.* 33, 217–222. doi: 10.7882/AZ.2005.018

Russell, B. G., and Banks, P. B. (2007). Do Australian small mammals respond to native and introduced predator odors? *Aust. Ecol.* 32, 277–286. doi: 10.1111/j.1442-9993.2007.01685.x

Schildknecht, H., and Birkner, C. (1983). *Struktur und Wirkung der Musteliden-Ökomene III. Analyse der Analbeutelsekrete Mitteleuropäischer Musteliden. Chemiker-Ztg* 107, 267–270.

Schildknecht, H., Birkner, C., and Krau,ß, D. (1981). *Struktur und Wirkung der Musteliden-Ökomene II. Erweiterte Analyse des Analbeutelsekrets des Nerzes. Mustela Vison* L. *Chemiker-Ztg* 105, 273–286.

Schildknecht, H., and Hiller, H. (1984). Struktur und wirkung der musteliden-ökomene IV. Analyse verhaltensaktiver Drüsensekrete des Dachses (*Meles meles* L.). *Chemiker- Ztg.* 108, 1–5.

Schildknecht, H., Wilz, I., Enzmann, F., Grund, N., and Ziegler, M. (1976). Mustelan, the malodorous substance from the anal gland of the mink (*Mustela vison*) and the polecat (*Mustela putorius*). *Angw. Chem. Int. Ed. Engl.* 4, 242–243. doi: 10.1002/anie.197602421

Schneider, D., Kasang, G., and Kaissling, K. E. (1968). Determination of the olfactory threshold of *Bombyx mori* with tritium-labelled bombycol. *Naturwissenschaften* 55, 395. doi: 10.1007/BF00593307

Spencer, E. E., Crowther, M. S., and Dickman, C. R. (2014). Risky business: do native rodents use habitat and odor cues to manage predation risk in Australian deserts? *PLoS ONE* 9:e90566. doi: 10.1371/journal.pone.0090566

Staples, L. G., and McGregor, I. S. (2006). Defensive responses of Wistar and Sprague-Dawley rats to cat odour and TMT. *Behav. Brain Res.* 172, 351–354. doi: 10.1016/j.bbr.2006.04.011

Sullivan, T. P., Crump, D. R., and Sullivan, D. S. (1988a). Use of predator odors as repellents to reduce feeding damage by herbivores: III. Northern Montane and meadow voles (*Microtus montanus* and *Microtus pennsylvanicus*). *J. Chem. Ecol.* 14, 363–377. doi: 10.1007/BF01022552

Sullivan, T. P., Crump, D. R., and Sullivan, D. S. (1988b). Use of predator odors as repellents to reduce feeding damage by herbivores: IV. Montane and meadow voles (*Microtus montanus* and *Microtus pennsylvanicus*). *J. Chem. Ecol.* 14, 379–389. doi: 10.1007/BF01022553

Takahashi, L. K. (2014). Olfactory systems and neural circuits that modulate predator odor fear. *Front. Behav. Neurosci* 8:72. doi: 10.3389/fnbeh.2014.00072

Trinh, K., and Storm, D. R. (2003). Vomeronasal organ detects odorants in absence of signaling through main olfactory epithelium. *Nat. Neurosci.* 6, 519–525. doi: 10.1038/nn1039

Vernet-Maury, E. (1980). Trimethylthiazoline in fox feces: a natural alarming substance for the rat. *Olfaction Taste* 7, 407.

Vet, L. E. M. (1999). From chemical to population ecology: infochemical use in an evolutionary context. *J. Chem. Ecol.* 25, 31–49. doi: 10.1023/A:1020833015559

Voznessenskaya, V. V. (2014). "Influence of cat odor on reproductive behavior and physiology in the house mouse (*Mus musculus*)", in *Neurobiology of Chemical Communication*, ed C. Mucignat-Caretta (Boca Raton, FL: CRC Press), 389–406.

Wallace, K. J., and Rosen, J. B. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behav. Neurosci.* 114, 912–922. doi: 10.1037/0735-7044.114.5.912

Whitten, W. K., Wilson, M. C., Jorgenson, J. W., Novotny, M. V., and Carmack, M. (1980). Induction of marking behavior in wild red foxes *(Vulpes vulpes L.)* by synthetic urinary constituents. *J. Chem. Ecol.* 6, 49–55. doi: 10.1007/BF00987526

Wilson, M. C., Whitten, W. K., Wilson, S. R., Jorgenson, J. W., Novotny, M., and Carmack, M. (1980). "Marking behavior in wild red foxes in response to synthetic volatile urinary compounds," in *Chemical Signals: Vertebrates and Aquatic Invertebrates*, eds D. Müller-Schwartze and R. M. Silverstein (New York, NY: Plenum Press), 29–38. doi: 10.1007/978-1-4684-1027-3_3

Woolfson, A., and Rothschild, M. (1990). Speculating about pyrazines. *Proc. R. Soc. Lond. B* 242, 113–119. doi: 10.1098/rspb.1990.0113

Woolhouse, A. D., and Morgan, D. R. (1995). An evaluation of repellents to suppress browsing by possums. *J. Chem. Ecol.* 21, 1571–1583. doi: 10.1007/BF02035153

Zhang, J. X., Soini, H. A., Bruce, K. E., Wiesler, D., Woodley, S. K., Baum, M. J., et al. (2005). Putative chemosignals of the ferret (*Mustela furo*) associated with individual and gender recognition. *Chem. Senses* 30, 727–737. doi: 10.1093/chemse/bji065

Zöttl, M., Lienert, R., Clutton-Brock, T., Millesi, E., and Manser, M. B. (2013). The effects of recruitment to direct predator cues on predator responses in meerkats. *Behav. Ecol.* 24, 198–204. doi: 10.1093/beheco/ars154

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 25 August 2015
doi: 10.3389/fnins.2015.00292

The smell of fear: innate threat of 2,5-dihydro-2,4,5-trimethylthiazoline, a single molecule component of a predator odor

*Jeffrey B. Rosen *, Arun Asok and Trisha Chakraborty*

Department of Psychological and Brain Sciences, University of Delaware, Newark, DE, USA

In the last several years, the importance of understanding what innate threat and fear is, in addition to learning of threat and fear, has become evident. Odors from predators are ecologically relevant stimuli used by prey animals as warnings for the presence of danger. Of importance, these odors are not necessarily noxious or painful, but they have innate threat-like properties. This review summarizes the progress made on the behavioral and neuroanatomical fundamentals of innate fear of the predator odor, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), a component of fox feces. TMT is one of several single molecule components of predator odors that have been isolated in the last several years. Isolation of these single molecules has allowed for rapid advances in delineating the behavioral constraints and selective neuroanatomical pathways of predator odor induced fear. In naïve mice and rats, TMT induces a number of fear and defensive behaviors, including robust freezing, indicating it is an innate threat stimulus. However, there are a number of behavioral constraints that we do not yet understand. Similarly, while some of the early olfactory sensory pathways for TMT-induced fear are being delineated, the pathways from olfactory systems to emotional and motor output regions are less well understood. This review will focus on what we know and what we still need to learn about the behavior and neuroanatomy of TMT-induced fear.

Keywords: predator, odor, fear, 2,5-dihydro-2,4,5-trimethylthiazoline, TMT, olfaction

OPEN ACCESS

Edited by:
Markus Fendt,
Otto-von-Guericke University
Magdeburg, Germany

Reviewed by:
Thomas Endres,
Otto-von-Guericke University,
Germany
John Wemmie,
University of Iowa, USA

****Correspondence:***
Jeffrey B. Rosen,
Department of Psychological and
Brain Sciences, University of
Delaware, 105 The Green Room 108,
Newark, DE 19716, USA
jrosen@psych.udel.edu

Specialty section:
This article was submitted to
Systems Biology,
a section of the journal
Frontiers in Neuroscience

Received: *10 July 2015*
Accepted: *04 August 2015*
Published: *25 August 2015*

Citation:
Rosen JB, Asok A and Chakraborty T
(2015) The smell of fear:
innate threat of
2,5-dihydro-2,4,5-trimethylthiazoline,
a single molecule component of a
predator odor. Front. Neurosci. 9:292.
doi: 10.3389/fnins.2015.00292

Understanding how environmental stimuli motivate and influence behavior is one of the fundamental questions in behavioral neuroscience. Over the last several decades there has been tremendous progress toward answering this question. One of the more successful endeavors has been the study of defensive behaviors produced by threatening, dangerous, and fearful stimuli in rodents (Davis et al., 2010; LeDoux, 2012). This has primarily been studied using associative fear conditioning paradigms whereby a neutral stimulus, typically a tone, light, or context, is paired with an aversive stimulus, usually a foot shock, to become a fear conditioned stimulus. Fear conditioning research has been extremely informative for understanding how threat (i.e., fear) is processed across different levels of analysis (e.g., behavior, neuroanatomy, neurochemistry, genetics). In particular, fear conditioning research has provided insight into how animals behaviorally adapt, anticipate, and respond to a vast number of aversive and threatening environmental stimuli and potentially threatening situations. Importantly, Pavlovian fear conditioning paradigms have been instrumental for delineating the neural circuits necessary for learning about threats and fears that route through the amygdala (these will be discussed later). These circuits and the cellular

and molecular mechanisms within these circuits important for learning and expressing fear are continually being refined (Herry and Johansen, 2014; Do-Monte et al., 2015; Janak and Tye, 2015).

Although the ability to adapt, anticipate, and learn during situations of threat and danger is paramount for survival, another way to increase survival in the face of danger is to have hardwired "learning-independent" systems. That is, systems for assessing threatening stimuli having prepotent qualities, which drive defensive behavior without prior learning. Many animals, including humans, appear to have species-specific innate sensitivity to the threat (and appetitive) qualia of various stimuli. In humans, fears, and phobias may cluster into at least three spheres: heights, blood/injection/injury, and situational/animal (de Jongh et al., 2011). Many people have a fear of spiders or of snakes (Oosterink et al., 2009), which may be innate or have prepotency for quick learning (Ohman and Mineka, 2001; Poulton and Menzies, 2002; Rachman, 2002). Predators (e.g., cats, foxes, weasels) of mice and rats, and odors from these predators, appear to have these prepotent qualities and have therefore been used to study behavior to innate threat or fear in the laboratory (Dielenberg and McGregor, 2001; Rosen, 2004; Masini et al., 2006; Blanchard et al., 2013; Takahashi, 2014; Brennan and Keverne, 2015). The innate quality of these predator stimuli is implied by the fact that the stimuli produce robust defensive behaviors upon the first exposure in laboratory rodent strains that have not encountered these predators for generations. While live predators are more natural and present the full complement of multimodal threat stimuli, their complexity hinders studying the neural circuitry from selective sensory input to the defensive behavioral outputs in the laboratory. The use of predator odors has been beneficial to this end in that they have allowed for the precise examination of defensive behaviors produced by stimulating a single sensory modality. Although attempts have been made to gain better control of the sensory features stimulated by live predators (e.g., a robotic "predator" has been developed to simulate a moving and looming threat Choi and Kim, 2010), they are limited in that they provide an artificial threat and it is unclear how selective or innate the threat actually is. Predator odors, particularly single molecule components of these natural odors, have high sensory selectivity and have therefore become increasingly studied in neuroscience laboratories.

Whereas live predators are innate fear-inducers, and thus are simplified models of fear relative to conditioned fear because no learning is necessary, predator odors are even simpler models because the odor primarily limits the sensory systems involved to olfaction. Predator odors such as those derived from bodily secretions (e.g., urine and feces) are kairomones—semiochemicals emitted by an organism that benefits an organism of another species—and trigger the expression of innate fear behaviors in prey. However, single molecule components of natural odors derived from predator secretions are the simplest models used to induce fear because they reduce the sensory system to fewer receptors than natural, complex predator odors. Furthermore, these single molecule components (referred to as single molecule predator odors throughout the rest of the manuscript) should facilitate the discovery of precise neurobiological and neuroanatomical circuitries of fear (Stowers and Logan, 2010).

There are a number of recently identified single molecule predator odors that have been shown to induce innate fear and defensive behaviors in rodents and other animals. In particular, research has identified a class of single molecule olfactory signals, the major urinary proteins (MUPs), which are capable in and of themselves in driving defensive behaviors in rats and mice. For example, Feld4, a homolog of MUPs, was recently isolated from cat fur—a predator stimulus that has been used for several decades as a fear and anxiety-inducing stimulus (Papes et al., 2010). Feld4 in cat fur likely comes from cat saliva deposited on the fur during self-grooming behavior. It induces defensive behavior in mice (Papes et al., 2010). The same researchers also found that another MUP from rat urine (rMUP13) induced defensive behavior in mice. Both Feld4 and rMUP13 bind to the transient receptor potential cation channel, subfamily C, member 2 (TrpC2) receptor in the vomeronasal organ, but not the main olfactory bulb.

Another single molecule predator odor, 2-phenylethylamine, was isolated from a large number of carnivore urines, including bobcat, weasel, ferret and fox urine, and shown to induce defensive behavior in both rats and mice (Ferrero et al., 2011). The presence of 2-phenylethylamine was found to be more than 50 times higher in carnivore urines relative to non-carnivorous species. Importantly, a single odor receptor, trace amine-associated receptor 4 (TAAR4), was identified as the primary olfactory receptor responsible for elicitation of defensive behavior to 2-phenylethylamine (Ferrero et al., 2011). Another trace amine-associated receptor (TAAR13c) is also important for defensive behavior in zebrafish elicited by cadaverine and putrescine, two amines produced in decaying animal flesh (Hussain et al., 2013). Finally, single molecule kairomones isolated from fox feces (2,4,5-trimethylthiazoline, TMT), in addition to weasel and ferret anal secretions (2-propylthietane), were first isolated in the 1980s (Crump, 1980; Vernet-Maury et al., 1984) and have since been used as predator threats in their synthesized forms. Given that TMT was discovered before other single molecule predator odors, it has been the most extensively studied and yielded the most progress toward understanding the neuroanatomical circuits necessary for producing defensive behaviors after encountering these types of odors (for reviews see Rosen, 2004; Fendt et al., 2005a; Fendt and Endres, 2008; Takahashi, 2014; Brennan and Keverne, 2015). The remainder of this paper will focus on that progress and some aspects still left to be determined about the fear-inducing qualities of TMT, the neural systems and circuitry for innate fear, and defensive behaviors elicited by TMT.

TMT as an Unconditioned Fear Stimulus in Rodents

TMT is a synthetic compound that was originally isolated from fox feces by Vernet-Maury in the 1980s (Vernet-Maury et al.,

1984). Of a number of compounds isolated from fox feces, TMT appeared to have the most robust effect on avoidance, and therefore was touted and marketed as a natural rodent repellent (Vernet-Maury et al., 1984). TMT is a highly volatile, water insoluble molecule that contains sulfur. Sulfur is an element in carnivores' diet and digestion metabolites, and is important for the threat-inducing qualities of urine and feces (Nolte et al., 1994; Brechbühl et al., 2013), as a meatless diet depletes sulfur in coyote urine, and the urine produced from a meatless diet does not elicit defensive responses from mice (Nolte et al., 1994). Cat feces from a meatless diet also reduced mouse defensive behavior compared to feces from a carnivorous diet, although sulfur content was not tested in this study (Berton et al., 1998). Interestingly, mouse alarm pheromones, which signal conspecific danger, contain another thiazoline compound that is chemically similar to TMT, suggesting that both conspecific odor alarm signals and predator kairomones are transduced by the same olfactory receptors (Brechbühl et al., 2013). The highly volatile nature of TMT indicates that it might be a long distance olfactory threat signal to rodents. TMT is thought to be an unconditioned threatening stimulus because naïve, laboratory bred, and raised rats and mice display fear-like responses on their first exposure to TMT (Wallace and Rosen, 2000). Examining freezing as a fear response, TMT elicits freezing to the same levels that footshock-induced conditioned freezing does (Wallace and Rosen, 2001). Also similar to footshock-induced freezing, exposure to TMT can be titrated to produce low to high amounts of freezing (Wallace and Rosen, 2000; Endres et al., 2005), demonstrating a dose-response relationship between the number of TMT molecules and the amount of freezing or unconditioned fear. The unconditioned quality of TMT is also evident from the lack of habituation and sensitization to repeated exposures to TMT, in addition to the difficulty of TMT to support contextual fear conditioning (Wallace and Rosen, 2000; McGregor et al., 2002; Blanchard et al., 2003). Although habituation and cue and contextual conditioning have been demonstrated to cat fur odor (Blanchard et al., 2001; Dielenberg and McGregor, 2001; Takahashi et al., 2008), a lack of behavioral habituation, sensitization, and contextual fear conditioning has been extended to cat feces and ferret fur odor (Blanchard et al., 2003; Masini et al., 2006), suggesting different sources of predator odors have varied danger or threat inducing properties.

The difficulty of producing contextual conditioning to TMT does not suggest that TMT cannot support conditioning. Indeed, in test chambers that have more than one compartment, TMT can support conditioned responses. In a chamber where TMT is introduced in one compartment, rats will avoid that compartment when tested later without TMT present (Endres and Fendt, 2007). Similarly, in a chamber with a hide box, TMT exposure produces a small, but significant, increase in conditioned freezing the day following exposure (Rosen et al., 2008b). Conditioned changes in other behaviors were also found—increased time spent in the hide box, less exploration, and fewer contacts with the odor source (Rosen et al., 2008b).

What We Know about TMT-induced Defensive Behavior

TMT is the most widely studied single molecule predator odor because it produces robust, invariant levels of freezing behavior—a well-documented fear behavior in rats, mice, and even humans (Blanchard and Blanchard, 1969; Roelofs et al., 2010; Hagenaars et al., 2014). Considering that freezing is also the most studied behavior for fear conditioning experiments, unconditioned freezing to TMT provides support for the notion that a fear response is due to the odor's threat or fear inducing properties instead of other properties, such as its unpleasant, even acrid, qualities that elicit avoidance (Fendt and Endres, 2008; Ayers et al., 2013). TMT also elicits or reduces other behaviors associated with threat, such as eliciting flat-back stretching and decreasing exploration and rearing—behaviors that have been documented to be defensive in rats and mice in response to a cat and cat fur odor (Fendt and Endres, 2008; Rosen et al., 2008b).

Interestingly, other single molecule predator odors have not been shown (or not been tested) to elicit freezing, but have been shown to induce avoidance (Ferrero et al., 2011). We think it is important to demonstrate that a predator odor elicits well-documented fear or defensive behaviors as well as avoidance because avoidance can be a result of the unpleasant or noxious qualities of the odor rather than its fear-inducing properties (Wallace and Rosen, 2000; Fendt and Endres, 2008). Odors not associated with predators, like butyric acid and caproic acid, which have noxious acrid qualities, have been shown to induce avoidance, similar to many predator odors, but not induce significant freezing (Wallace and Rosen, 2000). Measuring the elicitation of documented defensive and fear behaviors in addition to avoidance is critical for the study of innate predator odors.

We also know that TMT doesn't readily support context conditioning (Wallace and Rosen, 2000; Blanchard et al., 2003), and this differs from cat fur odor, which does serve as an unconditioned stimulus for context and auditory conditioning in single chamber tests (Blanchard et al., 2001; Dielenberg and McGregor, 2001; Takahashi et al., 2008). However, in two-compartment test chambers TMT does support context conditioning of a number of defensive and avoidance behaviors (Endres and Fendt, 2007; Rosen et al., 2008b), suggesting that although TMT induces robust freezing in a simple single chamber, it needs some environmental complexity to support contextual fear conditioning.

What We Still Need to Learn about TMT-induced Defensive Behavior

This leads us to ask what we do not know about TMT induced behavior. Since TMT-induced conditioning is so circumscribed, a major question is, what are the environmental constraints of TMT supported conditioning? Size of test chamber, lighting conditions, familiarity, and prior safe experience of the environment modulate innate TMT-induced fear behavior

(Morrow et al., 2002; Rosen et al., 2008b; Nikaido and Nakashima, 2009; Knox et al., 2012). Why are two (or more) compartment chambers necessary for supporting context-TMT conditioning? Furthermore, what types of neutral stimuli can be conditioned to TMT? An interesting study demonstrated that TMT supports flavor conditioned avoidance (Myers and Rinaman, 2005). Mice learned to avoid drinking flavor-infused water (combined taste and odor stimuli, e.g., almond or vanilla extract in water) that was previously paired with TMT compared to non-TMT paired flavors. This was selective for TMT, as avoidance did not occur when flavored-water was paired with exposure to banana extract, a non-predator odor. This study, in addition to studies needing complex test chambers for TMT-induced context conditioning (Endres and Fendt, 2007; Rosen et al., 2008b), suggests that more naturalistic stimuli or environments may produce more robust conditioning than context.

TMT, while not readily supporting contextual fear conditioning, might produce a generalized sensitization-like effect. Following exposure to TMT, adult mice were found to have enhanced acoustic startle responses, which lasted 3 days or more (Hebb et al., 2003). Rats and mice exposed to TMT also showed increased anxious behavior (less time spent in the open arms) on the elevated plus maze, in an open field test, and in a light-dark test lasting more than 3 days following TMT exposure (Hebb et al., 2002, 2004; Fendt et al., 2005a). This is similar to the well documented long-lasting anxiogenic effects of cat odors (Cohen et al., 2012). Further study of the generalized sensitization-like effects of TMT would be quite important for translational models of anxieties, phobias, and posttraumatic stress disorder (Rosen et al., 2008b).

However, another study did not find sensitization to TMT-induced freezing during chronic exposure to high levels of corticosterone (Rosen et al., 2008a). TMT induces corticosterone secretion (Day et al., 2004), which is known to enhance anxious behavior and fear conditioned freezing (Schulkin et al., 2005; Roozendaal et al., 2009), so it is difficult to reconcile the increase in prolonged sensitization following TMT on startle and the elevated-plus maze, and the lack of an effect on TMT-induced freezing with chronic corticosterone. Possibly, increased corticosterone associated with TMT needs an environmental context to produce long lasting anxiogenic effects. Or, increased corticosterone might be involved in potentiating defensive responses to other situations (i.e., generalized sensitization) after TMT exposure, like startle, elevated plus maze, and the other situations mentioned above. Clearly, there are still avenues and interesting features to discover about the defensive and fear behavior associated with TMT and TMT-induced physiological responses.

An additional question stems from the differences in the ability of TMT to induce fear responses in different strains of rats and mice. Sprague–Dawley and Long–Evans strains of rats display robust freezing to TMT, but Wistar rats do not (Rosen et al., 2006; Staples and McGregor, 2006). Similarly, CD-1 mice do not seem to respond to TMT with freezing behavior, but C57BL/6J mice do (Fortes-Marco et al., 2013). However, another lab has shown increases in TMT-induced freezing in CD-1 mice (Hebb et al., 2004), but didn't compare other mouse strains. Whether these strain differences are due to differences in the ability to perceive (smell) TMT would be important to answer, and would suggest that these differences are due to genetic variation in the olfactory receptor populations in these strains (Rosen et al., 2006). If olfactory receptor populations are different, then this can be exploited to discover the selective olfactory receptors responsible for TMT to induce fear responses.

What we know and still need to learn about TMT-induced fear behavior is summarized in **Table 1**.

Neuroanatomy of TMT-induced Fear

Studies investigating the neuroanatomy of predator odors have also made significant progress over the years. Most research has investigated cat and cat fur odor as innate predator stimuli (Blanchard et al., 2005; Gross and Canteras, 2012). The locus of attention has primarily been in the hypothalamus and amygdala using lesions, pharmacological inactivation, electrical stimulation, and markers of neuronal activation (for review see Gross and Canteras, 2012; Takahashi, 2014). Newer, molecular techniques (e.g., transgenic mice, optogenetics) are also being employed to identify the neuroanatomy and receptors for innate fear of predator odors (Root et al., 2014).

While some progress has been made on the "fear-associated" neuroanatomy of single molecule predator odors (that is, amygdala and hypothalamic circuitry), great progress has been made in elucidating the olfactory receptors and neuroanatomy for a variety of single molecule predator odors by starting at the sensory organ—the nasal epithelium. For example, individual olfactory receptors, or a class of olfactory receptors in the case of TMT, have been identified for Feld4, rMUP13, 2-phenylethylamine, and TMT. In mice, the transient receptor

TABLE 1 | TMT-induced defensive behavior.

What we know about the TMT-induced defensive behavior

- TMT produces robust, invariant levels of freezing with repeated exposure
  - Lack of habituation
  - Lack of sensitization
- TMT can induce a large array of species-specific defensive behaviors
  - Size of text chamber, lighting conditions, familiarity, prior safe experience modulate freezing, and other defensive behaviors
- TMT can support context and flavor-avoidance conditioning of defensive behaviors

What we still need to learn about TMT-induced defensive behavior

- What types of stimuli can and cannot be conditioned to TMT?
- What are the environmental constraints of TMT-induced fear conditioning?
  - TMT supports limited context conditioning, but good conditioning of flavor avoidance
- Why are more complex environments better for TMT-induced fear conditioning?
- How good is TMT as a model for anxiety disorders—phobias and posttraumatic stress disorder?
  - Study on TMT-induced generalized sensitization, habituation, and extinction is needed.

potential cation channel, subfamily C, member 2 (TrpC2) in the vomeronasal organ is critical for avoidance and risk assessment behavior to Feld4 and rMUP13 (Papes et al., 2010). In rats and mice the trace amine-associated receptor 4 (TAAR4) in the main olfactory bulb is important for avoidance behavior to 2-phenylethylamine (Ferrero et al., 2011; Dewan et al., 2013). In addition, sensory olfactory neurons with a class of olfactory receptors in zone II of the nasal epithelium, possibly with up to about 100 olfactory receptor genes called dorsal domain class II receptors (DII), synapse in the glomeruli of the dorsal portion of the main olfactory bulb. Most importantly this class of DII olfactory neurons are necessary for innate avoidance of TMT (Kobayakawa et al., 2007). This landmark study, which produced mutant mice lacking neurons with DII receptors and showed a lack of fear and anxious responses to TMT (Kobayakawa et al., 2007), has been a major impetus for the recent progress made in the neuroanatomical olfactory circuits necessary for processing single molecule predator odors and innate fear. It empirically demonstrated that a group of class II olfactory neurons in zone II of the nasal epithelium, which project to the dorsal part of the main olfactory bulb, was responsible for innate fear to TMT, a volatile predator odor.

A recent study also points to the Grueneberg ganglion as another olfactory system important for TMT-induced freezing (Brechbühl et al., 2013). The Grueneberg ganglion is an olfactory subsystem located at the tip of the nose close to the entry of the naris. It comprises neurons that are both sensitive to cold temperature and play an important role in the detection of alarm pheromones (Brechbühl et al., 2008, 2013). The olfactory receptors of the Grueneberg ganglion are particularly sensitive to methylated thiazolines, of which TMT is one. The neurons of the Grueneberg ganglion synapse in the dorsal main olfactory bulb (Brechbühl et al., 2013), possibly within the DII domain glomeruli (Kobayakawa et al., 2007). Transection of the axons of the Grueneberg ganglion cells blocks TMT-induced freezing (Brechbühl et al., 2013). Thus, it is likely that the elicitation of freezing by TMT is transduced through receptors in the nasal epithelium (Kobayakawa et al., 2007) and the Grueneberg ganglion (Brechbühl et al., 2013).

Although the studies just reviewed suggest that TMT's fear inducing qualities are transmitted to the brain via two olfactory systems, there is the possibility that the noxious property of TMT is transduced through the trigeminal nerve to drive the freezing, avoidance, and other behavioral effects of TMT (McGregor et al., 2002; Fendt and Endres, 2008; Galliot et al., 2012). To address this issue, our lab recently demonstrated that olfactory bulb ablation completely blocked the freezing response to TMT, whereas transection of the infraorbital and ethmoidal branches of the trigeminal nerve (eliminating noxious sensations from the nasal cavity, mouth region, and whiskers) had no effect of TMT-induced freezing (Ayers et al., 2013). This was complemented by a lack of an effect of olfactory bulb lesions on butyric acid-induced behavior, whereas the trigeminal transection reduced freezing to butyric acid (Ayers et al., 2013). These results indicate that TMT-elicited innate fear behavior is dependent on olfaction, but not its noxious properties. This effect of olfactory bulb ablation on TMT-induced freezing has recently been replicated (Taugher et al., 2015, this special issue).

Advances in circuitry beyond the olfactory bulb have been made from the DII TMT-responsive neurons in the dorsal main olfactory bulb (**Figures 1C–F**). Optical imaging of the olfactory bulb found TMT-responsive glomeruli clustered in the posterior part of the DII domain (called cluster J; Matsumoto et al., 2010). The neurons from this posterior part of DII domain of the olfactory bulb have further been traced to synapse on mitral and tufted cells that project to the cortical amygdala (Miyamichi et al., 2010; Sosulski et al., 2011). Both studies speculated that olfactory circuits projecting to the cortical amygdala are responsible for the generation of innate behavior to olfactory stimuli. Functional studies using optogenetic methods demonstrated that TMT-induced innate avoidance and freezing were reduced by inhibition of mitral cells projections from the dorsal olfactory bulb to the cortical amygdala (Root et al., 2014). Optogenetic activation of anterior cortical amygdala neurons induced avoidance and freezing behaviors similar to those induced by TMT (Root et al., 2014). Together, these studies identify a functional two-synapse olfactory circuit for innate fear behavior to TMT. This circuit initiates fear behavior from class II olfactory sensory neurons in the nasal epithelium or Grueneberg ganglion cells, which synapse on mitral cells in cluster J glomeruli in the posterior dorsal olfactory bulb. From there, mitral cells synapse in the anterior cortical nucleus of the amygdala.

Where the cortical amygdala neurons synapse in the next leg of an innate olfactory fear circuit is not known at present (**Figure 1D**). However, using the immediate-early gene, c-fos, as an activation marker, TMT has been shown to induce activation in the medial part of the anterodorsal medial nucleus of the amygdala (Day et al., 2004) and muscimol inactivation of the medial nucleus of the amygdala significantly reduced TMT-induced freezing behavior (Müller and Fendt, 2006).

Once transduction of the TMT fear-inducing signal gets beyond the olfactory cortical regions and medial amygdala, it is not clear how the circuit proceeds to motor control regions important for fear and defensive behavior, such as the periaqueductal gray (PAG) to generate escape and freezing responses (Vianna and Brandão, 2003) (**Figure 1E**). Circuitry through three hypothalamic nuclei—the anterior hypothalamic nucleus, the dorsomedial division of the ventromedial hypothalamic nucleus and the dorsal premammillary nucleus—have been delineated for fear and defensive behavior induced by a live cat and cat fur odor, called the medial hypothalamic defensive circuit (Gross and Canteras, 2012). The dorsomedial division of the ventromedial hypothalamus (VMHdm) has also been shown to be activated by a number of single molecule predator odors that are transduced through various olfactory organs, including vomeronasal organ (Feld4), nasal epithelium (2-phenylethylamine), and Grueneberg ganglion (2-propylthietane) (Pérez-Gómez et al., 2015). Most interestingly, TMT did not activate c-fos in the VMHdm in this study and another study (Staples et al., 2008; Pérez-Gómez et al., 2015), but see (Day et al., 2004). Furthermore, in dissociating the neurocircuitry for TMT-induced fear from the circuitry for other predator odors, a comprehensive excitotoxic and electrolytic lesion study

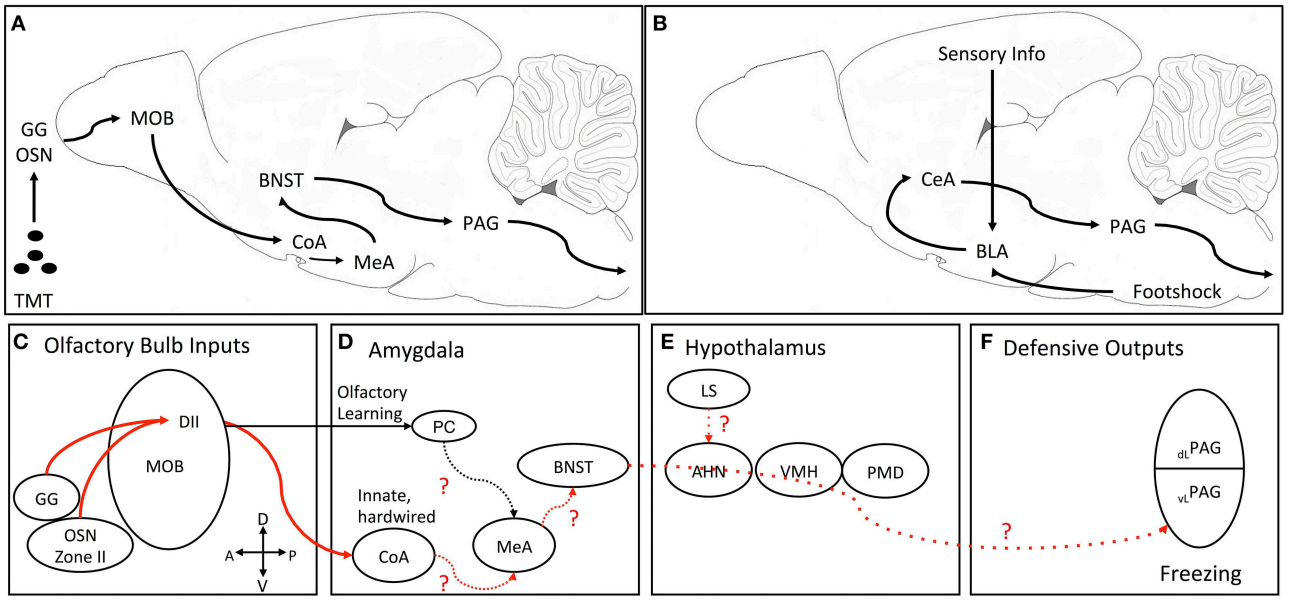


FIGURE 1 | Proposed neural processing pathway for TMT-induced freezing and pathway for fear conditioning. (A) Simplified schematic of pathway from olfaction to freezing output. **(B)** Simplified schematic of the classic fear conditioning pathway where conditioned and unconditioned information impinges on the BLA and then gets sent to CeA which projects to the PAG. **(C–F)** Detailed schematic of the pathway for TMT-induced freezing. Solid red lines are empirically determined neural pathways for innate TMT-induced freezing. The dotted red lines are the proposed connections through nuclei known to be important for TMT-induced freezing. **(C)** TMT inputs from type II olfactory sensory neurons and the Grueneberg ganglion project to domain II glomeruli in the dorsal main olfactory bulb. These glomeruli project through two pathways. **(D)** One pathway from the mitral/tufted cells to the PC for TMT-related learning and another to the CoA for innate defensive processing. For innate TMT-induced freezing hypothesized that information is then relayed to the MeA and then serially to ventral parts of the BNST. **(E)** TMT information is then sent from the BNST to the PAG passing through the medial hypothalamic defensive circuit (AHN, VMH). An additional input for TMT-induced freezing is from the LS possibly projecting to the AHN. **(F)** Finally, it is hypothesized that TMT information from the BNST reaches the vlPAG to mediate defensive freezing. AHN, anterior hypothalamic nucleus; BLA, basolateral amygdala complex; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; CoA, cortical nucleus of the amygdala; DI and DII, dorsal domain 1 and domain 2 glomeruli; GG, Grueneberg ganglion; LS, lateral septum; MeA, medial nucleus of the amygdala; MOB, main olfactory bulb; OSN, type II olfactory sensory neurons; dlPAG, dorsolateral periaqueductal gray; vlPAG, ventrolateral periaqueductal gray; PC, pyriform cortex; PMD, dorsal premammillary nucleus; VMH, ventromedial hypothalamus.

of the three nuclei of the medial hypothalamic defense circuit found that excitotoxic lesions of any of the three nuclei did not reduce TMT-induced freezing (Pagani and Rosen, 2009). However, the same study showed that electrolytic lesions in the anterior hypothalamic nucleus and VMHdm significantly reduced TMT-induced freezing. Interestingly, these electrolytic lesions reduced shock-induced contextual fear conditioning, identifying another dissociation of innate fear and learned fear neural circuitry.

Given that electrolytic lesions, but not excitotoxic lesions, of the VMHdm and anterior hypothalamic nucleus reduced TMT-induced freezing, the results suggest that axonal fibers passing through these regions are likely part of a circuit for TMT-induced freezing. One possible candidate is a pathway from the bed nucleus of the stria terminalis (BNST) to the PAG (Dong and Swanson, 2004, 2005, 2006), which passes through the anterior hypothalamic nucleus and VMHdm (**Figure 1F**). Inactivation of the ventral BNST with muscimol or norepinephrine antagonism with clonidine has been shown to block TMT-induced freezing (Fendt et al., 2003, 2005b; Fendt and Endres, 2008). Furthermore, TMT induces immediate-early gene activation in the medial and lateral aspects of the BNST (Day et al., 2004; Asok et al., 2013). Immediate-early gene expression is also blocked in DII olfactory sensory neuron knockout mice exposed to TMT (Kobayakawa et al., 2007). Because the medial nucleus of the amygdala innervates the medial parts of the BNST, a putative pathway for TMT-induced freezing could be zone II of the nasal epithelium or Grueneberg ganglion—dorsal main olfactory bulb—cortical amygdala—medial amygdala—medial bed nucleus of the stria terminalis—ventrolateral periaqueductal gray (**Figure 1A**). However, an alternative pathway might be through the lateral septum since inactivation of the lateral septum has been shown to block TMT-induced freezing (Endres and Fendt, 2008). The lateral septum has reciprocal connections with the amygdala, bed nucleus of the stria terminalis, and periaqueductal gray to be incorporated into the circuit described above. An interesting article on social aggregation of rats as a defense against prey, demonstrated that rats with a more active response to cat fur odor displayed less c-fos activation in the lateral septum suggesting that the septum may be involved in defensive aggregation (Bowen et al., 2013).

TMT-induced Fear: Neuroanatomical Pathways Compared to the Fear Conditioning Neuroanatomical Pathway

A putative, partial neuroanatomical circuit for TMT-induced innate fear behavior is laid out in **Figure 1A**. The neuroanatomical circuit for fear conditioning and fear-conditioned behavior is well delineated (**Figure 1B**) and will not be detailed here (see Ledoux, 2000; Davis and Whalen, 2001; Davis et al., 2010; Dejean et al., 2015, for detailed descriptions of the circuit). What is obvious about the TMT circuit is that it differs from the canonical circuit for fear conditioning and fear conditioned defensive behavior (freezing, startle), which contains the basolateral and central amygdala complexes. The basolateral complex (which contains the lateral and basal nuclei of the amygdala) and the central complex (which contains the lateral and medial divisions of the central nucleus of the amygdala) have been shown to be central and crucial for foot-shock induced fear conditioning with various conditioned stimuli including sounds, lights, contexts, and smells. Because TMT-induced freezing is not learned, the pathway appears to bypass the basolateral and central complexes, and instead looks as if it proceeds from the olfactory bulb through the amygdala cortex, medial amygdala nucleus, and medial bed nucleus of the stria terminalis to the periaqueductal gray (See previous section). Lesions or inactivation of the basolateral or central nuclei of the amygdala produce only small reductions or only a delay in TMT induced-freezing (Wallace and Rosen, 2001; Fendt et al., 2003; Rosen, 2004; Müller and Fendt, 2006), indicating these amygdala nuclei so important for fear conditioning, are not necessary for TMT-induced freezing, but seem to modulate TMT effects. Corroborating the minimal lesion and inactivation effects, immediate-early gene (c-fos, egr-1) expression is not increased following TMT exposure in the basolateral complex of the amygdala in rats (Day et al., 2004; Staples et al., 2008; Asok et al., 2013), but see Hebb et al. (2004) in mice.

The lack, or minimal effects, of inactivation and lesions of the basolateral and central complexes of the amygdala on TMT-induced freezing does not suggest that these regions are not involved in olfactory-motivated behavior. In fact, a number of studies demonstrate that the basolateral complex of the amygdala is important for olfactory fear conditioning, where once a non-predator odor is paired with a footshock, it elicits fear responses (Cousens and Otto, 1998; Sevelinges, 2004; Walker et al., 2005; Jones et al., 2007; Hegoburu et al., 2014). It appears likely that the basolateral complex is critical for olfactory-fear conditioning as it is for auditory and visual fear conditioning induced by footshock, but not for innate TMT-induced freezing. In contrast, as discussed above, the basolateral complex may only modulate TMT-induced freezing by delaying the full expression of freezing or slightly reducing the amount of freezing (Wallace and Rosen, 2001; Fendt et al., 2003).

The role of the central nucleus of the amygdala in olfactory fear conditioning is less certain because there are very few studies in which this nucleus is inhibited or lesioned. One study in infant rats showed that lesions of the central nucleus interfered with olfactory fear conditioning with shock (Sananes and Campbell, 1989). However, c-fos expression in the central nucleus of the amygdala was not increased in olfactory fear conditioning in adult rats (Schettino and Otto, 2001), nor was c-fos increased in the central nucleus of the amygdala with context conditioning induced by cat fur odor (Dielenberg et al., 2001; Staples et al., 2005). This contrasts with the very large increase in c-fos, egr-1, CART, and CRH in the central nucleus of the amygdala with exposure to TMT (Day et al., 2004; Staples et al., 2008; Asok et al., 2013; Sharma et al., 2014).

Another region of the extended amygdala—the bed nucleus of the stria terminalis—is not only important in TMT-induced freezing, but is also involved in sustained fear (Davis et al., 2010). Sustained fear would include contextual fear conditioning (Sullivan et al., 2004), fear conditioning with prolonged conditioned fear stimuli (Waddell et al., 2006; Davis et al., 2010), fear sensitization (Davis and Walker, 2014), fear generalization (Duvarci et al., 2009), and also innate fear of TMT (Fendt et al., 2003). Interestingly, if the basolateral amygdala complex is inactivated during contextual fear conditioning, the BNST can take over the function of the basolateral amygdala complex (Ponnusamy et al., 2007; Poulos et al., 2010; Zimmerman and Maren, 2011). Thus, the BNST is involved in more than just innate fear of predator odor, but has a functionally more rich involvement in fear and anxiety.

What We Know about the Neuroanatomy of TMT-induced Fear Behavior

Research on the neuroanatomy of TMT-induced fear has progressed to where there appears to be a specialized circuit from the nasal epithelium to the main olfactory bulb to the cortical amygdala. From there we don't quite know the circuit, but from the cortical amygdala it possibly continues to the medial amygdala nucleus, then bed nucleus of the stria terminalis, before finally reaching the periaqueductal gray for freezing. The lateral septum is also likely part of this pathway, but how it is connected is not known. We also know that the prelimbic cortex also modulates TMT-induced freezing, but inactivation enhances freezing, suggesting it plays an inhibitory role (Fitzpatrick et al., 2011).

Importantly, the basolateral and central complexes of the amygdala, so essential for fear conditioning, do not seem to play primary roles in TMT-induced fear. The data summarized above suggest there is a divergence of pathways for conditioned fear and innate fear. The dual neural pathways for learning about environmental contingencies of olfactory stimuli tracking through the basolateral and central complexes of the amygdala on the one hand, and hardwired pathways for innate sensory coding of ecologically significant odors on the other, is also evident in simpler animals than mammals (Vosshall and Stocker, 2007). For example, drosophila has two olfactory pathways, one through the antenna lobe (analogous to the olfactory bulbs) to the mushroom bodies, a region involved in olfactory learning and memory (possibly analogous to the pyriform cortex in mammals), and the lateral horn, a region mediating direct behavioral responses to odors (possibly analogous to the cortical amygdala) (Vosshall

and Stocker, 2007). This type of divergent neural circuitry for learning vs. innate coding for threat (and appetitive odors, Root et al., 2014) seems to be a common motif in the animal kingdom.

What We Still Need to Learn about the Neuroanatomy of TMT-induced Fear Behavior

Although the olfactory circuit through the cortical amygdala is fairly well delineated, the actual olfactory receptors responsible for TMT's innate properties are not well defined. The Domain II (DII) population of olfactory receptors comprises about 100 olfactory receptor genes (Kobayakawa et al., 2007), but which specific ones are responsible for TMT-induced freezing are not known. The identification of the specific olfactory receptor(s) that bind TMT and transduce TMT's innate fear properties, similar to what has been accomplished for olfactory receptors for 2-phenylethylamine (the TAAR4 receptor, Ferrero et al., 2011) and for Feld4 from cat fur/saliva and rMUP13 from rat urine (the TrpC2 receptor, Papes et al., 2010), would help propel the neuroanatomical circuitry research on TMT further.

Interestingly, there are also olfactory receptors in more ventral regions of the olfactory bulb. TMT can be used as a discriminative stimulus for positive reward in mutant mice devoid of the dorsal Domain II glomeruli, apparently through the TMT-responsive receptors in the ventral olfactory bulb (Kobayakawa et al., 2007). Possibly, these ventrally located neurons project to the pyriform cortex, which is activated by TMT (Illig and Haberly, 2003), as opposed to the cortical amygdala, to transmit TMT-induced olfactory information for involvement in olfactory learning (Kobayakawa et al., 2007; Miyamichi et al., 2010; Root et al., 2014).

An additional brain region that receives olfactory input from the main olfactory bulb is the entorhinal cortex (Insausti et al., 2002), which has major projections to the hippocampus. The hippocampus is particularly involved in contextual fear conditioning induced by coyote urine (Wang et al., 2012, 2013).

Another question also remains—how are the various regions of the circuit wired to each other? The part of the circuit from the nasal epithelium to cortical amygdala is pretty well characterized, but from there it is unclear how the circuit progresses to the PAG to elicit freezing behavior. Are projections from the cortical amygdala to the medial nucleus of the amygdala important? Is a direct pathway from the cortical amygdala to the bed nucleus of the stria terminalis involved? Or is the pathway from the medial nucleus of the amygdala to the ventral and medial divisions of the bed nucleus of the stria terminalis necessary? Does a direct pathway from the BNST to the PAG convey the TMT-induced fear signal for freezing?

Further delineation of the parts of the BNST that are involved in the various types of fear need to be addressed. The ventral and medial nuclei of the BNST are important for TMT-induced fear (Fendt et al., 2003; Müller and Fendt, 2006). However, the dorsolateral division of the BNST (also called the oval nucleus of the BNST) shows high levels of immediate-early gene expression following TMT exposure (Day et al., 2004; Asok et al., 2013). This is the region of the BNST that is implicated in sustaining learned fear and anxiety (Davis et al., 2010). How the dorsal and ventral divisions of the BNST interact for TMT-induced fear would be of particular interest for understanding the circuitry and modulation of innate predator fear and other types of fear.

There may also be other circuits involved in TMT-induced behaviors that route through areas outside of the BNST. Inactivation of the lateral septum significantly reduces freezing to TMT, but how it interacts with the proposed circuit (**Figure 1**) is not clear. The lateral septum strongly projects to the anterior nucleus of the hypothalamus, which is part of the cat and cat fur induced defensive circuit (Gross and Canteras, 2012), but the anterior nucleus of the hypothalamus does not seem to be part of the circuit for TMT-induced freezing (Pagani and Rosen, 2009). Furthermore, a number of neurotransmitter system modulate TMT-induced freezing, but we have not learned much beyond the initial studies demonstrating an effect. For example, norepinephrine infused into the ventral BNST enhanced TMT-induced freezing, while an α_2-antagonist blocks this norepinephrine enhancement and blocked TMT-induced freezing on its own (Fendt et al., 2005b). Chemical lesions

TABLE 2 | Neuroanatomy of TMT-induced fear.

What we know about the neuroanatomy of TMT-induced fear

- A pathway from olfactory neurons in the nasal epithelium and Grueneberg ganglion to the amygdala cortex has been delineated
  - Zone II receptors in nasal epithelium process innate fear to TMT
  - Genetic deletion of zone II olfactory sensory neurons eliminate fear responses to TMT
  - These olfactory sensory neurons synapse on mitral cells project to the amygdala cortex
  - Optogenetic inactivation of this projection to the amygdala cortex blocks TMT-induced freezing
- Olfaction, but not nociception, of TMT is critical for TMT-induce freezing
- Medial nucleus of the amygdala and bed nucleus of the stria terminalis are part of a proposed circuit for TMT-induced fear
  - Inactivation of these regions block TMT-induced freezing
- The medial hypothalamic defensive circuit is not critical for TMT-induced freezing
  - Fiber-sparing lesions of nuclei of the medial hypothalamic defensive circuit do not block TMT-induced freezing, but lesions that destroy fibers passing through this circuit block TMT-induced freezing

What we still need to learn about the neuroanatomy of TMT-induced fear

- What are the specific olfactory receptors for TMT-induced fear?
  - About 100 receptors are possible, further refinement is important
- What is the role of the pyriform and entorhinal cortex in TMT-induced fear and fear conditioning?
- How are the various regions of the circuit wired?
  - Projects from the amygdala cortex to the medial nucleus of the amygdala and bed nucleus of the stria terminalis are ill defined.
  - Is a direct project from the bed nucleus of the stria terminalis to the periaqueductal gray part of the circuit? Or is there an indirect pathway?
  - How are other regions that affect TMT-induced fear (e.g., lateral septum, prefrontal cortex) wired into the circuit?
  - How many circuits for TMT-induced fear are there?
  - How similar and divergent are circuits for fear to various predator odors?

of acetylcholine producing neurons also reduce TMT-induced freezing (Knox et al., 2008). However, more studies on the modulatory role of various neurotransmitters will be informative about autonomic and attentional mechanism of innate fear.

What we know and still need to learn about the anatomy of TMT-induced fear is summarized in **Table 2**.

Conclusions

Great progress has been made in the last two decades on understanding the neural basis of both learned fear and innate fear. Detailed progression on neural circuitries from olfaction to defensive behavior is within reach. Identification of single molecules that elicit defensive and fear responses allows for precise determination of the olfactory receptor, or receptors, involved in the transduction of the olfactory threat signals. Investigation using TMT as a threatening stimulus for rodents has been particularly important in this advancement. Behavioral studies have demonstrated the robustness of TMT-induced freezing, which has allowed for the use of traditional lesion/inactivation manipulations and modern optogenetic and molecular techniques to accurately localize olfactory neurons and the functional neural pathways responsible for perception of predator odor threat and the generation of defensive behavior. Comparison of the neural circuitry for TMT with other predator odors is critical for delineating the common and unique circuits for predator odors. This would not only be at the olfactory receptor stage, but at regions and synapses throughout the neural circuits, where differences between TMT and cat-derived odors are already evident (Pagani and Rosen, 2009; Gross and Canteras, 2012; Pérez-Gómez et al., 2015).

Finally, continued behavioral investigations on the generation of TMT-induced behaviors other than freezing will be important for broadening the scope of analysis into multiple circuitries for various species selective defensive responses. Further, examination of the innate properties of TMT-induced behavior and the constraints of fear learning supported by TMT and other predator odors will be important for understanding innate and learned fears and phobias (Rosen et al., 2008b), and then possible treatments.

Author Contributions

JR, AA, and TC all contributed to the conception, drafting, and revising of the work critically for important intellectual content. JR, AA, and TC gave their final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding

The work was partially funded by grants P20GM103653 and R01HD075066 from the National Institutes of Health.

References

Asok, A., Ayers, L. W., Awoyemi, B., Schulkin, J., and Rosen, J. B. (2013). Immediate early gene and neuropeptide expression following exposure to the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT). *Behav. Brain Res.* 248C, 85–93. doi: 10.1016/j.bbr.2013.03.047

Ayers, L. W., Asok, A., Heyward, F. D., and Rosen, J. B. (2013). Freezing to the predator odor 2,4,5 dihydro 2,5 trimethylthiazoline (TMT) is disrupted by olfactory bulb removal but not trigeminal deafferentation. *Behav. Brain Res.* 253C, 54–59. doi: 10.1016/j.bbr.2013.06.034

Berton, F., Vogel, E., and Belzung, C. (1998). Modulation of mice anxiety in response to cat odor as a consequence of predators diet. *Physiol. Behav.* 65, 247–254. doi: 10.1016/S0031-9384(98)00126-7

Blanchard, D. C., Canteras, N. S., Markham, C. M., Pentkowski, N. S., and Blanchard, R. J. (2005). Lesions of structures showing FOS expression to cat presentation: effects on responsivity to a Cat, Cat odor, and nonpredator threat. *Neurosci. Biobehav. Rev.* 29, 1243–1253. doi: 10.1016/j.neubiorev.2005.04.019

Blanchard, D. C., Markham, C., Yang, M., Hubbard, D., Madarang, E., and Blanchard, R. J. (2003). Failure to produce conditioning with low-dose trimethylthiazoline or cat feces as unconditioned stimuli. *Behav. Neurosci.* 117, 360–368. doi: 10.1037/0735-7044.117.2.360

Blanchard, D. C., Summers, C. H., and Blanchard, R. J. (2013). The role of behavior in translational models for psychopathology: functionality and dysfunctional behaviors. *Neurosci. Biobehav. Rev.* 37, 1567–1577. doi: 10.1016/j.neubiorev.2013.06.008

Blanchard, R. J., and Blanchard, D. C. (1969). Crouching as an index of fear. *J. Comp. Physiol. Psychol.* 67, 370–375. doi: 10.1037/h0026779

Blanchard, R. J., Yang, M., Li, C. I., Gervacio, A., and Blanchard, D. C. (2001). Cue and context conditioning of defensive behaviors to cat odor stimuli. *Neurosci. Biobehav. Rev.* 25, 587–595. doi: 10.1016/S0149-7634(01)00043-4

Bowen, M. T., Kevin, R. C., May, M., Staples, L. G., Hunt, G. E., and McGregor, I. S. (2013). Defensive aggregation (huddling) in *Rattus norvegicus* toward predator odor: individual differences, social buffering effects and neural correlates. *PLoS ONE* 8:e68483. doi: 10.1371/journal.pone.0068483

Brechbühl, J., Klaey, M., and Broillet, M.-C. (2008). Grueneberg ganglion cells mediate alarm pheromone detection in mice. *Science* 321, 1092–1095. doi: 10.1126/science.1160770

Brechbühl, J., Moine, F., Klaey, M., Nenniger-Tosato, M., Hurni, N., Sporkert, F., et al. (2013). Mouse alarm pheromone shares structural similarity with predator scents. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4762–4767. doi: 10.1073/pnas.1214249110

Brennan, P., and Keverne, E. B. (2015). Biological complexity and adaptability of simple mammalian olfactory memory systems. *Neurosci. Biobehav. Rev.* 50, 29–40. doi: 10.1016/j.neubiorev.2014.10.020

Choi, J. S., and Kim, J. J. (2010). Amygdala regulates risk of predation in rats foraging in a dynamic fear environment. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21773. doi: 10.1073/pnas.1010079108

Cohen, H., Kozlovsky, N., Alona, C., Matar, M. A., and Joseph, Z. (2012). Animal model for PTSD: from clinical concept to translational research. *Neuropharmacology* 62, 715–724. doi: 10.1016/j.neuropharm.2011.04.023

Cousens, G., and Otto, T. (1998). Both pre- and posttraining excitotoxic lesions of the basolateral amygdala abolish the expression of olfactory and contextual fear conditioning. *Behav. Neurosci.* 112:1092. doi: 10.1037/0735-7044.112.5.1092

Crump, D. R. (1980). Thietanes and dithiolanes from the anal gland of the stoat (*Mustela erminea*). *J. Chem. Ecol.* 6, 341–347. doi: 10.1007/BF01402912

Davis, M., and Walker, D. L. (2014). Role of bed nucleus of the stria terminalis and amygdala AMPA receptors in the development and expression of context conditioning and sensitization of startle by prior shock. *Brain Struct. Funct.* 219, 1969–1982. doi: 10.1007/s00429-013-0616-5

Davis, M., Walker, D. L., Miles, L., and Grillon, C. (2010). Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. *Neuropsychopharmacology* 35, 105–135. doi: 10.1038/npp.2009.109

Davis, M., and Whalen, P. J. (2001). The amygdala: vigilance and emotion. *Mol. Psychiatry* 6, 13–34. doi: 10.1038/sj.mp.4000812

Day, H. E. W., Masini, C. V., and Campeau, S. (2004). The pattern of brain c-fos mRNA induced by a component of fox odor, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), in rats, suggests both systemic and processive stress characteristics. *Brain Res.* 1025, 139–151. doi: 10.1016/j.brainres.2004.07.079

Dejean, C., Courtin, J., Rozeske, R. R., Bonnet, M. C., Dousset, V., Michelet, T., et al. (2015). Neuronal circuits for fear expression and recovery: recent advances and potential therapeutic strategies. *Biol. Psychiatry* 78, 298–306. doi: 10.1016/j.biopsych.2015.03.017

de Jongh, A., Oosterink, F. M. D., Kieffer, J. M., Hoogstraten, J., and Aartman, I. H. A. (2011). The structure of common fears: comparing three different models. *Am. J. Psychol.* 124, 141–149. doi: 10.5406/amerjpsyc.124.2.0141

Dewan, A., Pacifico, R., Zhan, R., Rinberg, D., and Bozza, T. (2013). Non-redundant coding of aversive odours in the main olfactory pathway. *Nature* 497, 486–489. doi: 10.1038/nature12114

Dielenberg, R. A., and McGregor, I. S. (2001). Defensive behavior in rats towards predatory odors: a review. *Neurosci. Biobehav. Rev.* 25, 597–609. doi: 10.1016/S0149-7634(01)00044-6

Dielenberg, R. A., Hunt, G. E., and McGregor, I. S. (2001). "When a rat smells a cat": the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor. *Neuroscience* 104, 1085–1097. doi: 10.1016/S0306-4522(01)00150-6

Do-Monte, F. H., Quiñones-Laracuente, K., and Quirk, G. J. (2015). A temporal shift in the circuits mediating retrieval of fear memory. *Nature* 519, 460–463. doi: 10.1038/nature14030

Dong, H.-W., and Swanson, L. W. (2004). Organization of axonal projections from the anterolateral area of the bed nuclei of the stria terminalis. *J. Comp. Neurol.* 468, 277–298. doi: 10.1002/cne.10949

Dong, H.-W., and Swanson, L. W. (2005). Projections from bed nuclei of the stria terminalis, dorsomedial nucleus: implications for cerebral hemisphere integration of neuroendocrine, autonomic, and drinking responses. *J. Comp. Neurol.* 494, 75–107. doi: 10.1002/cne.20790

Dong, H.-W., and Swanson, L. W. (2006). Projections from bed nuclei of the stria terminalis, anteromedial area: cerebral hemisphere integration of neuroendocrine, autonomic, and behavioral aspects of energy balance. *J. Comp. Neurol.* 494, 142–178. doi: 10.1002/cne.20788

Duvarci, S., Bauer, E. P., and Paré, D. (2009). The bed nucleus of the stria terminalis mediates inter-individual variations in anxiety and fear. *J. Neurosci.* 29, 10357–10361. doi: 10.1523/JNEUROSCI.2119-09.2009

Endres, T., Apfelbach, R., and Fendt, M. (2005). Behavioral changes induced in rats by exposure to trimethylthiazoline, a component of fox odor. *Behav. Neurosci.* 119, 1004–1010. doi: 10.1037/0735-7044.119.4.1004

Endres, T., and Fendt, M. (2007). Conditioned behavioral responses to a context paired with the predator odor trimethylthiazoline. *Behav. Neurosci.* 121, 594–601. doi: 10.1037/0735-7044.121.3.594

Endres, T., and Fendt, M. (2008). Inactivation of the lateral septum blocks fox odor-induced fear behavior. *Neuroreport* 19, 667–670. doi: 10.1097/WNR.0b013e3282fb78d9

Fendt, M., and Endres, T. (2008). 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox odor - just repugnant or really fear-inducing? *Neurosci. Biobehav. Rev.* 32, 1259–1266. doi: 10.1016/j.neubiorev.2008.05.010

Fendt, M., Endres, T., and Apfelbach, R. (2003). Temporary inactivation of the bed nucleus of the stria terminalis but not of the amygdala blocks freezing induced by trimethylthiazoline, a component of fox feces. *J. Neurosci.* 23, 23–28.

Fendt, M., Endres, T., Lowry, C. A., Apfelbach, R., and McGregor, I. S. (2005a). TMT-induced autonomic and behavioral changes and the neural basis of its processing. *Neurosci. Biobehav. Rev.* 29, 1145–1156. doi: 10.1016/j.neubiorev.2005.04.018

Fendt, M., Siegl, S., and Steiniger-Brach, B. (2005b). Noradrenaline transmission within the ventral bed nucleus of the stria terminalis is critical for fear behavior induced by trimethylthiazoline, a component of fox odor. *J. Neurosci.* 25, 5998–6004. doi: 10.1523/JNEUROSCI.1028-05.2005

Ferrero, D. M., Lemon, J. K., Fluegge, D., Pashkovski, S. L., Korzan, W. J., Datta, S. R., et al. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11235–11240. doi: 10.1073/pnas.1103317108

Fitzpatrick, C. J., Knox, D., and Liberzon, I. (2011). Inactivation of the prelimbic cortex enhances freezing induced by trimethylthiazoline, a component of fox feces. *Behav. Brain Res.* 221, 320–323. doi: 10.1016/j.bbr.2011.03.024

Fortes-Marco, L., Lanuza, E., and Martinez-Garcia, F. (2013). Of pheromones and kairomones: what receptors mediate innate emotional responses? *Anat. Rec.* 296, 1346–1363. doi: 10.1002/ar.22745

Galliot, E., Laurent, L., Hacquemand, R., Pourié, G., and Millot, J.-L. (2012). Fear-like behavioral responses in mice in different odorant environments: trigeminal versus olfactory mediation under low doses. *Behav. Processes* 90, 161–166. doi: 10.1016/j.beproc.2012.01.002

Gross, C. T., and Canteras, N. S. (2012). The many paths to fear. *Nat. Rev. Neurosci.* 13, 651–658. doi: 10.1038/nrn3301

Hagenaars, M. A., Oitzl, M., and Roelofs, K. (2014). Updating freeze: aligning animal and human research. *Neurosci. Biobehav. Rev.* 47, 165–176. doi: 10.1016/j.neubiorev.2014.07.021

Hebb, A. L. O., Zacharko, R. M., Dominguez, H., Trudel, F., Laforest, S., and Drolet, G. (2002). Odor-induced variation in anxiety-like behavior in mice is associated with discrete and differential effects on mesocorticolimbic cholecystokinin mRNA expression. *Neuropsychopharmacology* 27, 744–755. doi: 10.1016/S0893-133X(02)00354-8

Hebb, A. L. O., Zacharko, R. M., Gauthier, M., and Drolet, G. (2003). Exposure of mice to a predator odor increases acoustic startle but does not disrupt the rewarding properties of VTA intracranial self-stimulation. *Brain Res.* 982, 195–210. doi: 10.1016/S0006-8993(03)03008-7

Hebb, A. L. O., Zacharko, R. M., Gauthier, M., Trudel, F., Laforest, S., and Drolet, G. (2004). Brief exposure to predator odor and resultant anxiety enhances mesocorticolimbic activity and enkephalin expression in CD-1 mice. *Eur. J. Neurosci.* 20, 2415–2429. doi: 10.1111/j.1460-9568.2004.03704.x

Hegoburu, C., Parrot, S., Ferreira, G., and Mouly, A.-M. (2014). Differential involvement of amygdala and cortical NMDA receptors activation upon encoding in odor fear memory. *Learn. Mem.* 21, 651–655. doi: 10.1101/lm.036558.114

Herry, C., and Johansen, J. P. (2014). Encoding of fear learning and memory in distributed neuronal circuits. *Nat. Neurosci.* 17, 1644–1654. doi: 10.1038/nn.3869

Hussain, A., Saraiva, L. R., Ferrero, D. M., Ahuja, G., Krishna, V. S., Liberles, S. D., et al. (2013). High-affinity olfactory receptor for the death-associated odor cadaverine. *Proc. Natl. Acad. Sci. U.S.A.* 110, 19579–19584. doi: 10.1073/pnas.1318596110

Illig, K. R., and Haberly, L. B. (2003). Odor-evoked activity is spatially distributed in piriform cortex. *J. Comp. Neurol.* 457, 361–373. doi: 10.1002/cne.10557

Insausti, R., Marcos, P., Arroyo-Jiménez, M. M., Blaizot, X., and Martinez-Marcos, A. (2002). Comparative aspects of the olfactory portion of the entorhinal cortex and its projection to the hippocampus in rodents, nonhuman primates, and the human brain. *Brain Res. Bull.* 57, 557–560. doi: 10.1016/S0361-9230(01)00684-0

Janak, P. H., and Tye, K. M. (2015). From circuits to behaviour in the amygdala. *Nature* 517, 284–292. doi: 10.1038/nature14188

Jones, S. V., Stanek-Rattiner, L., Davis, M., and Ressler, K. J. (2007). Differential regional expression of brain-derived neurotrophic factor following olfactory fear learning. *Learn. Mem.* 14, 816–820. doi: 10.1101/lm.781507

Knox, D., Brothers, H., Norman, G. J., and Berntson, G. G. (2008). Nucleus basalis magnocellularis and substantia innominata corticopetal cholinergic lesions attenuate freezing induced by predator odor. *Behav. Neurosci.* 122, 601–610. doi: 10.1037/0735-7044.122.3.601

Knox, D., Fitzpatrick, C. J., George, S. A., Abelson, J. L., and Liberzon, I. (2012). Unconditioned freezing is enhanced in an appetitive context: implications for the contextual dependency of unconditioned fear. *Neurobiol. Learn. Mem.* 97, 386–392. doi: 10.1016/j.nlm.2012.03.002

Kobayakawa, K., Kobayakawa, R., Matsumoto, H., Oka, Y., Imai, T., Ikawa, M., et al. (2007). Innate versus learned odour processing in the mouse olfactory bulb. *Nature* 450, 503–508. doi: 10.1038/nature06281

LeDoux, J. (2012). Rethinking the emotional brain. *Neuron* 73, 653–676. doi: 10.1016/j.neuron.2012.02.004

Ledoux, J. E. (2000). Emotion circuits in the brain. *Annu. Rev. Neurosci.* 23, 155–184. doi: 10.1146/annurev.neuro.23.1.155

Masini, C. V., Sauer, S., White, J., Day, H. E. W., and Campeau, S. (2006). Non-associative defensive responses of rats to ferret odor. *Physiol. Behav.* 87, 72–81. doi: 10.1016/j.physbeh.2005.08.044

Matsumoto, H., Kobayakawa, K., Kobayakawa, R., Tashiro, T., Mori, K., Sakano, H., et al. (2010). Spatial arrangement of glomerular molecular-feature

clusters in the odorant-receptor class domains of the mouse olfactory bulb. *J. Neurophysiol.* 103, 3490–3500. doi: 10.1152/jn.00035.2010

McGregor, I., Schrama, L., Ambermoon, P., and Dielenberg, R. (2002). Not all "predator odours" are equal: cat odour but not 2,4,5 trimethylthiazoline (TMT; fox odour) elicits specific defensive behaviours in rats. *Behav. Brain Res.* 129, 1–16. doi: 10.1016/S0166-4328(01)00324-2

Miyamichi, K., Amat, F., Moussavi, F., Wang, C., Wickersham, I., Wall, N. R., et al. (2010). Cortical representations of olfactory input by trans-synaptic tracing. *Nature* 472, 191–196. doi: 10.1038/nature09714

Morrow, B. A., Elsworth, J. D., and Roth, R. H. (2002). Fear-like biochemical and behavioral responses in rats to the predator odor, TMT, are dependent on the exposure environment. *Synapse* 46, 11–18. doi: 10.1002/syn.10109

Müller, M., and Fendt, M. (2006). Temporary inactivation of the medial and basolateral amygdala differentially affects TMT-induced fear behavior in rats. *Behav. Brain Res.* 167, 57–62. doi: 10.1016/j.bbr.2005.08.016

Myers, E. A., and Rinaman, L. (2005). Trimethylthiazoline supports conditioned flavor avoidance and activates viscerosensory, hypothalamic, and limbic circuits in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 288, R1716–R1726. doi: 10.1152/ajpregu.00479.2004

Nikaido, Y., and Nakashima, T. (2009). Effects of environmental novelty on fear-related behavior and stress responses of rats to emotionally relevant odors. *Behav. Brain Res.* 199, 241–246. doi: 10.1016/j.bbr.2008.11.043

Nolte, D. L., Mason, J. R., Epple, G., Aronov, E., and Campbell, D. L. (1994). What are predator urines aversive to prey? *J. Chem. Ecol.* 20, 1505–1516. doi: 10.1007/BF02059876

Ohman, A., and Mineka, S. (2001). Fears, phobias, and preparedness: toward an evolved module of fear and fear learning. *Psychol. Rev.* 108, 483–522. doi: 10.1037/0033-295X.108.3.483

Oosterink, F. M. D., de Jongh, A., and Hoogstraten, J. (2009). Prevalence of dental fear and phobia relative to other fear and phobia subtypes. *Eur. J. Oral Sci.* 117, 135–143. doi: 10.1111/j.1600-0722.2008.00602.x

Pagani, J. H., and Rosen, J. B. (2009). The medial hypothalamic defensive circuit and 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) induced fear: comparison of electrolytic and neurotoxic lesions. *Brain Res.* 1286, 133–146. doi: 10.1016/j.brainres.2009.06.062

Papes, F., Logan, D. W., and Stowers, L. (2010). The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. *Cell* 141, 692–703. doi: 10.1016/j.cell.2010.03.037

Pérez-Gómez, A., Bleymehl, K., Stein, B., Pyrski, M., Birnbaumer, L., Munger, S. D., et al. (2015). Innate predator odor aversion driven by parallel olfactory subsystems that converge in the ventromedial hypothalamus. *Curr. Biol.* 25, 1340–1346. doi: 10.1016/j.cub.2015.03.026

Ponnusamy, R., Poulos, A. M., and Fanselow, M. S. (2007). Amygdala-dependent and amygdala-independent pathways for contextual fear conditioning. *Neuroscience* 147, 919–927. doi: 10.1016/j.neuroscience.2007.04.026

Poulos, A. M., Ponnusamy, R., Dong, H.-W., and Fanselow, M. S. (2010). Compensation in the neural circuitry of fear conditioning awakens learning circuits in the bed nuclei of the stria terminalis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14881–14886. doi: 10.1073/pnas.1005754107

Poulton, R., and Menzies, R. G. (2002). Non-associative fear acquisition: a review of the evidence from retrospective and longitudinal research. *Behav. Res. Ther.* 40, 127–149. doi: 10.1016/S0005-7967(01)00045-6

Rachman, S. (2002). Fears born and bred: non-associative fear acquisition? *Behav. Res. Ther.* 40, 121–126. doi: 10.1016/S0005-7967(01)00044-4

Roelofs, K., Hagenaars, M. A., and Stins, J. (2010). Facing freeze: social threat induces bodily freeze in humans. *Psychol. Sci.* 21, 1575–1581. doi: 10.1177/0956797610384746

Root, C. M., Denny, C. A., Hen, R., and Axel, R. (2014). The participation of cortical amygdala in innate, odour-driven behaviour. *Nature* 515, 269–273. doi: 10.1038/nature13897

Roozendaal, B., McEwen, B. S., and Chattarji, S. (2009). Stress, memory and the amygdala. *Nat. Rev. Neurosci.* 10, 423–433. doi: 10.1038/nrn2651

Rosen, J. B. (2004). The neurobiology of conditioned and unconditioned fear: a neurobehavioral system analysis of the amygdala. *Behav. Cogn. Neurosci. Rev.* 3, 23–41. doi: 10.1177/1534582304265945

Rosen, J. B., Donley, M. P., Gray, D., West, E. A., Morgan, M. A., and Schulkin, J. (2008a). Chronic corticosterone administration does not potentiate unconditioned freezing to the predator odor, trimethylthiazoline. *Behav. Brain Res.* 194, 32–38. doi: 10.1016/j.bbr.2008.06.019

Rosen, J. B., Pagani, J. H., Rolla, K. L. G., and Davis, C. (2008b). Analysis of behavioral constraints and the neuroanatomy of fear to the predator odor trimethylthiazoline: a model for animal phobias. *Neurosci. Biobehav. Rev.* 32, 1267–1276. doi: 10.1016/j.neubiorev.2008.05.006

Rosen, J. B., West, E. A., and Donley, M. P. (2006). Not all rat strains are equal: differential unconditioned fear responses to the synthetic fox odor 2,4,5-trimethylthiazoline in three outbred rat strains. *Behav. Neurosci.* 120, 290–297. doi: 10.1037/0735-7044.120.2.290

Sananes, C. B., and Campbell, B. A. (1989). Role of the central nucleus of the amygdala in olfactory heart rate conditioning. *Behav. Neurosci.* 103:519. doi: 10.1037/0735-7044.103.3.519

Schettino, L. F., and Otto, T. (2001). Patterns of Fos expression in the amygdala and ventral perirhinal cortex induced by training in an olfactory fear conditioning paradigm. *Behav. Neurosci.* 115:1257. doi: 10.1037/0735-7044.115.6.1257

Schulkin, J., Morgan, M. A., and Rosen, J. B. (2005). A neuroendocrine mechanism for sustaining fear. *Trends Neurosci.* 28, 629–635. doi: 10.1016/j.tins.2005.09.009

Sevelinges, Y. (2004). Olfactory fear conditioning induces field potential potentiation in rat olfactory cortex and amygdala. *Learn. Mem.* 11, 761–769. doi: 10.1101/lm.83604

Sharma, A., Rale, A., Utturwar, K., Ghose, A., and Subhedar, N. (2014). Identification of the CART neuropeptide circuitry processing TMT-induced predator stress. *Psychoneuroendocrinology* 50, 194–208. doi: 10.1016/j.psyneuen.2014.08.019

Sosulski, D. L., Bloom, M. L., Cutforth, T., Axel, R., and Datta, S. R. (2011). Distinct representations of olfactory information in different cortical centres. *Nature* 472, 213–216. doi: 10.1038/nature09868

Staples, L. G., Hunt, G. E., Cornish, J. L., and McGregor, I. S. (2005). Neural activation during cat odor-induced conditioned fear and "trial 2" fear in rats. *Neurosci. Biobehav. Rev.* 29, 1265–1277. doi: 10.1016/j.neubiorev.2005.04.009

Staples, L. G., and McGregor, I. S. (2006). Defensive responses of Wistar and Sprague-Dawley rats to cat odour and TMT. *Behav. Brain Res.* 172, 351–354. doi: 10.1016/j.bbr.2006.04.011

Staples, L. G., McGregor, I. S., Apfelbach, R., and Hunt, G. E. (2008). Cat odor, but not trimethylthiazoline (fox odor), activates accessory olfactory and defense-related brain regions in rats. *Neuroscience* 151, 937–947. doi: 10.1016/j.neuroscience.2007.11.039

Stowers, L., and Logan, D. W. (2010). Olfactory mechanisms of stereotyped behavior: on the scent of specialized circuits. *Curr. Opin. Neurobiol.* 20, 274–280. doi: 10.1016/j.conb.2010.02.013

Sullivan, G. M., Apergis, J., Bush, D. E. A., Johnson, L. R., Hou, M., and Ledoux, J. E. (2004). Lesions in the bed nucleus of the stria terminalis disrupt corticosterone and freezing responses elicited by a contextual but not by a specific cue-conditioned fear stimulus. *Neuroscience* 128, 7–14. doi: 10.1016/j.neuroscience.2004.06.015

Takahashi, L. K. (2014). Olfactory systems and neural circuits that modulate predator odor fear. *Front. Behav. Neurosci.* 8:72. doi: 10.3389/fnbeh.2014.00072

Takahashi, L. K., Chan, M. M., and Pilar, M. L. (2008). Predator odor fear conditioning: current perspectives and new directions. *Neurosci. Biobehav. Rev.* 32, 1218–1227. doi: 10.1016/j.neubiorev.2008.06.001

Taugher, R. J., Ghobbeh, A., Sowers, L. P., Fan, R., and Wemmie, J. A. (2015). ASIC1A in the bed nucleus of the stria terminalis mediates TMT-evoked freezing. *Front. Neurosci.* 9:239. doi: 10.3389/fnins.2015.00239

Vernet-Maury, E., Polak, E. H., and Demael, A. (1984). Structure-activity relationship of stress-inducing odorants in the rat. *J. Chem. Ecol.* 10, 1007–1018. doi: 10.1007/BF00987509

Vianna, D. M. L., and Brandão, M. L. (2003). Anatomical connections of the periaqueductal gray: specific neural substrates for different kinds of fear. *Braz. J. Med. Biol. Res.* 36, 557–566. doi: 10.1590/S0100-879X2003000500002

Vosshall, L. B., and Stocker, R. F. (2007). Molecular architecture of smell and taste in Drosophila. *Annu. Rev. Neurosci.* 30, 505–533. doi: 10.1146/annurev.neuro.30.051606.094306

Waddell, J., Morris, R. W., and Bouton, M. E. (2006). Effects of bed nucleus of the stria terminalis lesions on conditioned anxiety: aversive conditioning with long-duration conditional stimuli and reinstatement of extinguished fear. *Behav. Neurosci.* 120:324. doi: 10.1037/0735-7044.120.2.324

Walker, D. L., Paschall, G. Y., and Davis, M. (2005). Glutamate receptor antagonist infusions into the basolateral and medial amygdala reveal differential contributions to olfactory vs. context fear conditioning and expression. *Learn. Mem.* 12, 120–129. doi: 10.1101/lm.87105

Wallace, K. J., and Rosen, J. B. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behav. Neurosci.* 114, 912–922. doi: 10.1037/0735-7044.114.5.912

Wallace, K. J., and Rosen, J. B. (2001). Neurotoxic lesions of the lateral nucleus of the amygdala decrease conditioned fear but not unconditioned fear of a predator odor: comparison with electrolytic lesions. *J. Neurosci.* 21, 3619–3627.

Wang, M. E., Fraize, N. P., Yin, L., Yuan, R. K., Petsagourakis, D., Wann, E. G., et al. (2013). Differential roles of the dorsal and ventral hippocampus in predator odor contextual fear conditioning. *Hippocampus* 23, 451–466. doi: 10.1002/hipo.22105

Wang, M. E., Wann, E. G., Yuan, R. K., Ramos Álvarez, M. M., Stead, S. M., and Muzzio, I. A. (2012). Long-term stabilization of place cell remapping produced by a fearful experience. *J. Neurosci.* 32, 15802–15814. doi: 10.1523/JNEUROSCI.0480-12.2012

Zimmerman, J. M., and Maren, S. (2011). The bed nucleus of the stria terminalis is required for the expression of contextual but not auditory freezing in rats with basolateral amygdala lesions. *Neurobiol. Learn. Mem.* 95, 199–205. doi: 10.1016/j.nlm.2010.11.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

HYPOTHESIS AND THEORY
published: 06 August 2015
doi: 10.3389/fnins.2015.00276

Olfactory instruction for fear: neural system analysis

Newton S. Canteras[1], Eloisa Pavesi[2] and Antonio P. Carobrez[2]*

[1] Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil, [2] Department of Pharmacology, CCB, Federal University of Santa Catarina, Florianópolis, Brazil

Different types of predator odors engage elements of the hypothalamic predator-responsive circuit, which has been largely investigated in studies using cat odor exposure. Studies using cat odor have led to detailed mapping of the neural sites involved in innate and contextual fear responses. Here, we reviewed three lines of work examining the dynamics of the neural systems that organize innate and learned fear responses to cat odor. In the first section, we explored the neural systems involved in innate fear responses and in the acquisition and expression of fear conditioning to cat odor, with a particular emphasis on the role of the dorsal premammillary nucleus (PMd) and the dorsolateral periaqueductal gray (PAGdl), which are key sites that influence innate fear and contextual conditioning. In the second section, we reviewed how chemical stimulation of the PMd and PAGdl may serve as a useful unconditioned stimulus in an olfactory fear conditioning paradigm; these experiments provide an interesting perspective for the understanding of learned fear to predator odor. Finally, in the third section, we explored the fact that neutral odors that acquire an aversive valence in a shock-paired conditioning paradigm may mimic predator odor and mobilize elements of the hypothalamic predator-responsive circuit.

Keywords: predator odor, innate fear, fear conditioning, hypothalamic circuits, amygdala

OPEN ACCESS

Edited by:
Markus Fendt,
Otto-von-Guericke University
Magdeburg, Germany

Reviewed by:
Fernando Martinez-Garcia,
Universitat Jaume I de Castelló, Spain
Kerstin E. A. Wernecke,
Otto-von-Guericke University
Magdeburg, Germany

****Correspondence:***
Newton S. Canteras,
Department of Anatomy, Institute of Biomedical Sciences, University of Sao Paulo, Av. Lineu Prestes, 2415, CEP 05508-000 Sao Paulo, SP, Brazil
newton@icb.usp.br

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *01 July 2015*
Accepted: *22 July 2015*
Published: *06 August 2015*

Citation:
Canteras NS, Pavesi E and Carobrez AP (2015) Olfactory instruction for fear: neural system analysis. Front. Neurosci. 9:276. doi: 10.3389/fnins.2015.00276

General Concepts regarding Predator Odor and Its Impact on Defensive Behavior Research

Olfactory cues are effective stimuli for the retrieval of stressful memories (Wiemers et al., 2014). Notably, when exposed to a trauma-associated olfactory cue, post-traumatic stress disorder-vulnerable victims have been shown to exhibit clinical symptoms in addition to increased regional blood flow in several regions related to fear and anxiety processing, including the amygdala, insula, medial prefrontal cortex, and anterior cingulate cortex (Vermetten et al., 2007). Thus, understanding of the neural circuits involved in fear encoding and expression in response to an olfactory stimulus seems to be an important step for translational studies that are attempting to uncover the neural mediation of pathological fear memory encoding in anxiety disorders.

Predator odors are semiochemical cues that evolved and were incorporated as species memory, or phyletic memory (Fuster, 1997). For this reason, regardless of any previous contact with a real cat, lab rodents will promptly recognize a cat odor and exhibit defensive behavior, such as increased immobility and the stereotyped stretched attend/approach postures that are characteristic of risk assessment behavior (Van Der Poel, 1979; Pinel et al., 1989). These instinctive fear responses are also present when subjects are re-exposed to a predator odor-associated environment, suggesting the acquisition and consolidation of contextual memory to predator odor (Hubbard et al., 2004).

Cat odor has been used as a valuable tool to study fear and anxiety. Compared to live cat exposure, cat odor exposure induces less intense instinctive fear responses, which can be reduced by anxiolytic drugs and are generally related to the approach-avoidance conflict—a general hallmark in anxiety (Blanchard et al., 1990, 1993; Zangrossi and File, 1992; Dielenberg and Mcgregor, 2001). However, it is difficult to standardize the amount of cat odor stimulus presented, and different degrees of freezing and avoidance may be found depending on the strength of the stimulus (Takahashi et al., 2005). When a hiding place is available, cat odor exposure increases the time the animal spends in an enclosed compartment and the time spent in the stretched-out posture within the enclosed compartment, which serves as an index of risk assessment behavior (Mcgregor et al., 2002; Dielenberg et al., 2004). Using a similar paradigm, Do Monte et al. (2008) found comparable defensive responses during exposure to the cat odor-associated context on the day following cat odor exposure, including an increased duration of time spent in the enclosed compartment and in stretched-out behavior.

A great deal has been learned about the olfactory pathways that mediate defensive responses to a number of predator odors (Takahashi, 2014; Pérez-Gómez et al., 2015). Of particular relevance for the present review, a number of the other predator odors that have been tested, excluding TMT (2,5-dihydro-2,4,5-trimethylthiazole), which is present in fox feces, have been shown to engage neural elements that are also responsive to cat odor (Pérez-Gómez et al., 2015). This finding suggests that multiple parallel mechanisms for the detection of different predator odors seem to converge in the brain to facilitate a common behavioral response. In this way, cat odor may be used as a good model to understand the neural mediation of defensive responses to other types of predator odor. In the present review, we will focus on three lines of work examining the dynamics of the neural systems that organize innate and learned fear responses to cat odor. In the first section, we will explore the neural systems involved in innate and learned fear responses to cat odor by examining the role of glutamatergic and beta-adrenergic transmission in the expression of innate fear and in the acquisition of contextual fear learning, with particular emphasis on the role of the dorsal premammillary nucleus (PMd) and the dorsolateral periaqueductal gray (PAGdl) as key sites that influence innate fear and contextual conditioning. In the second section, we will explore how chemical stimulation of these critical sites (i.e., the PMd and PAGdl) may be used as a useful unconditioned stimulus (US) in an olfactory fear conditioning paradigm and is likely to mimic predatory threats in instructing prosencephalic sites of higher order processing. Finally, in the third section, we will explore our findings regarding the use of a neutral odor as a conditioned stimulus (CS) in shock-based fear conditioning and determine how elements of the hypothalamic circuit that are responsive to predator odor may be engaged in the expression of shock-based olfactory fear conditioning.

Neural Mediation of Innate Fear Responses to Predator Odor

Predator odors are thought to work as kairomones, which are semiochemicals that are released by one species and have a favorable adaptive effect on a different "receiving" species but no favorable effect on the transmitting species (Dicke and Grostal, 2001; Wyatt, 2014). A number of studies have shown that the detection of predator odors relies on distinct olfactory subsystems for chemodetection, namely the vomeronasal organ (VNO) (McGregor et al., 2004), the Grueneberg ganglion (GG) (Brechbuhl et al., 2013), and subsets of sensory neurons within the main olfactory epithelium (MOE) that express trace amine-associated receptors (TAARs) (Liberles, 2015). Nasal detection of different predator odors has been associated with distinct olfactory subsystems, i.e., 2-phenylethylamine, which is found in carnivore urine, activates TAAR4 neurons in the MOE (Ferrero et al., 2011; Dewan et al., 2013), cat fur odor activates the VNO (McGregor et al., 2004), and 2-propylthietane, which is extracted from the stoat anal gland, is a GG activator (see Pérez-Gómez et al., 2015). Exposure to all of these different types of predator odors results in increased Fos expression in the posteroventral part of the medial amygdalar nucleus (MEApv) and in the dorsomedial part of the ventromedial hypothalamic nucleus (VMHdm; see Pérez-Gómez et al., 2015). Thus, an important concept emerges from this analysis: the detection of different predator odors converges in a pathway formed by the MEApv and VMHdm (**Figure 1**). The MEApv is part of the vomeronasal amygdala and is known to provide dense input to the VMHdm (Canteras et al., 1995), which, in turn, plays a critical role in the integration of predator-related defensive responses.

Optogenetic activation of steroidogenic factor 1 (SF1)-expressing neurons in the dorsomedial and central parts of the VMH (VMHdm/c) initiates a range of context-dependent

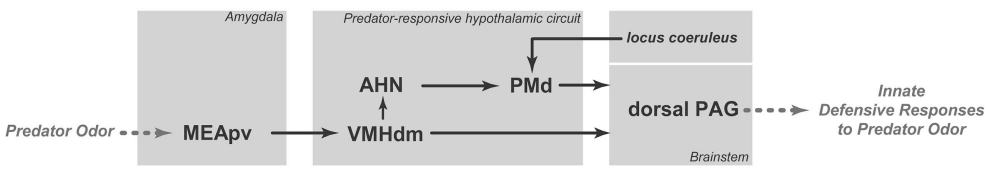


FIGURE 1 | Schematic diagram showing the putative brain circuit involved in organizing innate defensive responses to predator odor. AHN, anterior hypothalamic nucleus; MEApv, medial amygdalar nucleus, posteroventral part; PAG, periaqueductal gray; PMd, dorsal premammillary nucleus; VMHdm, ventromedial hypothalamic nucleus, dorsomedial part. See text for discussion.

somatomotor and autonomic responses that resemble animals' natural defensive behaviors (Wang et al., 2015). As shown by Wang et al. (2015), during VMHdm/c stimulation, if a hiding place was available, animals went to the hiding box and stayed inside of the box despite continued stimulation. Conversely, in the absence of the hiding box, VMHdm/c stimulation evoked either freezing or running and jumping, depending on the intensity of the stimulation. Moreover, VMHdm/c stimulation promoted avoidance, and the animals tended to avoid the place where they had received the stimulation. Taken together, these results indicate that VMHdm/c stimulation can induce complex defensive behavioral responses, including immobility, escape jumping, hiding, and avoidance. The main targets of the VMHdm/c are the dorsal periaqueductal gray (dPAG) and the anterior hypothalamic nucleus (AHN; **Figure 1**). Activation of the VMHdm/c projection to the dPAG induces inflexible immobility, whereas stimulation of the pathway from the VMHdm/c to the AHN promotes avoidance as well as immobility, running, and escape jumping (Wang et al., 2015). Thus, the direct pathway to the dPAG may be responsible for a rapid freezing response upon detection of a predator, whereas the AHN pathway may integrate a more complete range of complex defensive responses.

At least a portion of the complex defensive behaviors that have been described for the AHN are likely to be mediated through its projections to the dorsal premammillary nucleus (PMd; **Figure 1**). The AHN provides a dense bilateral projection to the PMd (Risold et al., 1994), which is the most responsive brain site to a predator or its odor (Canteras et al., 1997; Dielenberg et al., 2001; Cezario et al., 2008). Notably, PMd lesions have been shown to be highly effective in reducing anti-predator defensive responses (Canteras et al., 1997; Blanchard et al., 2003; Cezario et al., 2008). Pharmacological blockade of the PMd has also been shown to reduce innate defensive responses to cat odor (Canteras et al., 2008; Do Monte et al., 2008). In fact, the AHN, VMHdm and PMd form a partially segregated circuit in the medial zone of the hypothalamus; this circuit has been coined the predator-responsive circuit (Gross and Canteras, 2012). The PMd is likely to amplify neural processing in the predator-responsive hypothalamic circuit (Cezario et al., 2008). In line with this view, the PMd is particularly responsive to predator cues, and lesions or pharmacological blockade of this region drastically reduce anti-predator defensive responses. Conversely, PMd lesions are ineffective in other threatening situations, such as the elevated plus maze and exposure to post-shock contextual cues, suggesting the PMd may play a differential role in anti-predatory defensive responses (Blanchard et al., 2003).

The PAG is the major target of the predator-responsive circuit; the PMd and the VMHdm, albeit to a lesser extent, provide extensive projections to the dorsolateral and dorsomedial parts of the PAG. The projections are mostly aimed at the rostral and intermediate rostro-caudal levels of the PAG, and at caudal levels of the PAG, a moderate input is also centered in the lateral and ventrolateral divisions (Canteras, 2002). Notably, the spatial distribution of the axonal projections from the predator-responsive circuit to the PAG closely matches the pattern of Fos activation elicited in the PAG following exposure to predator odor (Dielenberg et al., 2001). Thus, rats exposed to cat odor present a particularly large increase in Fos activation in the dorsomedial and dorsolateral regions of the rostral two-thirds of the PAG in addition to somewhat sparser Fos labeling in the lateral and ventrolateral PAG at caudal levels (Dielenberg et al., 2001). The PAGdl seems to be critical for the integration of forebrain limbic information related to predator odor. On the efferent side, the PAGdl appears to control the entire range of defensive responses to predator odor, including flight, immobility, hiding and risk assessment behaviors (Blanchard et al., 1990; Carrive, 1993; Keay and Bandler, 1993). In support of this view, lesions in the dorsal PAG have also been shown to block all types of fear responses to predator odor (Sukikara et al., 2010).

Moreover, exposure to cat odor up-regulates Fos expression in the locus coeruleus (Dielenberg et al., 2001; McGregor et al., 2004). Firing rates in the locus coeruleus increase during stressful situations, and the locus coeruleus has been associated with fear responses (Bremner et al., 1996; Schenberg et al., 2001). Importantly, locus coeruleus lesions decrease both unconditioned and conditioned fear responses (Neophytou et al., 2001). In line with this view, systemic administration of propranolol (a centrally acting beta-blocker) reduced unconditioned defensive behaviors and PMd Fos expression in response to cat odor (Do Monte et al., 2008). This effect of noradrenergic transmission on fear responses should be, at least in part, mediated by noradrenergic projections from the locus coeruleus to the PMd (Sobrinho and Canteras, 2011; **Figure 1**), where beta-adrenoceptor blockade before cat odor exposure has been shown to reduce defensive responses to cat odor (Do Monte et al., 2008).

In summary, as shown in **Figure 1**, predator odors are processed by the MEApv, which engages the medial hypothalamic predator-responsive circuit. The predator-responsive circuit is further modulated by significant noradrenergic input from the locus coeruleus (which likely plays a critical role in the response to predator odors) and projects to the dorsal PAG, which is involved in the expression of innate defensive responses.

Neural Mediation of Learned Fear Responses to Predator Odor

Exposure to a predator or its odor evokes robust contextually conditioned defensive responses (Blanchard et al., 2001; Mcgregor et al., 2002). Contextual defensive responses are characterized by freezing, risk assessment behavior, and avoidance of the environment where a predator or its odor had been previously encountered.

The PMd influences the memory processes that link predatory threats to the associated context. Injection of an NMDA receptor antagonist (2-amino-5-phosphonopentanoic acid) into the PMd during cat odor exposure has been shown to impair conditioned defensive responses to the associated environment (Canteras et al., 2008). Moreover, beta adrenergic blockade in the PMd also reduces contextual fear responses to cat odor (Do Monte et al., 2008). As we shall discuss below, the projection from

the PMd to the ventral anteromedial thalamic nucleus (AMv; Canteras and Swanson, 1992; **Figure 2A**) is a likely pathway to influence mnemonic mechanisms linking predatory threats to their associated context. Recent findings from our group showed that AMv pharmacological inactivation prior to cat exposure did not interfere with innate fear responses but drastically reduced contextual conditioning to the predator-associated environment (De Lima et al., 2013). Thus, the PMd–AMv pathway may be involved in the acquisition of predator-related contextual fear memories. As shown in **Figure 2A**, the AMv is in a strategic position to convey predator cues to cortico-hippocampal-amygdalar sites involved in the acquisition of contextual fear memories. Previous studies have revealed that the AMv projects to the prelimbic, anterior cingulate, retrosplenial, ectorhinal, and perirhinal cortices as well as to the ventral subiculum and presubiculum (Shibata, 1993; Van Groen et al., 1999). Based on the projection pattern of the AMv, it is clear that this nucleus should exert an important influence on hippocampal processing, either through direct projections to the ventral subiculum and presubiculum or through indirect pathways mediated by the anterior cingulate and retrosplenial areas (Wyss and Van Groen, 1992; Jones and Witter, 2007). The hippocampus is known to be involved in mediating contextual fear memory to predatory threats, and findings from the Blanchard laboratory have shown that ventral hippocampal lesions impair conditioned defensive behaviors associated with either direct exposure to a cat or to its odor (Pentkowski et al., 2006). Moreover, the AMv is also in a position to influence amygdalar sites involved in fear conditioning. As previously noted, the AMv provides moderate input to perirhinal and ectorhinal areas, which are known to provide massive projections to the lateral amygdalar nucleus (Shi and Cassell, 1999). Synapses in the lateral amygdalar nucleus exhibit plasticity that is crucial for fear conditioning (Johansen et al., 2011), and cytotoxic lesions to this region have been shown to block contextual conditioning to predatory threats (Martinez et al., 2011). Taken together, these anatomical findings indicate that the AMv occupies a strategic position to influence both the hippocampus and the lateral amygdalar nucleus, supporting its role in the acquisition of contextual fear memory.

The PMd is also mobilized in response to cat-odor-associated contexts (Staples et al., 2005). Interestingly, there is overlap between the neural systems that integrate unconditioned and contextually conditioned anti-predator defensive responses. Rats exposed to a predator-associated environment exhibit increased Fos expression in elements of the predator-responsive hypothalamic circuit, including the AHN, PMd and, to a lesser extent, the VMHdm (Cezario et al., 2008). As shown in **Figure 2B**, during exposure to a predator-related context, the AHN is in a position to receive contextual information from the septo-hippocampal system, while the VMHdm is targeted by amygdalar sites involved in predator-related contextual memory. On the efferent side, pharmacological PMd inactivation with the $GABA_A$ receptor agonist muscimol blocks conditioned defensive responses during exposure to a predator-associated environment (Cezario et al., 2008).

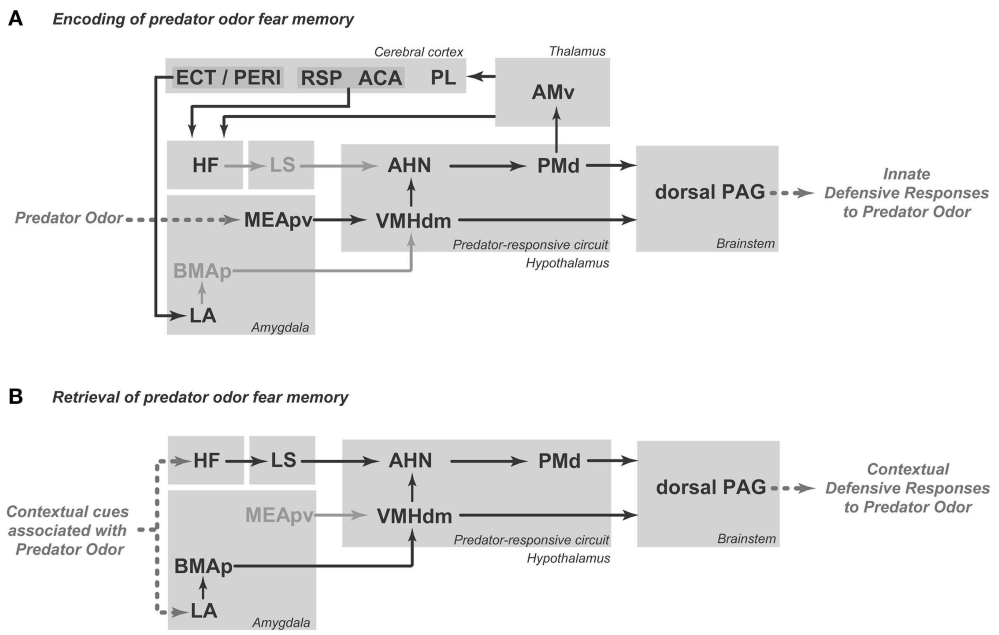


FIGURE 2 | Schematic diagrams showing the putative brain systems that support the encoding (A) and retrieval (B) of learned fear to predator odor. ACA, anterior cingulate area; AHN, anterior hypothalamic nucleus; AMv, anteromedial thalamic nucleus, ventral part; BMAp, basomedial amygdalar nucleus, posterior part; ECT, ectorhinal area; HF, hippocampal formation; LA, lateral amygdalar nucleus; LS, lateral septum; MEApv, medial amygdalar nucleus, posteroventral part; PAG, periaqueductal gray; PERI, perirhinal area; PL, prelimbic area; PMd, dorsal premammillary nucleus; RSP, retrosplenial area; VMHdm, ventromedial hypothalamic nucleus, dorsomedial part. See text for discussion.

As for unconditioned anti-predatory defensive responses, the PAG plays a critical role in the expression of contextually conditioned anti-predator responses (**Figure 2B**). The same pattern of PAG activation observed in response to a live predator, but considerably less intense, was found in animals exposed to an environment previously associated with a predator (Cezario et al., 2008).

Stimulation of the PMd and Dorsal PAG Supports Fear Learning

As previously discussed, the PMd and PAGdl are particularly responsive to predator threats, and we have considered whether stimulation of these sites would be able to mimic internal state changes related to predator threats and therefore serve as a reliable unconditioned stimulus (US) in an olfactory fear conditioning (OFC) paradigm.

The PMd is known to present a substantial plexus of noradrenergic fibers, and beta-adrenoceptor blockade therein results in a clear reduction of innate defensive responses to cat odor. Therefore, we have explored whether beta-adrenergic stimulation of the PMd could serve as a reliable US in an OFC paradigm (Pavesi et al., 2011). Rats were conditioned by pairing the US—an intra-PMd microinjection of isoproterenol (a beta-adrenoceptor agonist)—with the conditioned stimulus (CS)—amyl acetate odor. Beta-adrenoceptor stimulation in the PMd did not produce overt defensive responses but was able to support olfactory conditioning (Pavesi et al., 2011). Accordingly, the subjects that received isoproterenol in the PMd paired with amyl acetate odor as the CS showed marked defensive behavior toward the amyl acetate odor alone 48 h after the conditioning. As described above, the PMd is likely to influence fear learning through a thalamic pathway involving the AMv and its associated cortico-hippocampal-amygdalar circuits. Interestingly, infusion of an NMDA receptor antagonist into another important target of the PMd—the dorsal PAG—was able to block the acquisition of OFC elicited by isoproterenol injection into the PMd (Pavesi et al., 2011), suggesting that the dorsal PAG would also serve for the PMd to provide instructive signals to prosencephalic circuits related to fear learning.

To address the putative role of the dorsal PAG in fear learning, we tested whether glutamatergic activation of the dorsal PAG could serve as a reliable US in an OFC paradigm (Kincheski et al., 2012). Using the same olfactory fear conditioning test described above, rats were subjected to CS/US pairings, with NMDA infusion into the dorsal PAG serving as the US. In contrast to what was observed for PMd beta-adrenergic activation, immediately after the NMDA injection into the dorsal PAG, the rats exhibited flight and jumping behaviors during the first minute of observation. This behavior was followed by an increased amount of freezing, which was limited to the first 5 min (Kincheski et al., 2012). Notably, when the subjects were removed from the conditioning box during the first 5 min of odor exposure, which is when the flight, jumping and freezing responses were predominant, no learning occurred. Conversely, when the rats were removed 5 min after the initial period of vigorous defensive responses, when most of the overt defensive behavior had waned, fear learning was more effective; these results support the idea that NMDA applied directly to the dorsal PAG can serve as a reliable US and is capable of supporting olfactory fear conditioning (Kincheski et al., 2012).

To understand how the dorsal PAG is able to influence prosencephalic circuits related to fear learning, we revisited the ascending connections of the dorsolateral PAG (Kincheski et al., 2012; **Figure 3**). The main ascending target of the dorsolateral PAG is the anterior hypothalamic nucleus, which, as described above, is part of the medial hypothalamic predator-responsive circuit. In line with this view, electrical stimulation of the dorsolateral PAG up-regulates Fos expression in the PMd (Vianna et al., 2003), supporting the idea that the PAGdl and the predator-responsive circuit operate in concert. Notably, blockade of PMd beta-adrenergic receptors with atenolol has been shown to impair the acquisition of the olfactory fear learning that is promoted by NMDA stimulation of the dorsal PAG (Kincheski et al., 2012). Thus, the projection from the PAGdl to the medial hypothalamic predator-responsive circuit may serve as an important link by which the prosencephalic sites involved in fear learning can be influenced. In addition, as shown in **Figure 3**, the PAGdl may potentially influence fear learning through a number of parallel thalamic pathways. The PAGdl provides a substantial projection to the intralaminar nuclei. The intralaminar nuclei, in turn, project to the anterior cingulate area, forming a pathway that is involved in fear learning (Furlong et al., 2010). In addition, the PAGdl also projects to a number of other thalamic sites, including the nucleus reuniens, the lateral

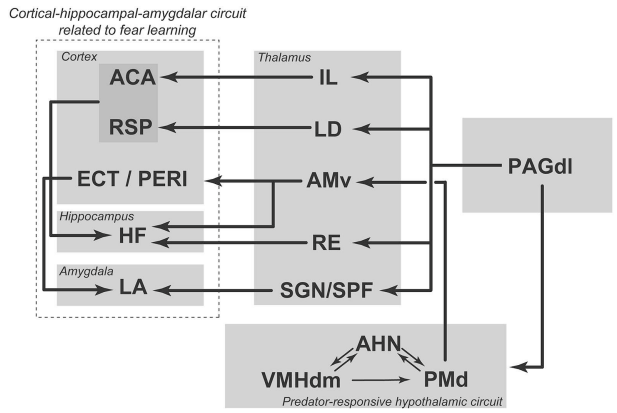


FIGURE 3 | Summary diagram illustrating ascending projections from the dorsolateral PAG to hypothalamic and thalamic targets; these projections influence cortical-hippocampal-amygdalar circuits related to fear learning. ACA, anterior cingulate area; AHN, anterior hypothalamic nucleus; AMv, anteromedial thalamic nucleus, ventral part; ECT, ectorhinal area; HF, hippocampal formation; IL, intralaminar nuclei; LA, lateral amygdalar nucleus; LD, laterodorsal thalamic nucleus; PAGdl, periaqueductal gray, dorsolateral part; PERI, perirhinal area; PMd, dorsal premammillary nucleus; RE, nucleus reuniens; RSP, retrosplenial area; SGN, supragenicular nucleus; SPF, subparafascicular thalamic nucleus; VMHdm, ventromedial hypothalamic nucleus, dorsomedial part. See text for discussion. Modified from Kincheski et al. (2012).

dorsal nucleus, the supragenicular nucleus and the parvicellular subparafascicular nucleus, all of which are known to project to elements of the cortical-hippocampal-amygdalar circuit that is involved in fear conditioning, as described above (Van Groen and Wyss, 1992; Linke et al., 2000; Vertes et al., 2006). An important concept emerges from the present analysis: subcortical regions that process elemental fear to predator threats, such as the PMd and the dorsal PAG, may work as critical nodes that serve to instruct prosencephalic sites to promote fear learning. Accordingly, the processing of primal emotional states related to predatory threats should take place in these subcortical nodes and exert a critical influence on fear learning.

Shock-based Fear Conditioning Using Olfactory Cues Engages Elements of the Hypothalamic Circuit Responsive to Predator Odor

The most common experimental approach to investigate fear processing is shock-based Pavlovian conditioning. However, shock-based fear conditioning to non-olfactory cues does not appear to engage elements of the hypothalamic predator-responsive system and therefore does not seem to be suitable for investigations of the neural basis of fear responses to predator odor (Gross and Canteras, 2012). Accordingly, we have tested whether shock-based fear conditioning to olfactory cues would be a useful system to model predator odor and engage elements of the hypothalamic circuit responsive to cat odor.

Data from our laboratory have confirmed that a neutral olfactory stimulus, such as coffee odor or amyl acetate, can serve as a reliable CS in a fear conditioning paradigm (Canteras et al., 2008). As shown in **Figure 4**, the experimental paradigm consisted of two consecutive phases: the acquisition of olfactory fear conditioning (days 1 and 2) and the expression of olfactory fear conditioning (days 3–5). The expression of olfactory fear conditioning (second phase) was performed in an odor box (**Figure 4**) and consisted of three sessions: familiarization (day 3), CS-neutral odor exposure (day 4; test session), and context (day 5). During the familiarization session, the animals did not exhibit fear responses to the odor box, indicating that they did not generalize the fear response to a different context. As the animals were re-exposed to the CS-neutral odor in the odor box, they displayed clear defensive responses and spent most of the time either hiding or engaged in "head-out" behavior. They also avoided approaching the odor source. When the animals were placed in the same context without the CS-neutral odor, the animals exhibited the same sort of defensive behaviors displayed on the previous day during exposure to the CS-neutral odor. These results are important because they demonstrate that the CS-neutral odor was able to mimic a predator odor and produced clear contextually conditioned defensive behavior (Canteras et al., 2008).

Interestingly, the shock-paired neutral odor also up-regulated Fos expression in the PMd. Therefore, similar to cat odor, the shock-paired neutral odor also mobilized the PMd (Canteras et al., 2008). Moreover, the PMd has been shown to be involved in the expression of conditioned responses to a shock-paired

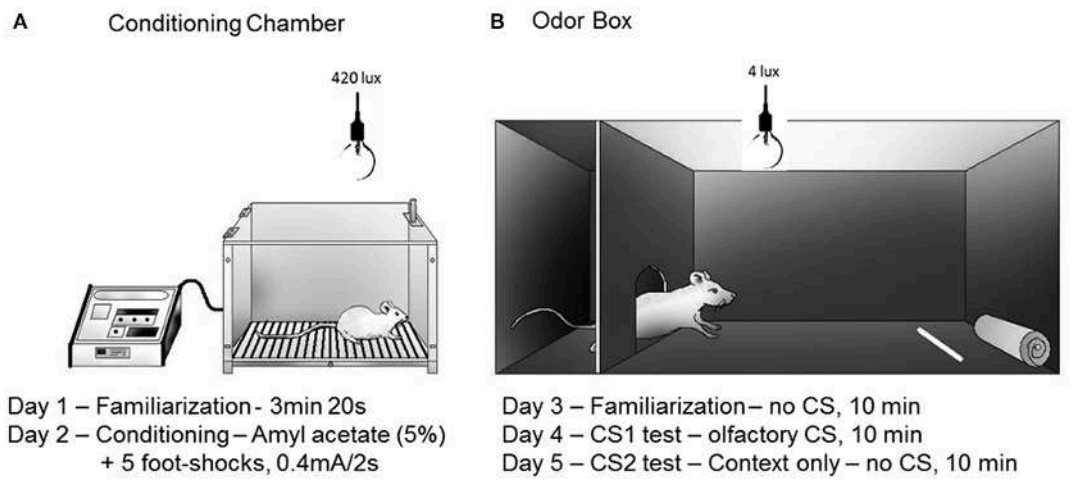


FIGURE 4 | Schematic drawings representing the conditioning chamber (A) and the odor box (B) used in the olfactory fear conditioning protocol. Rats were placed inside a stainless steel box located under a fume hood with lighting conditions of 420 lux **(A)** on day 1 (5 min) and day 2 (conditioning). A filter paper saturated with amyl acetate (5%, 250 μl) was used as the olfactory CS. Five electrical foot shocks (0.5 mA, 2 s, 40 s inter-trial interval) were used as the US. The retention of the CS-US association was tested in a Plexiglass box **(B)** that was also located under a fume hood with lighting conditions of 4 lux. The Plexiglass box consisted of a roof-enclosed compartment (left side) and an open (unroofed) compartment (right side). Retention was tested in 10 min sessions on the following three consecutive days (day 3, no-odor, habituation; day 4, olfactory CS exposure test; day 5, associated context, no-odor). The odor source was positioned in the opposite side of the enclosed compartment. The parameters analyzed included the percentage of time spent in the following behaviors: approaching the CS, hiding in the enclosed compartment and stretching out from the enclosed compartment toward the open compartment (head-out). The protocol was based on Dielenberg and McGregor (1999) and Kroon and Carobrez (2009).

neutral odor and to influence the acquisition of CS1-CS2 second-order contextual conditioning. Accordingly, NMDA receptor blockade in the PMd during re-exposure to the footshock-paired neutral odor significantly reduced conditioned fear responses to the CS-neutral odor and resulted in clear impairment of contextual defensive responses on the following day (Canteras et al., 2008).

We have further tested the role of beta-adrenergic transmission in the PMd to investigate its participation in the expression of shock-based olfactory fear conditioning. Animals were subjected to the shock-based olfactory fear conditioning protocol as previously described. On day 4, prior to the expression of olfactory fear conditioning, the rats were divided into four groups: the control group (PBS), atenolol

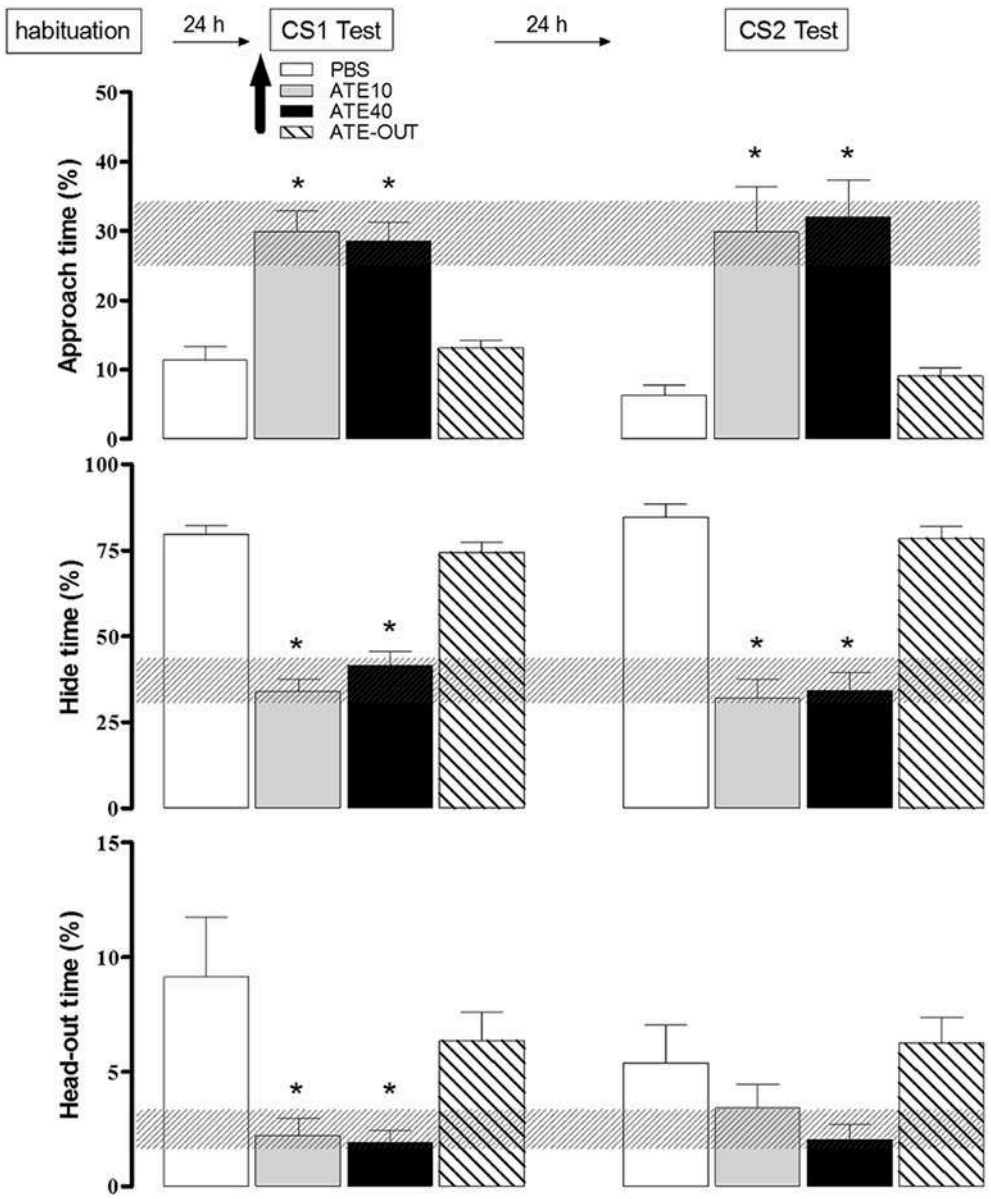


FIGURE 5 | Effects of dorsal premammillary nucleus (PMd) application of the beta-adrenoceptor antagonist atenolol (ATE; 10–40 nmol; 0.3 μl on the defensive behavior of rats exposed to an olfactory conditioned stimulus (CS). The familiarization session and the olfactory (CS1) and context (CS2) sessions (10 min in duration) were conducted over three consecutive days. The parameters analyzed were plotted as the mean (+SEM) and were represented in histograms as the percentage of time spent approaching the odor source (top panel), hiding in the enclosed compartment (middle) and stretching out from the enclosed compartment toward the open compartment (head-out; bottom) during the CS1 or CS2 test session. The hatched horizontal bars represent the confidence limit intervals (within 95%) obtained during the familiarization session. The subjects ($n = 8$–12) received PBS ($n = 9$) or 10 ($n = 8$) or 40 ($n = 8$) nmol ATE, which was administered into the PMd 10 min before the rats were exposed to the CS1 (amyl acetate 5%). ATE OUT ($n = 12$) represents rats in which the cannula was placed outside the PMd. *$p < 0.05$, compared with the PBS control group (repeated measures ANOVA followed by Newman–Keuls' *post-hoc* test).

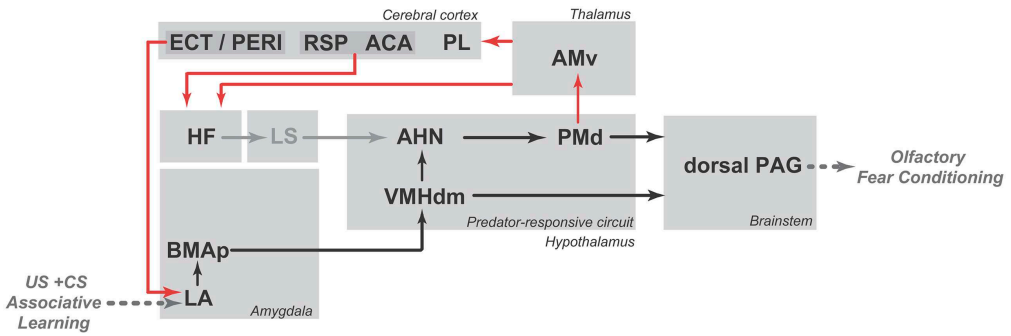


FIGURE 6 | Schematic diagram showing the putative circuit involved in the expression of olfactory fear conditioning to a neutral odor that was previously paired with footshock (shown in black lines) as well as the PMd–AMv path and the cortical-hippocampal-amygdalar circuit that are putatively involved in second-order contextual conditioning (shown in red lines). ACA, anterior cingulate area; AHN, anterior hypothalamic nucleus; AMv, anteromedial thalamic nucleus, ventral part; BMAp, basomedial amygdalar nucleus, posterior part; ECT, ectorhinal area; HF, hippocampal formation; LA, lateral amygdalar nucleus; LS, lateral septum; PAG, periaqueductal gray; PERI, perirhinal area; PL, prelimbic area; PMd, dorsal premammillary nucleus; RSP, retrosplenial area; VMHdm, ventromedial hypothalamic nucleus, dorsomedial part. See text for discussion.

outside the PMd (ATE-out), and atenolol in the PMD (10 nmol of ATE-10 or 40 nmol of ATE-40). The rats were microinjected into the PMd, and 10 min later, were placed in the apparatus in the presence of the CS-neutral odor. As shown in **Figure 5**, in contrast to the animals of the other experimental groups (the PBS and the ATE-out groups), the animals in which atenolol was injected into the PMd (the ATE-10 and ATE-40 groups) showed a significant increase in approach time and a significant decrease in hiding and head-out times during exposure to the footshock-paired odor (CS-neutral odor) as well as decreased contextual defensive responses on the following day, as demonstrated by a significant increase in the time the animals spent approaching a neutral cloth and a decreased hide time. Taken together, the experimental data suggest that the PMd may influence both the expression of olfactory fear conditioning and CS1-CS2 second-order contextual conditioning.

As shown in **Figure 6**, associative learning between conditioned and unconditioned stimuli is likely to occur in the lateral amygdalar nucleus. In support of this view, tetrodotoxin infusion in the region of the lateral amygdalar nucleus has been shown to impair the acquisition of olfactory fear conditioning (Kilpatrick and Cahill, 2003). Notably, the lateral amygdalar nucleus may influence the medial hypothalamic predator-responsive circuit through its dense projections to the posterior part of the basomedial amygdalar nucleus, which provides substantial input to the ventromedial hypothalamic nucleus (Petrovich et al., 1996). Alternatively, as pointed by Cádiz-Moretti et al. (2014), information about pain stimuli (relayed through the posterior intralaminar thalamic complex and the parabrachial area) and neutral odors (relayed through indirect projections from the piriform cortex and cortical amygdala) may converge in the MEApv, a critical site for responses to predator odors, and this association at the MEApv could easily explain how a neutral odor that was previously paired with foot shock would engage elements of the predator-responsive hypothalamic circuit. However, further studies are needed to provide support for this hypothesis.

As discussed here, the PMd seems to be critical for the expression of olfactory fear conditioning as well as for second-order contextual conditioning. As summarized in **Figure 6**, the expression of olfactory fear conditioning may depend on PMd projections to the PAG (shown in black lines), whereas second-order contextual conditioning may involve the projection to the AMv and the associated cortico-hippocampal-amygdalar circuits that are related to fear learning (shown in red lines). However, further studies are needed to increase understanding of these pathways and their relationships with olfactory fear conditioning.

Concluding Remarks

Different types of predator odors engage elements of the hypothalamic predator-responsive circuit, which has been primarily investigated in studies using exposure to a live cat or its odor. These studies have provided the general basis of our understanding of how innate and learned fear responses to predator threats are organized. Of particular relevance, primal responses to predator odors involve a neural system formed by the medial amygdalar nucleus, the medial hypothalamic predator-responsive circuit and the dorsal PAG, and elements of this neural system (i.e., the PMd and dorsal PAG) seem to be critical in supplying instructive signals to cortico-hippocampal-amygdalar circuits related to fear conditioning to promote fear learning. According to the present view, the medial amygdalar nucleus processes predator olfactory cues and transmits this information to the medial hypothalamic predator-responsive circuit and the dorsal PAG, where primal emotional states related to predatory threats are processed. This idea provides an interesting perspective of the role of the hypothalamus and dorsal PAG, as opposed to telencephalic sites, in the processing of emotional states in response to predatory threats. Conversely, fear learning depends on instructive signals

from these subcortical nodes to cortico-hippocampal-amygdalar circuits for the association of a single cue or contextual information with the emotional state related to the predatory threat.

Interestingly, neutral olfactory stimuli that acquire an aversive valence in a shock-based fear conditioning paradigm may mobilize elements of the hypothalamic-predator responsive circuit, raising interesting possibilities related to how aversive learned olfactory stimuli can mimic predator odor. At this point, future studies are needed to explore how and where associative learning occurs and transforms neutral olfactory stimuli that have been paired with foot shocks into predator-like odors that have the same properties of natural predator odors in inducing innate and learned defensive responses.

Acknowledgments

This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Research Grant #2014/05432-9 to NC and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Research Grant #484407/2013-9 to AC. NC and AC received CNPq fellowships, and EP was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) fellowship grant BJT A055/2013.

References

Blanchard, D. C., Li, C. I., Hubbard, D., Markham, C. M., Yang, M., Takahashi, L. K., et al. (2003). Dorsal premammillary nucleus differentially modulates defensive behaviors induced by different threat stimuli in rats. *Neurosci. Lett.* 345, 145–148. doi: 10.1016/S0304-3940(03)00415-4

Blanchard, R. J., Blanchard, D. C., Weiss, S. M., and Meyer, S. (1990). The effects of ethanol and diazepam on reactions to predatory odors. *Pharmacol. Biochem. Behav.* 35, 775–780. doi: 10.1016/0091-3057(90)90357-N

Blanchard, R. J., Shepherd, J. K., Rodgers, R. J., Magee, L., and Blanchard, D. C. (1993). Attenuation of antipredator defensive behavior in rats following chronic treatment with imipramine. *Psychopharmacology (Berl)* 110, 245–253. doi: 10.1007/BF02246981

Blanchard, R. J., Yang, M., Li, C. I., Gervacio, A., and Blanchard, D. C. (2001). Cue and context conditioning of defensive behaviors to cat odor stimuli. *Neurosci. Biobehav. Rev.* 25, 587–595. doi: 10.1016/S0149-7634(01)00043-4

Brechbühl, J., Moine, F., Klaey, M., Nenniger-Tosato, M., Hurni, N., Sporkert, F., et al. (2013). Mouse alarm pheromone shares structural similarity with predator scents. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4762–4767. doi: 10.1073/pnas.1214249110

Bremner, J. D., Krystal, J. H., Southwick, S. M., and Charney, D. S. (1996). Noradrenergic mechanisms in stress and anxiety: I. Preclinical studies. *Synapse* 23, 28–38.

Cádiz-Moretti, B., Otero-García, M., Martínez-García, F., and Lanuza, E. (2014). Afferent projections to the different medial amygdala subdivisions: a retrograde tracing study in the mouse. *Brain Struct. Funct.* doi: 10.1007/s00429-014-0954-y. [Epub ahead of print].

Canteras, N. S. (2002). The medial hypothalamic defensive system: hodological organization and functional implications. *Pharmacol. Biochem. Behav.* 71, 481–491. doi: 10.1016/S0091-3057(01)00685-2

Canteras, N. S., and Swanson, L. W. (1992). The dorsal premammillary nucleus: an unusual component of the mammillary body. *Proc. Natl. Acad. Sci. U.S.A.* 89, 10089–10093. doi: 10.1073/pnas.89.21.10089

Canteras, N. S., Chiavegatto, S., Ribeiro do Valle, L. E., and Swanson, L. W. (1997). Severe reduction of rat defensive behavior to a predator by discrete hypothalamic chemical lesions. *Brain Res. Bull.* 44, 297–305. doi: 10.1016/S0361-9230(97)00141-X

Canteras, N. S., Kroon, J. A., Do-Monte, F. H., Pavesi, E., and Carobrez, A. P. (2008). Sensing danger through the olfactory system: the role of the hypothalamic dorsal premammillary nucleus. *Neurosci. Biobehav. Rev.* 32, 1228–1235. doi: 10.1016/j.neubiorev.2008.05.009

Canteras, N. S., Simerly, R. B., and Swanson, L. W. (1995). Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. *J. Comp. Neurol.* 360, 213–245. doi: 10.1002/cne.903600203

Carrive, P. (1993). The periaqueductal gray and defensive behavior: functional representation and neuronal organization. *Behav. Brain Res.* 58, 27–47. doi: 10.1016/0166-4328(93)90088-8

Cezario, A. F., Ribeiro-Barbosa, E. R., Baldo, M. V., and Canteras, N. S. (2008). Hypothalamic sites responding to predator threats–the role of the dorsal premammillary nucleus in unconditioned and conditioned antipredatory defensive behavior. *Eur. J. Neurosci.* 28, 1003–1015. doi: 10.1111/j.1460-9568.2008.06392.x

De Lima, M. A. X., Rufino, R. A., Mota-Ortiz, S. R., Baldo, M. V. C., and Canteras, N. S. (2013). "The ventral part of the anteromedial thalamic nucleus is involved in the acquisition, but not the expression of contextual responses to predatory threats," in *Abstract Presented at Society for Neuroscience Meeting*, Abstract number: 862.19.

Dewan, A., Pacifico, R., Zhan, R., Rinberg, D., and Bozza, T. (2013). Non redundant coding of aversive odours in the main olfactory pathway. *Nature* 497, 486–489. doi: 10.1038/nature12114

Dicke, M., and Grostal, P. (2001). Chemical detection of natural enemies by arthropods: an ecological perspective. *Annu. Rev. Ecol. Syst.* 32, 1–23. doi: 10.1146/annurev.ecolsys.32.081501.113951

Dielenberg, R. A., and McGregor, I. S. (1999). Habituation of the hiding response to cat odor in rats (*Rattus norvegicus*). *J. Comp. Psychol.* 113, 376–387. doi: 10.1037/0735-7036.113.4.376

Dielenberg, R. A., and Mcgregor, I. S. (2001). Defensive behavior in rats towards predatory odors: a review. *Neurosci. Biobehav. Rev.* 25, 597–609. doi: 10.1016/S0149-7634(01)00044-6

Dielenberg, R. A., Hunt, G. E., and Mcgregor, I. S. (2001). "When a rat smells a cat": the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor. *Neuroscience* 104, 1085–1097. doi: 10.1016/S0306-4522(01)00150-6

Dielenberg, R. A., Leman, S., and Carrive, P. (2004). Effect of dorsal periaqueductal gray lesions on cardiovascular and behavioral responses to cat odor exposure in rats. *Behav. Brain Res.* 153, 487–496. doi: 10.1016/j.bbr.2004.01.015

Do Monte, F. H., Canteras, N. S., Fernandes, D., Assreuy, J., and Carobrez, A. P. (2008). New perspectives on beta-adrenergic mediation of innate and learned fear responses to predator odor. *J. Neurosci.* 28, 13296–13302. doi: 10.1523/JNEUROSCI.2843-08.2008

Ferrero, D. M., Lemon, J. K., Fluegge, D., Pashkovski, S. L., Korzan, W. J., Datta, S. R., et al. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11235–11240. doi: 10.1073/pnas.1103317108

Furlong, T. M., Cole, S., Hamlin, A. S., and McNally, G. P. (2010). The role of prefrontal cortex in predictive fear learning. *Behav. Neurosci.* 124, 574–586. doi: 10.1037/a0020739

Fuster, J. M. (1997). Network memory. *Trends Neurosci.* 20, 451–459. doi: 10.1016/S0166-2236(97)01128-4

Gross, C. T., and Canteras, N. S. (2012). The many paths to fear. *Nat. Rev. Neurosci.* 13, 651–658. doi: 10.1038/nrn3301

Hubbard, D. T., Blanchard, D. C., Yang, M., Markham, C. M., Gervacio, A., Chun-I, L., et al. (2004). Development of defensive behavior and conditioning to cat odor in the rat. *Physiol. Behav.* 80, 525–530. doi: 10.1016/j.physbeh.2003.10.006

Johansen, J. P., Cain, C. K., Ostroff, L. E., and LeDoux, J. E. (2011). Molecular mechanisms of fear learning and memory. *Cell* 147, 509–524. doi: 10.1016/j.cell.2011.10.009

Jones, B. F., and Witter, M. P. (2007). Cingulate cortex projections to the parahippocampal region and hippocampal formation in the rat. *Hippocampus* 17, 957–976. doi: 10.1002/hipo.20330

Keay, K. A., and Bandler, R. (1993). Deep and superficial noxious stimulation increases Fos-like immunoreactivity in different regions of the midbrain periaqueductal grey of the rat. *Neurosci. Lett.* 154, 23–26. doi: 10.1016/0304-3940(93)90162-E

Kilpatrick, L., and Cahill, L. (2003). Modulation of memory consolidation for olfactory learning by reversible inactivation of the basolateral amygdala. *Behav. Neurosci.* 117, 184–188. doi: 10.1037/0735-7044.117.1.184

Kincheski, G. C., Mota-Ortiz, S. R., Pavesi, E., Canteras, N. S., and Carobrez, A. P. (2012). The dorsolateral periaqueductal gray and its role in mediating fear learning to life threatening events. *PLoS ONE* 7:e50361. doi: 10.1371/journal.pone.0050361

Kroon, J. A., and Carobrez, A. P. (2009). Olfactory fear conditioning paradigm in rats: effects of midazolam, propranolol or scopolamine. *Neurobiol. Learn. Mem.* 91, 32–40. doi: 10.1016/j.nlm.2008.10.007

Liberles, S. D. (2015). Trace amine-associated receptors: ligands, neural circuits, and behaviors. *Curr. Opin. Neurobiol.* 34C, 1–7. doi: 10.1016/j.conb.2015.01.001

Linke, R., Braune, G., and Schwegler, H. (2000). Differential projection of the posterior paralaminar thalamic nuclei to the amygdaloid complex in the rat. *Exp. Brain Res.* 134, 520–532. doi: 10.1007/s002210000475

Martinez, R. C., Carvalho-Netto, E. F., Ribeiro-Barbosa, E. R., Baldo, M. V., and Canteras, N. S. (2011). Amygdalar roles during exposure to a live predator and to a predator-associated context. *Neuroscience* 172, 314–328. doi: 10.1016/j.neuroscience.2010.10.033

McGregor, I. S., Hargreaves, G. A., Apfelbach, R., and Hunt, G. E. (2004). Neural correlates of cat odor-induced anxiety in rats: region-specific effects of the benzodiazepine midazolam. *J. Neurosci.* 24, 4134–4144. doi: 10.1523/JNEUROSCI.0187-04.2004

Mcgregor, I. S., Schrama, L., Ambermoon, P., and Dielenberg, R. A. (2002). Not all 'predator odours' are equal: cat odour but not 2,4,5 trimethylthiazoline (TMT; fox odour) elicits specific defensive behaviours in rats. *Behav. Brain Res.* 129, 1–16. doi: 10.1016/S0166-4328(01)00324-2

Neophytou, S. I., Aspley, S., Butler, S., Beckett, S., and Marsden, C. A. (2001). Effects of lesioning noradrenergic neurones in the locus coeruleus on conditioned and unconditioned aversive behaviour in the rat. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 25, 1307–1321. doi: 10.1016/S0278-5846(01)00181-6

Pavesi, E., Canteras, N. S., and Carobrez, A. P. (2011). Acquisition of Pavlovian fear conditioning using beta-adrenoceptor activation of the dorsal premammillary nucleus as an unconditioned stimulus to mimic live predator-threat exposure. *Neuropsychopharmacology* 36, 926–939. doi: 10.1038/npp.2010.231

Pentkowski, N. S., Blanchard, D. C., Lever, C., Litvin, Y., and Blanchard, R. J. (2006). Effects of lesions to the dorsal and ventral hippocampus on defensive behaviors in rats. *Eur. J. Neurosci.* 23, 2185–2196. doi: 10.1111/j.1460-9568.2006.04754.x

Pérez-Gómez, A., Bleymehl, K., Stein, B., Pyrski, M., Birnbaumer, L., Munger, S. D., et al. (2015). Innate predator odor aversion driven by parallel olfactory subsystems that converge in the ventromedial hypothalamus. *Curr. Biol.* 25, 1340–1346. doi: 10.1016/j.cub.2015.03.026

Petrovich, G. D., Risold, P. Y., and Swanson, L. W. (1996). Organization of projections from the basomedial nucleus of the amygdala: a PHAL study in the rat. *J. Comp. Neurol.* 374, 387–420.

Pinel, J. P., Symons, L. A., Christensen, B. K., and Tees, R. C. (1989). Development of defensive burying in *Rattus norvegicus*: experience and defensive responses. *J. Comp. Psychol.* 103, 359–365. doi: 10.1037/0735-7036.103.4.359

Risold, P. Y., Canteras, N. S., and Swanson, L. W. (1994). Organization of projections from the anterior hypothalamic nucleus: a *Phaseolus vulgaris*-leucoagglutinin study in the rat. *J. Comp. Neurol.* 348, 1–40. doi: 10.1002/cne.903480102

Schenberg, L. C., Bittencourt, A. S., Sudré, E. C., and Vargas, L. C. (2001). Modeling panic attacks. *Neurosci. Biobehav. Rev.* 25, 647–659. doi: 10.1016/S0149-7634(01)00060-4

Shi, C. J., and Cassell, M. D. (1999). Perirhinal cortex projections to the amygdaloid complex and hippocampal formation in the rat. *J. Comp. Neurol.* 406, 299–328.

Shibata, H. (1993). Efferent projections from the anterior thalamic nuclei to the cingulate cortex in the rat. *J. Comp. Neurol.* 330, 533–542. doi: 10.1002/cne.903300409

Sobrinho, C. R., and Canteras, N. S. (2011). A study of the catecholaminergic inputs to the dorsal premammillary nucleus. *Neurosci. Lett.* 501, 157–162. doi: 10.1016/j.neulet.2011.07.006

Staples, L. G., Hunt, G. E., Cornish, J. L., and McGregor, I. S. (2005). Neural activation during cat odor-induced conditioned fear and 'trial 2' fear in rats. *Neurosci. Biobehav. Rev.* 29, 1265–1277. doi: 10.1016/j.neubiorev.2005.04.009

Sukikara, M. H., Mota-Ortiz, S. R., Baldo, M. V., Felicio, L. F., and Canteras, N. S. (2010). The periaqueductal gray and its potential role in maternal behavior inhibition in response to predatory threats. *Behav. Brain Res.* 209, 226–233. doi: 10.1016/j.bbr.2010.01.048

Takahashi, L. K. (2014). Olfactory systems and neural circuits that modulate predator odor fear. *Front. Behav. Neurosci.* 8:72. doi: 10.3389/fnbeh.2014.00072

Takahashi, L. K., Nakashima, B. R., Hong, H., and Watanabe, K. (2005). The smell of danger: a behavioral and neural analysis of predator odor-induced fear. *Neurosci. Biobehav. Rev.* 29, 1157–1167. doi: 10.1016/j.neubiorev.2005.04.008

Van Der Poel, A. M. (1979). A note on "stretched attention", a behavioural element indicative on an approach-avoidance conflict in rats. *Anim. Behav.* 27, 446–450. doi: 10.1016/0003-3472(79)90181-7

Van Groen, T., and Wyss, J. M. (1992). Projections from the laterodorsal nucleus of the thalamus to the limbic and visual cortices in the rat. *J. Comp. Neurol.* 324, 427–448. doi: 10.1002/cne.903240310

Van Groen, T., Kadish, I., and Wyss, J. M. (1999). Efferent connections of the anteromedial nucleus of the thalamus of the rat. *Brain Res. Brain Res. Rev.* 30, 1–26. doi: 10.1016/S0165-0173(99)00006-5

Vermetten, E., Schmahl, C., Southwick, S. M., and Bremner, J. D. (2007). Positron tomographic emission study of olfactory induced emotional recall in veterans with and without combat-related posttraumatic stress disorder. *Psychopharmacol. Bull.* 40, 8–30.

Vertes, R. P., Hoover, W. B., Do Valle, A. C., Sherman, A., and Rodriguez, J. J. (2006). Efferent projections of reuniens and rhomboid nuclei of the thalamus in the rat. *J. Comp. Neurol.* 499, 768–796. doi: 10.1002/cne.21135

Vianna, D. M., Borelli, K. G., Ferreira-Netto, C., Macedo, C. E., and Brandão, M. L. (2003). Fos-like immunoreactive neurons following electrical stimulation of the dorsal periaqueductal gray at freezing and escape thresholds. *Brain Res. Bull.* 62, 179–189. doi: 10.1016/j.brainresbull.2003.09.010

Wang, L., Chen, I. Z., and Lin, D. (2015). Collateral pathways from the ventromedial hypothalamus mediate defensive behaviors. *Neuron* 85, 1344–1358. doi: 10.1016/j.neuron.2014.12.025

Wiemers, U. S., Sauvage, M. M., and Wolf, O. T. (2014). Odors as effective retrieval cues for stressful episodes. *Neurobiol. Learn. Mem.* 112, 230–236. doi: 10.1016/j.nlm.2013.10.004

Wyatt, T. D. (2014). *Pheromones and Animal Behavior, 2nd Edn*. Cambridge, UK: Cambridge University Press.

Wyss, J. M., and Van Groen, T. (1992). Connections between the retrosplenial cortex and the hippocampal formation in the rat: a review. *Hippocampus* 2, 1–11. doi: 10.1002/hipo.450020102

Zangrossi, H. Jr., and File, S. E. (1992). Chlordiazepoxide reduces the generalised anxiety, but not the direct responses, of rats exposed to cat odor. *Pharmacol. Biochem. Behav.* 43, 1195–1200. doi: 10.1016/0091-3057(92)90502-7

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 07 October 2015
doi: 10.3389/fnins.2015.00363

The scent of wolves: pyrazine analogs induce avoidance and vigilance behaviors in prey

Kazumi Osada[1]*, *Sadaharu Miyazono*[2] *and Makoto Kashiwayanagi*[2]

[1] Division of Physiology, Department of Oral Biology, School of Dentistry, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Japan, [2] Department of Sensory Physiology, Asahikawa Medical University, Asahikawa, Japan

The common gray wolf (*Canis lupus*) is an apex predator located at the top of the food chain in the Northern Hemisphere. It preys on rodents, rabbits, ungulates, and many other kinds of mammal. However, the behavioral evidence for, and the chemical basis of, the fear-inducing impact of wolf urine on prey are unclear. Recently, the pyrazine analogs 2, 6-dimethylpyrazine, 2, 3, 5-trimethylpyrazine and 3-ethyl-2, 5-dimethyl pyrazine were identified as kairomones in the urine of wolves. When mice were confronted with a mixture of purified pyrazine analogs, vigilance behaviors, including freezing and excitation of neurons at the accessory olfactory bulb, were markedly increased. Additionally, the odor of the pyrazine cocktail effectively suppressed the approach of deer to a feeding area, and for those close to the feeding area elicited fear-related behaviors such as the "tail-flag," "flight," and "jump" actions. In this review, we discuss the transfer of chemical information from wolf to prey through the novel kairomones identified in wolf urine and also compare the characteristics of wolf kairomones with other predator-produced kairomones that affect rodents.

Keywords: fear, Hokkaido deer, kairomone, pyrazine analogs, wolf

OPEN ACCESS

Edited by:
Markus Fendt,
Otto-von-Guericke University
Magdeburg, Germany

Reviewed by:
Gerard Coureaud,
Centre National de la Recherche
Scientifique, France
Raimund Apfelbach,
University of Tübingen, Germany

***Correspondence:**
Kazumi Osada,
Division of Physiology, Department of
Oral Biology, School of Dentistry,
Health Sciences University of
Hokkaido, 1757 Kanazawa,
Ishikari-Tobetsu,
Hokkaido 061-0293, Japan
osadak@hoku-iryo-u.ac.jp

Specialty section:
This article was submitted to
Systems Biology,
a section of the journal
Frontiers in Neuroscience

Received: *25 June 2015*
Accepted: *22 September 2015*
Published: *07 October 2015*

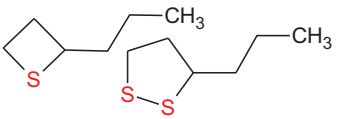
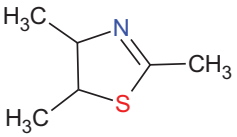
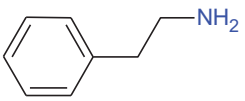
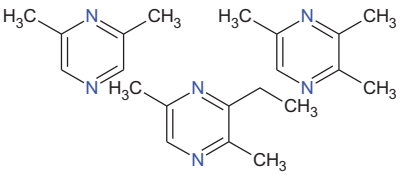
Citation:
Osada K, Miyazono S and
Kashiwayanagi M (2015) The scent of
wolves: pyrazine analogs induce
avoidance and vigilance behaviors in
prey. Front. Neurosci. 9:363.
doi: 10.3389/fnins.2015.00363

Introduction

The common gray wolf (*Canis lupus*) is an apex predator at the top of the food chain in the Northern Hemisphere. It preys on rodents, rabbits, ungulates, and many other kinds of mammal. The detection of predator phenotypic traits by prey species is a vitally important function of communication between mammals. How prey species discern predators is an important question. For prey animals that rely on chemical communication to regulate social and sexual interactions, it is possible that the presence of a predator can be detected by its scent. These scents and some non-volatile molecules that affect the vomeronasal organ (VNO) (Hurst et al., 2001; Kimoto et al., 2005; Papes et al., 2010; Kaur et al., 2014) are defined as semiochemicals. Semiochemicals are divided into two major groups: pheromones (for conspecific communication) and allelochemicals (for interspecific communication) (Nielsen et al., 2015). Kairomones are allelochemicals that transfer unidirectionally from an emitter to a receiver and provide a benefit to the receiver organism (Brown et al., 1970; Liberles, 2014; Nielsen et al., 2015; Wernecke et al., 2015). Therefore, when a prey animal benefits, the chemical signal produced by a predator is a kairomone. The known kairomones produced by predators that affect rodents are summarized in the **Table 1**.

Wolf urine contains several volatile chemicals that could be used for predator-prey chemosignaling. Wolves use scent-marking to inform neighboring wolf packs of their existence, and herbivores may also use the signals. Moreover, wolf urine is artificially applied by humans to

TABLE 1 | Rodent kairomones and the source materials from which they were derived.

Kairomones	Structure	Source	References
2-propylthietane, 3-propyl-1,2-dithiolane		Anal grand secretions from stoats (*Mustela erminea*) and ferrets (*M. putorius*)	Crump, 1978, 1980; Crump and Moors, 1985; Sullivan et al., 1988a,b
Trimethylthiazoline		Feces from red foxes (*Vulpes vulpes*)	Vernet-Maury et al., 1984; Wallace and Rosen, 2000; Fendt et al., 2005
MUP-13, MUP Feld4	Major urinary proteins: MW 18,729 kD (MUP-13)	Urine from rats *(Rattus norvegicus*), and saliva from cats (*Felis catus*)	Papes et al., 2010
Phenylethylamine		Urine from various kinds of carnivore	Ferrero et al., 2011
Alkylpyrazine analogs		Urine from wolves (*Canis lupus*)	Osada et al., 2013

keep many kinds of herbivore and other animals at bay (Sullivan et al., 1985a,b; Pyare and Berger, 2003; Chamaillé-Jammes et al., 2014). Thus, wolf urine possibly includes unidentified molecules that are used in predator-prey chemosignaling. Actually, the behavioral evidence for, and the chemical basis of, the fear-inducing impact of wolf urine on prey species were recently unveiled (Osada et al., 2013).

Wild animals frequently invade human habitats and can cause serious problems. For example, deer cause large amounts of damage and economic losses in agricultural, horticultural, and forest resources around the world (Trdan and Vidrih, 2008; Killian et al., 2009; Kimball et al., 2009; Baasch et al., 2010; Gheysen et al., 2011; Masuko et al., 2011). Rather than hunting deer, it may be preferable to control their behavior using kairomones so that they can coexist with other wild animals without destroying human habitats or natural environments. Here, we discuss the transfer of chemical information from wolf to prey through novel kairomones identified in wolf urine and compare the characteristics of the wolf kairomones with those of the other predator-produced kairomones that affect rodents.

The Study of Chemical Communication via Wolf Urine

The Scent of Wolf Urine is Used for Social Communication

Because wolves are gregarious carnivores, their olfactory-based communication system is much more complex than that of comparatively solitary species such as foxes and cats (Fox and Cohen, 1977). In addition to olfactory cues, wolves use visual, vocal, and tactile cues to communicate with each other. However, urine is an important mode of chemical communication for the wolf (Peters and Mech, 1975; Mech, 1977).

Using radio-tracking techniques to study natural wolf packs, Peters and Mech (1975) identified detailed conditions for urine scent-marking in wolves. Wolf packs living in the Superior National Forest of northeastern Minnesota are territorial, and most stable territories range in size from 125 to 310 km^2. These territories seem to be stable and exclusive, and over several months there is a "buffer zone" (about 2 km wide) along the borders. Interpack conflict is rare or non-existent in the area.

During four winter seasons, scent marking was studied to clarify the role played by marking in the information flow that is integral to maintaining the organization of wolf populations. One of the most important spatial differences was the difference in the urination rate between the centers and the edges of wolf territories. The frequency of urination (number of urinations/km track) along the edges of territories was approximately 2.1-fold higher than that in the center of territories. The wolves engaged in scent marking along the edges of their territories to inform members of the neighboring wolf packs of their presence, particularly during the breeding season (Peters and Mech, 1975). Similarly, coyotes (*Canis latrans*), which are smaller close relatives of the wolves, scent-mark in the same manner to maintain their territories (Gese and Ruff, 1997). These

observations suggest that alarm pheromones used by conspecifics exist in wolf urine.

It is also conceivable that wolf urine contains chemosignals used for communication between individuals belonging to different species. For example, densities of white-tailed deer (*Odocoileus virginianus*) are higher in buffer zones between territories held by wolf packs than inside the territories (Mech, 1977). Wolves apparently avoid hunting in buffer zones between the edges of territories that are indicated by scent-marks. Therefore, the survival of deer should be greater in buffer zones due to lower rates of predation by wolves. Importantly, deer voluntarily migrate to buffer zones in winter (Hoskinson and Mech, 1976; Rogers et al., 1980), suggesting that deer utilize chemo-olfactory cues and other sensory cues to reach these safety zones. Deer must be able to assess the quality and the intensity of odor emitted from the urine of wolves. Research therefore clearly suggests that urination by wolves and the semiochemicals in the urine are involved not only in conspecific pheromone perception, but also in the interspecific detection of kairomones.

The Urine of Predators, Including Wolves, Causes Avoidance Behavior in Various Types of Herbivore

Research shows that exposure to predator odor induces avoidance behavior in many kinds of prey animal, including ungulates. For example, white-tailed deer and/or black-tailed deer (*Odocoileus hemionus columbianus*) avoid the urinary odor of predators, including wolf, coyote, red fox (*Vulpes vulpes*), wolverine (*Gulo gulo*), lynx (*Lynx canadensis*), and bobcat (*Lynx rufus*), and the odor of the feces of cougar (*Puma concolor*), coyote, and wolf (Sullivan et al., 1985b; Swihart et al., 1991).

Specifically, Swihart et al. (1991) demonstrated that the topical application of coyote and bobcat urine (6 ml per plant) at weekly intervals to Japanese yew (*Taxus cuspidate*) and eastern hemlock (*Tsuga canadensis*) trees deterred white-tailed deer for at least 8 weeks. Non-predator (human and rabbit *Sylvilagus floridanus*) urine had no repellent effect. Coyote urine prevented damage slightly less well than bobcat urine. On the other hand, Sullivan et al. (1985b) conducted a bioassay to study the effect of dispensed predator urines in vials attached to salal (*Gaultheria shallon*) branches on black-tailed deer. The study demonstrated that the odor of wolf, coyote, and fox urine was more effective in suppressing the feeding of deer on salal than control or bobcat urine; this effect lasted for at least 6 days. Moreover, cattle (*Bos taurus*) exposed to the odor of wolf or dingo (*Canis lupus dingo*) showed defensive or avoidance responses (Kluever et al., 2009). These studies suggest that predator urine contains kairomone(s), which induce robust avoidance behavior not only in wild deer, but also in ungulate livestock.

In addition, hares (*Lepus americanus*) leave or avoid areas treated with odors derived from several kinds of predator (Sullivan et al., 1985a). American beavers (*Castor canadensis*) and marsupials show defensive or avoidance responses to the odor of wolves or dingoes (Lindgren et al., 1995; Parsons and Blumstein, 2010). Importantly, exposure to the urine of predators, but not to that of herbivores or conspecifics, induces defensive behaviors in laboratory rats (*Rattus norvegicus*), suggesting that laboratory rats detect a predisposed-active cue in predator odors (Fendt, 2006).

Research clearly indicates that the urine and feces of many carnivores, including wolves, contains kairomones that repel their prey animals. In a practical application, the urine of wolves or other predators can be used to drive away these animals without killing them (Sullivan et al., 1985a,b; Lindgren et al., 1995; Severud et al., 2011).

Chemical Physiology of the Volatile Constituents in Wolf Urine and that of Other Wild Canids

The urine of the wolf (Raymer et al., 1984), coyote (Nolte et al., 1994) and red fox (Jorgenson et al., 1978) contains numerous chemicals that emit a strong stench. Raymer et al. (1984) analyzed the chemical components of wolf urine that change with gender. Gas chromatography-mass spectrometry (GC-MS) and gas chromatography with a flame ionization detector (FID-GC) were used to identify and quantify typical wolf urinary components. The profile of volatiles resulting from GC separation was obtained through a headspace sampling procedure by thermal desorption of volatiles from a porous polymer (Tenax). Several compounds including Δ^3-isopentenyl methyl sulfide (IMS), 3, 5-dimethyl-2-octanone, and acetophenone were clearly associated with the gender of the animal, and also changed seasonally (Raymer et al., 1984). Therefore, it is postulated that the production of these wolf urinary chemicals depends on reproductive hormones. In castrated male wolves, testosterone induces the formation of some compounds typically associated with the intact male (several types of middle chain alkyl ketones and alkyl sulfides), while reducing the levels of other compounds (i.e., 3-ethylcyclo-pentanone and acetophenone) associated with castrated males and females (Raymer et al., 1986). Similarly, four volatile chemicals (IMS, 2-phenylethyl methyl sulfide, 6-methyl-heptene-2-one, and geranylacetone) were identified as constituents of the urine of red foxes (both sexes), with greater production during the winter season when mating occurred (Jorgenson et al., 1978).

In addition to the reproductive hormones and the season, the diet of a predator (the coyote) affected the ability of the urine to cause avoidance behavior in prey (Nolte et al., 1994). The authors used four species of rodent, namely the mountain beaver (*Aplodontia rufa*), the house mouse (*Mus musculus*), the deer mouse (*Peromyscus maniculatus*), and the guinea pig (*Cavia porcellus*) as subjects in behavioral experiments. Urine samples were collected from four urine donor coyotes that each ate only cantaloupe melon (*Cucumis melo*) (FU) or only minced raw meat (MU) for 5 days while housed in metabolic chambers. After acclimatization, the four species of rodent were given 24 h two-choice tests between apple cubes associated with either the FU or the MU. In this choice test, all four types of rodent ingested significantly more apple cubes from bowls scented with FU than they did from bowls that contained MU. Thus, all four species of rodent avoided the MU odor in favor of the FU odor. The results from high-performance liquid chromatography analysis of the urine showed that two unidentified chemical peaks existed only in MU. When MU was treated with mercuric chloride, these two peaks disappeared and the avoidance behavior evoked

by the MU odor also decreased (Nolte et al., 1994). These data suggest that sulfurous metabolites of meat digestion are important for the repellent nature of predator odors for potential prey. Additionally, although these authors found that the FU and the sulfur-deprived MU (SR) were both less aversive to prey than the MU, the intake of food was reduced in the presence of FU (and SR) relative to a control. This avoidance, therefore, might be attributed to other non-sulfurous compounds (Nolte et al., 1994).

When combined, it is conceivable that IMS (Wilson et al., 1978) and its derivatives are the candidates of predator urinary kairomones of wild canids, including the wolf. However, the capacity of these synthesized chemicals to induce vigilance behaviors in prey is limited, at least in a field experimental setting. For example, Sullivan et al. (1988a) demonstrated that when IMS or the analog (3-methyl-3-butenyl methyl sulfide; MBMS) was dispensed within capillary tubes and attached to apple trees with a twist-tie, there was no significant reduction in feeding damage to these trees from meadow voles (*Microtus pennsylvanicus*). Similarly, IMS and its analog did not cause significant avoidance and/or vigilance behaviors by pocket gophers (*Thomomys talpoides*) (Sullivan et al., 1988b), Mountain beavers (*Aplodontia rufa*) (Epple et al., 1995), or ungulates (Lindgren et al., 1995; Nolte et al., 2001). Only the snowshoe hare (*Lepus americanus*) effectively avoided the MBMS (Sullivan and Crump, 1986). Therefore, wolf urine likely contained additional kairomones that were used in predator-prey chemosignaling.

Identification of Wolf Kairomones by Mice

Alkyl Pyrazine Analogs are Wolf Kairomones

In a previous study (Osada et al., 2013), the avoidance (Fendt, 2006; Ferrero et al., 2011) and freezing behaviors (Wallace and Rosen, 2000; Fendt et al., 2005; Buron et al., 2007; Fendt and Endres, 2008) of female house mice in response to wolf urine were systematically analyzed. Three sets of commercially available urine samples, which were harvested approximately in November 2009, January 2010, and March 2010, from both genders of wolves that belonged to the same pack ($n > 10$) were obtained. As mentioned in the previous sections, the odor and the chemical components contained in wolf urine depend on the diet, the season, and the hormonal status of the animal. Therefore, differences might be expected in the avoidance behavior induced in mice by these different urine samples. However, all undiluted samples induced significant avoidance behavior in the mice when compared with the control (**Figure 1A**).

Predator scents comprise complex cocktails of volatiles, some of which emit a strong stench. Endres and Fendt (2009) showed that trimethylthiazoline (TMT), a kairomone derived from fox feces, induces freezing behavior in prey at very low concentrations. However, butyric acid, which is a repugnant, non-predator odor, did not induce such behaviors. Therefore, to determine which of the urine samples contained the most kairomones, avoidance, and freezing behavior bioassays were conducted using diluted urine samples. Osada et al. (2013) found that one group of urine samples, those harvested in March, induced the strongest vigilance behavior in mice (**Figures 1A,B**); these results indicate that the levels of kairomones in wolf urine might also increase near the end of the breeding season.

To identify potential novel kairomones in wolf urine, GC-quadrupole MS in conjunction with headspace solid phase micro-extraction was conducted. From over 50 representative peaks, 2, 6-dimethylpyrazine (DMP), 2, 3, 5,-trimethylpyrazine (TMP), and 3-ethyl-2, 5-dimethyl pyrazine (EDMP) were among several volatiles present at higher concentrations in the urine sample collected in March than in samples collected at other times (**Figure 1C**; peaks (14), (16), and (17), respectively). The concentration of 1-(methylthio)-2-methylbut-2-ene (peak number 9) tended to be highest in the March urine samples, although the difference was not statistically significant. Although these volatiles were characterized by a strong odor, there were no previous reports suggesting that they facilitate conspecific communication among canines (Jorgenson et al., 1978; Raymer et al., 1984). Therefore, these volatiles were hypothesized to be novel kairomones in the urine of wolves (Osada et al., 2013).

The results of additional behavioral and immunohistochemical studies indicate that these pyrazine analogs, especially a cocktail thereof, elicit significant freezing behavior in mice, at least in part by stimulating the murine accessory olfactory bulb (AOB). Thus, the pyrazine analogs identified in wolf urine represent a set of novel kairomones that initiate fear-related behavior in mice.

The Putative Sensory System Involved in Inducing Freezing and Avoidance Behavior in Response to Kairomones for Rodents

For several reasons, it is likely that these pyrazine analogs stimulated the main olfactory epithelia (MOE) to induce the freezing behavior. First, most of the alkyl pyrazine analogs are volatile compounds that emit a pungent odor (Tsantili-Kakoulidou and Kier, 1992). Second, measured as the uptake of [^{14}C] 2-deoxyglucose, Johnson et al. (2005) demonstrated that 2,3-dimethylpyrazine caused a robust stimulation of the glomerular layer of the rat main olfactory bulb (MOB). Third, the freezing behaviors are only observed in response to repugnant predator odors, such as TMT (Papes et al., 2010). In addition to the pyrazine analogs, there are several other predator odorants that elicit significant vigilance behaviors in rodents (**Table 1**). For example, Vernet-Maury (1980) reported that TMT is the primary component of the odor of fox feces, and that it induces autonomic and behavioral anti-predator responses in rodents. For example, experimental rats and mice exposed to the odor of foxes or to TMT (Vernet-Maury et al., 1984; Fendt et al., 2005) showed fear-related response behaviors, such as freezing-in-place (Wallace and Rosen, 2000; Buron et al., 2007; Fendt and Endres, 2008; Janitzky et al., 2009). Kobayakawa et al. (2007) demonstrated that TMT is mainly detected in the dorsal domain of the MOB. Similarly, rodents exposed to cat-derived odors displayed dose-dependent vigilance responses, including freezing, avoidance, and the increased production of stress hormones (Takahashi et al., 2005, 2007, 2008). Although little is known about the chemical basis underlying cat odor-induced freezing behavior, volatile compounds containing 3-mercapto-3-methyl-1-butanol have been identified as species-specific odorants in cat urine.

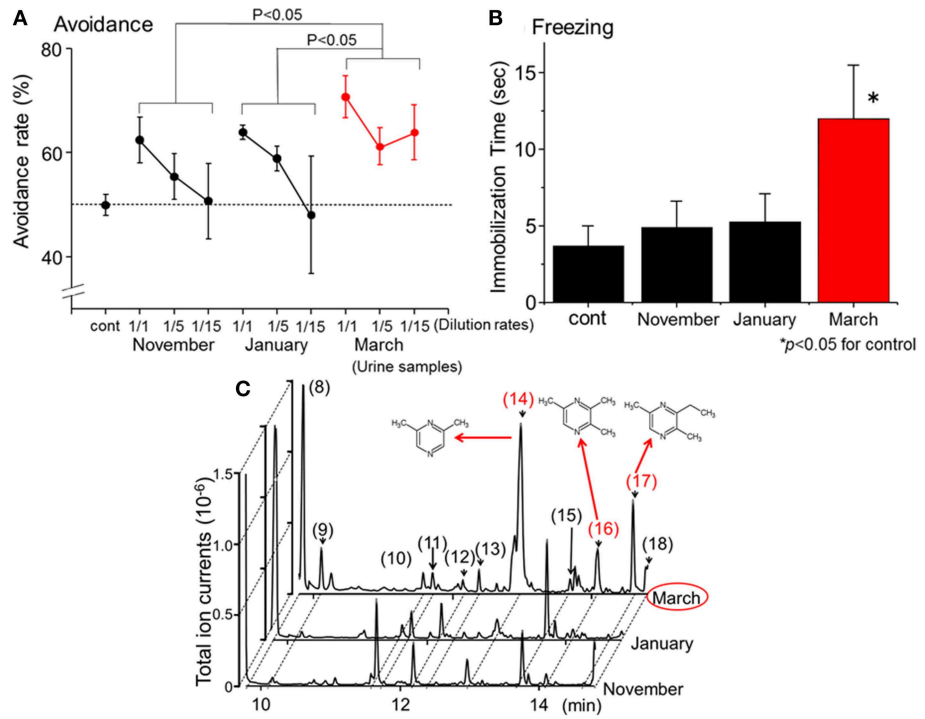


FIGURE 1 | Identification of novel kairomones (pyrazine analogs) in wolf urine. (A) Avoidance rates observed during exposure of mice to wolf urine samples harvested in approximately November 2009, January 2010, and March 2010. The avoidance rate was defined as the amount of time spent in the short arm of a Y maze in the presence of the control odor (water), divided by the total amount of time spent in both short arms in the presence of the wolf urine odor or the control odor. The statistical significance of the differences between the avoidance rates elicited by each of the wolf urine samples was assessed by repeated-measures ANOVA followed by Fisher's PLSD *post-hoc* test. **(B)** Comparison of the duration of "freezing" (immobilization behavior) by mice during a 3 min exposure to five-fold diluted wolf urine samples. The statistical significance of the differences between the freezing duration in response to wolf urine samples was compared with control (water) by means of ANOVA followed by Dunnett's *post-hoc* test. **(C)** Chromatograms from GC-MS analyses of wolf urine samples. Numbers refer to the following compounds: (8) Δ^3-isopentenyl methyl sulfide; (9) 1-(methylthio)-2-methylbut-2-ene; (10) 3-buten-1-ol, 3-methyl-; (11) 4-methyl-3-heptanone; (12) 2,4-dithiapentane*; (13) 1-pentanol, 2-methyl-; (14) pyrazine, 2,6-dimethyl- (DMP)*; (15) dimethyl trisulfide*; (16) pyrazine, trimethyl- (TMP)*; (17) pyrazine, 3-ethyl-2,5-dimethyl (EDMP)*; (18) acetic acid*. *Identified by GC-MS ($n = 6$) and by comparison with the retention times of identified chemicals. All figures modified from Osada et al. (2013).

These sex- and age-dependent cat-specific volatile compounds (Miyazaki et al., 2006) are detected as territorial markers and are used in conspecific recognition or in female attraction by mature male cats (Miyazaki et al., 2008). To detect predator signals, rodents may also use these volatiles. Using a reporter gene assay with trace amine-associated receptors (TAARs), Ferrero et al. (2011) found that the mouse TAAR4 selectively responded to the urine of several carnivores. Then, bobcat urine was fractionated with silica gel chromatography and analyzed with the reporter gene assay. The results showed that 2-phenylethylamine, a common component of the urine of various carnivores was a key component of an odorant blend that triggers spontaneous aversion via the olfactory sensory neurons (**Table 1**). In contrast to the TMT and the predator-derived lipocalins (Papes et al., 2010; see below), 2-phenylethylamine was identified in the urine of many species of carnivore and therefore might enable prey to avoid novel and dangerous predators (Liberles, 2014). Taken together, most of the above-mentioned volatile chemicals act by stimulating the MOE.

In addition to the primary olfactory system, most mammals have a vomeronasal system; this system contributes to the detection of certain conspecific pheromones, and it also perceives common volatile odorants (Trinh and Storm, 2003; Brennan and Keverne, 2004). Moreover, the vomeronasal system is thought to detect interspecific kairomones. For example, Ben-Shaul et al. (2010) identified a significant set of murine AOB neurons that respond robustly and selectively to predator cues. In addition, the exposure of rodents to cat odors increased the number of Fos-positive cells in the AOB (Staples et al., 2008). However, the chemical composition of these kairomones in predators remains difficult to determine. Papes et al. (2010) showed that in VNO-defective animals, TrpC2$^{-/-}$, the odor from mice predators (urine from rats, neck swab of cats, shed skin of snakes) did induce avoidance and risk assessment behaviors. The authors then purified the kairomones using size extraction fractionation and anion exchange FPLC techniques and identified the kairomones using behavioral and Ca^{2+} imaging assays. They demonstrated that the major urinary protein of rat (lipocalin) and recombinant feline Mup (based on Mup Feld4 in cat saliva) (**Table 1**) are sufficient to activate VNO and AOB neurons and initiate both defensive behavior and the ACTH response.

Osada et al. (2013) suggested that pyrazine analogs stimulated the AOB; therefore, they examined the immunoreactivity of Fos, a marker of neuronal excitation, primarily in the AOB. They found that the immunoreactivity of Fos was different in the AOB, particularly in the posterior granule cell layer, after mice were exposed to fresh wolf urine samples. These results suggest that substances in wolf urine cause excitation in part of the vomeronasal system. In this regard, pyrazine analogs might be the first identified volatile urinary chemosignals that evoke fear-associated immobilization and stimulate the rodent AOB and perhaps the MOB as well. Further studies are in progress to clarify the precise neurophysiological mechanisms underlying hard-wired fear-related responses evoked by pyrazine analogs.

In addition to the MOE and the VNO, the Gruenberg Ganglion is a detector of kairomones. According to previous studies, principal anal gland compounds from the stoat (*Mustela erminea*) and ferret (*M. putorius*) markedly alter the distribution of gophers (*Thomomys talpoides*) and clearly reduce the feeding of meadow voles on apple trees (Crump, 1978, 1980; Crump and Moors, 1985; Sullivan et al., 1988a,b). The predator odor chemicals involved comprise alkylthietanes and dithiolane, which were ether-extracted from excretions of the anal gland. Additionally, Brechbühl et al. (2013) demonstrated that 2-propylthietane, TMT, and 2-sec-butyl-4, 5-dihydrothiazole elicit freezing behavior in C57BL/6J mice by stimulating the Gruenberg Ganglion. Interestingly, several alkyl pyrazines, including DMP and TMP, can induce Fos-positive Gruenberg Ganglion cells in a dose-dependent manner (Mamasuew et al., 2011). Therefore, it is conceivable that these pyrazine analogs also induce robust fear-related behaviors by stimulating the Gruenberg Ganglion.

Putative Mechanism for the Production of Pyrazine Analogs in Predator Urine

The mechanism(s) by which pyrazine analogs are produced in wolf urine is unknown. However, an intriguing possibility is related to glycation, which occurs in all living animals (McPherson et al., 1988; Fu et al., 1992). Alkylpyrazine analogs are a typical class of glycation compound (Adams et al., 2008), which are formed between reducing sugars and glycine oligopeptides (Lu et al., 2005). Actually, food-derived oligopeptides can be detected in the blood after oral ingestion of meat and collagen (Iwai et al., 2005; Bauchart et al., 2007). Therefore, it is conceivable that the blood glucose and amino compounds derived from foods containing meat or connective tissue may be the source of pyrazine analogs generated in the urine of wolves and, perhaps, other carnivores.

The Effect of Putative Kairomones in Wolf Urine on Ungulates

Aversion and Vigilance Behaviors in Hokkaido Deer Exposed to Pyrazine Analogs—a Field Experiment

As mentioned above, Osada et al. (2013) identified a set of pyrazine analogs as wolf urinary kairomones that induce aversive and freezing behaviors in mice. A cocktail of these compounds had a greater effect than any one component alone. Because the wolf preys on various kinds of mammal, including ungulates, the authors thought that the pyrazine analogs might be kairomones that induce vigilance and fear in large mammals. Therefore, to investigate the ability of the pyrazine cocktail to act as a kairomone in prey animals other than mice, Osada et al. (2014) performed a field experiment on Hokkaido deer (*Cervus nippon yesoensis*). The experiment was conducted in a seminatural deer park (44°12′ N and 142°48′ E, Nishiokoppe, Hokkaido, Japan). Approximately 30 deer inhabited an enclosed area and had free access to herbage, bamboo grass, tree leaves, bark, and water. The feeding experiments were conducted twice, from summer to autumn, 2013. When individual male and female deer were followed, the pyrazine cocktail suppressed the duration and frequency of access to the feeding area by half. The cocktail also led to deer taking twice as long to reach the feeding area. Moreover, the cocktail elicited vigilance behaviors around the feeding areas, such as tail-flag (deer lift up their tail), flight (deer rapidly escape with their necks retracted), and jump (deer spring back; **Figure 2**). These behaviors might indicate fear and may act as alarm signals to warn conspecifics of impending danger (Caro, 1995, 2005; Eilam, 2005; Stankowich and Coss, 2006); thus, the results from this field study suggest that the pyrazine cocktail acted as a wolf kairomone, eliciting fear-associated aversive behaviors in deer (Osada et al., 2014).

Hokkaido Deer Have "Remembered" the Scent of a Predator, the Wolf, for 100 Years

The observation that the pyrazine cocktail acted as a wolf kairomone leads to the following question: why do Hokkaido deer show fear-associated aversive responses to the pyrazine analogs that form part of the scent of wolf urine? Japanese wolves (Hokkaido wolf, *C. l. hattai*; Honshu wolf, *C. l. hodophilax*) have been extinct for the last 100 years (Walker, 2005), so the individual deer used in the experiment have never been under threat of predation by wolves. Thus, the pyrazine cocktail may elicit a predisposed fear response in deer. Numerous studies demonstrate the persistence of responses to the scent of predators, and to their kairomones, in laboratory strains of rodents that have not experienced predation for several hundred generations (Apfelbach et al., 2005; Fendt, 2006; Osada et al., 2013; Takahashi, 2014).

Berger (1999) and Pyare and Berger (2003) reported that female moose (*Alces alces*) from a region that overlapped with the territory of a wolf pack (mainland Alaska) showed significantly stronger vigilance behavior when exposed to the odors of wolves than female moose from a region in which wolves were absent for at least 60 years until the 1990s (Wyoming). However, notably, the vigilance behavior was not higher than that of moose in a predator-free region (Kenai Peninsula) suggesting that learning was not a necessary component of the avoidance behavior induced by wolf urine. Additionally, Chamaillé-Jammes et al. (2014) obtained a similar result in a field study conducted on the Haida Gwaii archipelago in Canada, where black-tailed deer exhibit a fear response to wolf urine, even after more than 100 years of wolf absence. Interestingly, the response to wolf urine is greater than the response to urine derived from the black bear

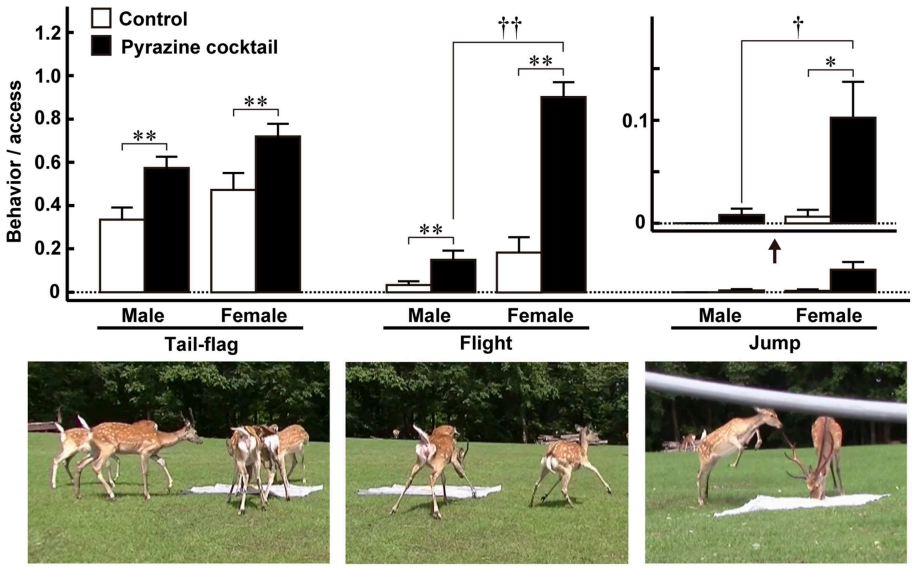


FIGURE 2 | Fear-related behaviors of male and female Hokkaido deer evoked by pyrazine analogs in the feeding experiment. The proportion of tail-flag, flight, and jump actions were estimated by calculating the ratio of the number of times each action was performed by males ($n = 27$) and females ($n = 19$) to the number of deer accessing the feeding areas. Open and closed bars indicate control and pyrazine cocktail feeding areas, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, Wilcoxon signed-rank test. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, Mann-Whitney U-test. Lower panels show typical photographs of each of the fear-related behaviors. Modified from Osada et al. (2014).

(*Ursus americanus*), which is currently present but is potentially a less dangerous predator for black-tailed deer. Moreover, wolves are more efficient predators than black bears, which typically attack fawns and rarely attack adults, and have little success when they do (Zager and Beecham, 2006). The authors concluded that the result is in accordance with "the hypothesis of the innate threat-sensitive foraging," which states that fear responses of prey species to predator cues should be adjusted to the level of danger posed by the predator. Additionally, based on "the multipredator hypothesis" proposed by Blumstein and colleagues (Blumstein, 2006; Blumstein et al., 2006, 2008), the authors speculated that the response to a more dangerous predator (the wolf) might be maintained by encountering a less dangerous predator if both predators' cues are similar, or if the responses are genetically linked (Chamaillé-Jammes et al., 2014). Therefore, Hokkaido deer may have maintained their recognition of, and response to, wolf scent by having contact with current existing predators such as the Hokkaido brown bear (*Ursus arctos yesoensis*) and the red fox (*Vulpes vulpes schrencki*).

Pyrazine analogs are not only found in the urine of predators but also in a wide variety of plants (Bohman et al., 2012, 2014), insects (Tentschert et al., 2000; Sharma et al., 2011), terrestrial vertebrates (Novotny et al., 1986; Woolfson and Rothschild, 1990; Zhang et al., 2005) and foods (EFSA Panel on Food Contact Materials Enzymes Flavourings and Processing Aids (CEF), 2011). This widespread distribution suggests that pyrazine analogs are of special significance as semiochemicals for different types of organism. However, we cannot preclude the possibility that these deer were previously exposed to other substrates that contain identical or similar components and, therefore, might display a learned fear response to the pyrazine mixture. Further research is required to clarify this point.

Sexual Dimorphism in Vigilance Behaviors in Response to Wolf Scent

Of the fear-related vigilance behaviors of deer quantified in the field experiment, flight and jump behaviors occurred frequently in females in the presence of the pyrazine cocktail (**Figure 2**; Osada et al., 2014). Although more data are needed before we can draw a firm conclusion, we also believe that the male deer with the largest antlers were less likely to show vigilance behaviors in response to the pyrazine cocktail than the other males and females. Thus, the response of deer to the pyrazine cocktail representing wolf scent is partly dependent on their sex, and, possibly, on the position of males in the social hierarchy of the herd.

Sexual dimorphism in the fear response has been studied in laboratory animals. For instance, in laboratory rats, mice, and meadow voles, females exhibit stronger responses to the odor of a predator (the red fox) than males (Perrot-Sinal et al., 1996; Hubbard et al., 2004; Buron et al., 2007). Age-related and hormonal variation has been also reported in the response to predator odors (Hubbard et al., 2004; King et al., 2005). At present, genetic and pharmacological studies have begun to be implemented to investigate the neural circuits that regulate fear of predator odors. For example, Choi et al. (2005) identified LIM (Lin-11, Is1-1, Mec-3) homeodomain transcription factors as molecular markers for the sub-nuclei of the medial amygdala, which is responsive to predator odors. Moriceau et al. (2004) indicated that corticosterone might control

the fear response of infant rats to conspecific predator odor, and that the response might be mediated by activation of the basolateral/lateral amygdala. Do Monte et al. (2008) suggested that noradrenergic transmission may modulate the expression of the fear response of rats to cat odor through the dorsal pre-mammillary nucleus. This knowledge is likely to facilitate further studies on sexual dimorphism in fear of predator odor in laboratory animals, and also in wild animals.

Potential for the Novel Wolf Kairomones to Act as Repellents for Ungulates

Researchers have identified various kairomones derived from odors of predators of rodents (**Table 1**). To the best of our knowledge, the pyrazine cocktail is the first example of kairomones that elicit aversive behavior in both rodents and ungulates. Excessively large deer populations may cause economic losses in agricultural, horticultural, and forest resources. To minimize such losses, natural odor sources are frequently used as chemical repellents for ungulates (Apfelbach et al., 2005; Kimball et al., 2009). Synthetic odors, however, have had little or no effect so far (Apfelbach et al., 2005). Because of its aversive effect on deer, the pyrazine cocktail might be effective as a chemical repellent for deer. Clearly, an excellent repellent must have a persistent effect. Studies confirm that the pyrazine cocktail had a good repellent effect when tested on the same herd of deer, and the effect lasted at least 1 month after the first day of the experiment (Osada et al., 2014).

An ideal repellent should also be a natural product. The pyrazine analogs in the cocktail are natural, non-carcinogenic, and of low acute toxicity; indeed, they are responsible for the characteristic roasted aromas in foods such as coffee, peanuts, beef, and potato (EFSA Panel on Food Contact Materials Enzymes Flavourings and Processing Aids (CEF), 2011). Actually, some kinds of alkyl pyrazine are widely used as flavoring ingredients in foods (Burdock and Carabin, 2008). Therefore, the pyrazine cocktail is expected to be an effective deer repellent that will not damage the natural environment.

Conclusions

We first reviewed historical studies relating to chemical communication between wolves. Urinary chemical communication between conspecifics in wolf packs is important in wild habitats. Moreover, the urine of wolves is also used as kairomones by prey animals. Next, we discussed the identification, chemical basis, and putative sensory system of wolf kairomones compared with other kairomones that affect rodents. We presented the possibility that wolf urine, and the pyrazine analogs contained therein, provoke a fear response by stimulating three different sensory systems, namely, the murine vomeronasal system, the main olfactory system and, perhaps, the Gruenberg Ganglion. In future, a number of novel semiochemicals which stimulate the three sensory systems may be found. We then discussed studies showing that these pyrazine analogs elicited vigilance behaviors not only in rodents, but also in an ungulate, the Hokkaido deer. In this section we discussed how Hokkaido deer have remembered the scent of wolves for 100 years or more. In addition, the sexual dimorphism in vigilance behavior and the potential of wolf kairomones to act as repellents were mentioned. Further studies are required to determine whether the vigilance response of deer to the pyrazine cocktail is predisposed or learned. Moreover, the discovery that pyrazine analogs evoke vigilance behaviors in prey animals provides a strong rationale for additional studies of odorant-induced behaviors and the neurophysiological mechanisms underlying them.

Acknowledgments

This work was supported by Japan Society for the Promotion of Science KAKENHI Grant (26450141 and 15K18573); by the A-step feasibility study program from the Japan Science and Technology Agency (No. AS251Z00533M); and by Grants-in-Aid for Regional R&D Proposal-Based Program from Northern Advancement Center for Science & Technology of Hokkaido Japan.

References

Adams, A., Polizzi, V., Van Boekel, M., and De Kimpe, N. (2008). Formation of pyrazines and a novel pyrrole in Maillard model systems of 1,3-dihydroxyacetone and 2-oxopropanal. *J. Agric. Food Chem.* 56, 2147–2153. doi: 10.1021/jf0726785

Apfelbach, R., Blanchard, C. D., Blanchard, R. J., Hayes, R. A., and McGregor, I. S. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neurosci. Biobehav. Rev.* 29, 1123–1144. doi: 10.1016/j.neubiorev.2005.05.005

Baasch, D. M., Fischer, J. W., Hygnstrom, S. E., VerCauteren, K. C., Tyre, A. J., Millspaugh, J. J., et al. (2010). Resource selection by elk in an agro-forested landscape of northwestern Nebraska. *Environ. Manage.* 46, 725–737. doi: 10.1007/s00267-010-9559-2

Bauchart, C., Savary-Auzeloux, I., Patureau Mirand, P., Thomas, E., Morzel, M., and Rémond, D. (2007). Carnosine concentration of ingested meat affects carnosine net release into the portal vein of minipigs. *J. Nutr.* 137, 589–593.

Ben-Shaul, Y., Katz, L. C., Mooney, R., and Dulac, C. (2010). *In vivo* vomeronasal stimulation reveals sensory encoding of conspecific and allospecific cues by the mouse accessory olfactory bulb. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5172–5177. doi: 10.1073/pnas.0915147107

Berger, J. (1999). Anthropogenic extinction of top carnivores and interspecific animal behaviour: implications of the rapid decoupling of a web involving wolves, bears, moose and ravens. *Proc. Biol. Sci.* 266, 2261–2267. doi: 10.1098/rspb.1999.0917

Blumstein, D. T. (2006). The multipredator hypothesis and the evolutionary persistence of antipredator behavior. *Ethology* 112, 209–217. doi: 10.1111/j.1439-0310.2006.01209.x

Blumstein, D. T., Barrow, L., and Luterra, M. (2008). Olfactory predator discrimination in yellow-bellied marmots. *Ethology* 114, 1135–1143. doi: 10.1111/j.1439-0310.2008.01563.x

Blumstein, D. T., Bitton, A., and Da Veiga, J. (2006). How does the presence of predators influence the persistence of antipredator behavior? *J. Theor. Biol.* 239, 460–468. doi: 10.1016/j.jtbi.2005.08.011

Bohman, B., Jeffares, L., Flematti, G., Phillips, R. D., Dixon, K. W., Peakall, R., et al. (2012). The discovery of 2-hydroxymethyl-3-(3-methylbutyl)-5-methylpyrazine: a semiochemical in orchid pollination. *Org. Lett.* 14, 2576–2578. doi: 10.1021/ol300864u

Bohman, B., Phillips, R. D., Menz, M. H., Berntsson, B. W., Flematti, G. R., Barrow, R. A., et al. (2014). Discovery of pyrazines as pollinator sex pheromones and orchid semiochemicals: implications for the evolution of sexual deception. *New Phytol.* 203, 939–952. doi: 10.1111/nph.12800

Brechbühl, J., Moine, F., Klaey, M., Nenniger-Tosato, M., Hurni, N., Sporkert, F., et al. (2013). Mouse alarm pheromone shares structural similarity with predator scents. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4762–4767. doi: 10.1073/pnas.1214249110

Brennan, P. A., and Keverne, E. B. (2004). Something in the air? New insights into mammalian pheromones. *Curr. Biol.* 14, R81–R89. doi: 10.1016/j.cub.2003.12.052

Brown, W. L. Jr., Eisner, T., and Whittaker, R. H. (1970). Allomones and kairomones: transspecific chemical messengers. *Bioscience* 20, 21. doi: 10.2307/1294753

Burdock, G. A., and Carabin, I. G. (2008). Safety assessment of 2-ethyl-3,(5 or 6) dimethylpyrazine as a food ingredient. *Regul. Toxicol. Pharmacol.* 50, 303–312. doi: 10.1016/j.yrtph.2007.12.007

Buron, G., Hacquemand, R., Pourie, G., Lucarz, A., Jacquot, L., and Brand, G. (2007). Comparative behavioral effects between synthetic 2,4,5-trimethylthiazoline (TMT) and the odor of natural fox (*Vulpes vulpes*) feces in mice. *Behav. Neurosci.* 121, 1063–1072. doi: 10.1037/0735-7044.121.5.1063

Caro, T. M. (1995). Pursuit-deterrence revisited. *Trends Ecol. Evol.* 10, 500–503. doi: 10.1016/S0169-5347(00)89207-1

Caro, T. M. (2005). *Antipredator Defenses in Birds and Mammals.* Chicago, IL: University of Chicago Press.

Chamaillé-Jammes, S., Malcuit, H., Le Saout, S., and Martin, J.-L. (2014). Innate threat-sensitive foraging: black-tailed deer remain more fearful of wolf than of the less dangerous black bear even after 100 years of wolf absence. *Oecologia* 174, 1151–1158. doi: 10.1007/s00442-013-2843-0

Choi, G. B., Dong, H. W., Murphy, A. J., Valenzuela, D. M., Yancopoulos, G. D., Swanson, L. W., et al. (2005). Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus. *Neuron* 46, 647–660. doi: 10.1016/j.neuron.2005.04.011

Crump, D. R. (1978). 2-Propylthietane, the major malodorous substance from the anal gland of the stoat (*Mustela erminea*). *Tetrahedron Lett.* 19, 5233–5234. doi: 10.1016/S0040-4039(01)85858-5

Crump, D. R. (1980). Thietanes and dithiolanes from the anal gland of the stoat (*Mustela erminea*). *J. Chem. Ecol.* 6, 341–347. doi: 10.1007/BF01402912

Crump, D. R., and Moors, P. J. (1985). Anal gland secretions of the stoat (*Mustela erminea*) and the ferret (*Mustela putorius formafuro*): some additional thietane components. *J. Chem. Ecol.* 11, 1037–1043. doi: 10.1007/BF01020673

Do Monte, F. H., Canteras, N. S., Fernandes, D., Assreuy, J., and Carobrez, A. P. (2008). New perspectives on beta-adrenergic mediation of innate and learned fear responses to predator odor. *J. Neurosci.* 28, 13296–13302. doi: 10.1523/JNEUROSCI.2843-08.2008

EFSA Panel on Food Contact Materials Enzymes Flavourings and Processing Aids (CEF). (2011). Scientific opinion on flavouring group evaluation 17, revision 3. (Fge.17rev3): pyrazine derivatives from chemical group 24. *EFSA J.* 9, 2456–2522. doi: 10.2903/j.efsa.2011.2456

Eilam, D. (2005). Die hard: a blend of freezing and fleeing as a dynamic defense–implications for the control of defensive behavior. *Neurosci. Biobehav. Rev.* 29, 1181–1191. doi: 10.1016/j.neubiorev.2005.03.027

Endres, T., and Fendt, M. (2009). Aversion- vs fear-inducing properties of 2,4,5-trimethyl-3-thiazoline, a component of fox odor, in comparison with those of butyric acid. *J. Exp. Biol.* 212, 2324–2327. doi: 10.1242/jeb.028498

Epple, G. J. R., Mason, E., Aronov, D. L., Nolte, R. A., Hartz, R., Kaloostian, D., et al. (1995). Feeding response to predator-based repellents in the mountain beaver (*Aplodontia rufa*). *Ecol. Appl.* 5, 1163–1170. doi: 10.2307/2269363

Fendt, M. (2006). Exposure to urine of canids and felids, but not of herbivores, induces defensive behavior in laboratory rats. *J. Chem. Ecol.* 32, 2617–2627. doi: 10.1007/s10886-006-9186-9

Fendt, M., and Endres, T. (2008). 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox odor—just repugnant or really fear-inducing? *Neurosci. Biobehav. Rev.* 32, 1259–1266. doi: 10.1016/j.neubiorev.2008.05.010

Fendt, M., Endres, T., Lowry, C. A., Apfelbach, R., and McGregor, I. S. (2005). TMT-induced autonomic and behavioral changes and the neural basis of its processing. *Neurosci. Biobehav. Rev.* 29, 1145–1156. doi: 10.1016/j.neubiorev.2005.04.018

Ferrero, D. M., Lemon, J. K., Fluegge, D., Pashkovski, S. L., Korzan, W. J., Datta, S. R., et al. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11235–11240. doi: 10.1073/pnas.1103317108

Fox, M., and Cohen, J. (1977). *How Animals Communicate.* Bloomington, IN: Indiana University Press.

Fu, M. X., Knecht, K. J., Thorpe, S. R., and Baynes, J. W. (1992). Role of oxygen in cross-linking and chemical modification of collagen by glucose. *Diabetes* 41, 42–48. doi: 10.2337/diab.41.2.S42

Gese, E. M., and Ruff, R. L. (1997). Scent-marking by coyotes, *Canis latrans*: the influence of social and ecological factors. *Anim. Behav.* 54, 1155–1166. doi: 10.1006/anbe.1997.0561

Gheysen, T., Brostaux, Y., Hébert, J., Ligot, G., Rondeux, J., and Lejeune, P. (2011). A regional inventory and monitoring setup to evaluate bark peeling damage by red deer (*Cervus elaphus*) in coniferous plantations in Southern Belgium. *Environ. Monit. Assess.* 181, 335–345. doi: 10.1007/s10661-010-1832-6

Hoskinson, R. L., and Mech, L. D. (1976). White-tailed deer migration and its role in wolf predation. *J. Wildl. Manage.* 40, 429–441. doi: 10.2307/3799944

Hubbard, D. T., Blanchard, D. C., Yang, M., Markham, C. M., Gervacio, A., Chun, I. L., et al. (2004). Development of defensive behavior and conditioning to cat odor in the rat. *Physiol. Behav.* 80, 525–530. doi: 10.1016/j.physbeh.2003.10.006

Hurst, J. L., Payne, C. E., Nevison, C. M., Marie, A. D., Humphries, R. E., Robertson, D. H., et al. (2001). Individual recognition in mice mediated by major urinary proteins. *Nature* 414, 631–634. doi: 10.1038/414631a

Iwai, K., Hasegawa, T., Taguchi, Y., Morimatsu, F., Sato, K., Nakamura, Y., et al. (2005). Identification of food-derived collagen peptides in human blood after oral ingestion of gelatin hydrolysates. *J. Agric. Food Chem.* 53, 6531–6536. doi: 10.1021/jf050206p

Janitzky, K., Stork, O., Lux, A., Yanagawa, Y., Schwegler, H., and Linke, R. (2009). Behavioral effects and pattern of brain c-fos mRNA induced by 2,5-dihydro-2,4,5-trimethylthiazoline, a component of fox feces odor in GAD67-GFP knock-in C57BL/6 mice. *Behav. Brain Res.* 202, 218–224. doi: 10.1016/j.bbr.2009.03.038

Johnson, B. A., Farahbod, H., and Leon, M. (2005). Interactions between odorant functional group and hydrocarbon structure influence activity in glomerular response modules in the rat olfactory bulb. *J. Comp. Neurol.* 483, 205–216. doi: 10.1002/cne.20409

Jorgenson, J. W., Novotny, M., Carmack, M., Copland, G. B., Wilson, S. R., Katona, S., et al. (1978). Chemical scent constituents in the urine of the Red Fox (*Vulpes vulpes L.*) during the winter season. *Science* 199, 796–798. doi: 10.1126/science.199.4330.796

Kaur, A. W., Ackels, T., Kuo, T. H., Cichy, A., Dey, S., Hays, C., et al. (2014). Murine pheromone proteins constitute a context-dependent combinatorial code governing multiple social behaviors. *Cell* 157, 676–688. doi: 10.1016/j.cell.2014.02.025

Killian, G., Kreeger, T. J., Rhyan, J., Fagerstone, K., and Miller, L. (2009). Observations on the use of GonaCon in captive female elk (*Cervus elaphus*). *J. Wildl. Dis.* 45, 184–188. doi: 10.7589/0090-3558-45.1.184

Kimball, B. A., Taylor, J., Perry, K. R., and Capelli, C. (2009). Deer responses to repellent stimuli. *J. Chem. Ecol.* 35, 1461–1470. doi: 10.1007/s10886-009-9721-6

Kimoto, H., Haga, S., Sato, K., and Touhara, K. (2005). Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature* 437, 898–901. doi: 10.1038/nature04033

King, J. A., De Oliveira, W. L., and Patel, N. (2005). Deficits in testosterone facilitate enhanced fear response. *Psychoneuroendocrinology* 30, 333–340. doi: 10.1016/j.psyneuen.2004.09.005

Kluever, B. M., Howery, L. D., Breck, S. W., and Bergman, D. L. (2009). Predator and heterospecific stimuli alter behaviour in cattle. *Behav. Process.* 81, 85–91. doi: 10.1016/j.beproc.2009.02.004

Kobayakawa, K., Kobayakawa, R., Matsumoto, H., Oka, Y., Imai, T., Ikawa, M., et al. (2007). Innate versus learned odour processing in the mouse olfactory bulb. *Nature* 450, 503–508. doi: 10.1038/nature06281

Liberles, S. D. (2014). Mammalian pheromones. *Annu. Rev. Physiol.* 76, 151–175. doi: 10.1146/annurev-physiol-021113-170334

Lindgren, P. M. F., Sullivan, T. P., and Crump, D. R. (1995). "Review of synthetic predator odor semiochemicals as repellents for wildlife management in the Pacific Northwest," in *USDA National Wildlife Research Center Repellents Conference* (Denver, CO), 217–230.

Lu, C. Y., Hao, Z., Payne, R., and Ho, C. T. (2005). Effects of water content on volatile generation and peptide degradation in the maillard reaction of glycine, diglycine, and triglycine. *J. Agric. Food. Chem.* 53, 6443–6447. doi: 10.1021/jf050534p

Mamasuew, K., Hofmann, N., Breer, H., and Fleischer, J. (2011). Grueneberg ganglion neurons are activated by a defined set of odorants. *Chem. Senses* 36, 271–282. doi: 10.1093/chemse/bjq124

Masuko, T., Souma, K., Kudo, H., Takasaki, Y., Fukui, E., Kitazawa, R., et al. (2011). Effects of the feeding of wild Yeso sika deer (*Cervus nippon yesoensis*) on the prevention of damage due to bark stripping and the use of feeding sites. *Anim. Sci. J.* 82, 580–586. doi: 10.1111/j.1740-0929.2010.00863.x

McPherson, J. D., Shilton, B. H., and Walton, D. J. (1988). Role of fructose in glycation and cross-linking of proteins. *Biochemistry* 27, 1901–1907. doi: 10.1021/bi00406a016

Mech, L. D. (1977). Wolf-pack buffer zones as prey reservoirs. *Science* 198, 320–321. doi: 10.1126/science.198.4314.320

Miyazaki, M., Yamashita, T., Suzuki, Y., Saito, Y., Soeta, S., Taira, H., et al. (2006). A major urinary protein of the domestic cat regulates the production of felinine, a putative pheromone precursor. *Chem. Biol.* 13, 1071–1079. doi: 10.1016/j.chembiol.2006.08.013

Miyazaki, M., Yamashita, T., Taira, H., and Suzuki, A., (eds.). (2008). "The biological function of cauxin, a major urinary protein of the domestic cat (*Felis catus*)," in *Chemical Signals in Vertebrates*, eds R. J. Beynon, J. L. Hurst, S. C. Roberts and T. D. Wyatt (New York, NY: Springer), 51–60.

Moriceau, S., Roth, T. L., Okotoghaide, T., and Sullivan, R. M. (2004). Corticosterone controls the developmental emergence of fear and amygdala function to predator odors in infant rat pups. *Int. J. Dev. Neurosci.* 22, 415–422. doi: 10.1016/j.ijdevneu.2004.05.011

Nielsen, B. L., Rampin, O., Meunier, N., and Bombail, V. (2015). Behavioral responses to odors from other species: introducing a complementary model of allelochemics involving vertebrates. *Front. Neurosci.* 9:226. doi: 10.3389/fnins.2015.00226

Nolte, D. L., Mason, J. R., Epple, G., Aronov, E., and Campbell, D. L. (1994). Why are predator urines aversive to prey? *J. Chem. Ecol.* 20, 1505–1516. doi: 10.1007/BF02059876

Nolte, D. L., Shipley, L. A., and Wagner, K. K. (2001). Efficacy of wolfin to repel black-tailed deer. *West J. Appl. For.* 16, 182–186.

Novotny, M., Jemiolo, B., Harvey, S., Wiesler, D., and Marchlewska-Koj, A. (1986). Adrenal-mediated endogenous metabolites inhibit puberty in female mice. *Science* 231, 722–725. doi: 10.1126/science.3945805

Osada, K., Kurihara, K., Izumi, H., and Kashiwayanagi, M. (2013). Pyrazine analogues are active components of wolf urine that induce avoidance and freezing behaviours in mice. *PLoS ONE* 8:e61753. doi: 10.1371/journal.pone.0061753

Osada, K., Miyazono, S., and Kashiwayanagi, M. (2014). Pyrazine analogs are active components of wolf urine that induce avoidance and fear-related behaviors in deer. *Front. Behav. Neurosci.* 8:276. doi: 10.3389/fnbeh.2014.00276

Papes, F., Logan, D. W., and Stowers, L. (2010). The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. *Cell* 141, 692–703. doi: 10.1016/j.cell.2010.03.037

Parsons, M. H., and Blumstein, D. T. (2010). Familiarity breeds contempt: kangaroos persistently avoid areas with experimentally deployed dingo scents. *PLoS ONE* 5:e10403. doi: 10.1371/journal.pone.0010403

Perrot-Sinal, T. S., Heale, V. R., Ossenkopp, K.-P., and Kavaliers, M. (1996). Sexually dimorphic aspects of spontaneous activity in meadow voles (*Microtus pennsylvanicus*): effects of exposure to fox odor. *Behav. Neurosci.* 110, 1126–1132. doi: 10.1037/0735-7044.110.5.1126

Peters, R. P., and Mech, L. D. (1975). Scent-marking in wolves. *Am. Sci.* 63, 628–637.

Pyare, S., and Berger, J. (2003). Beyond demography and delisting: ecological recovery for Yellowstone's grizzly bears and wolves. *Biol. Concerv.* 113, 63–73. doi: 10.1016/S0006-3207(02)00350-6

Raymer, J., Wiesler, D., Novotny, M., Asa, C., Seal, U. S., and Mech, L. D. (1984). Volatile constituents of wolf (*Canis lupus*) urine as related to gender and season. *Experientia* 40, 707–709. doi: 10.1007/BF01949734

Raymer, J., Wiesler, D., Novotny, M., Asa, C., Seal, U. S., and Mech, L. D. (1986). Chemical scent constituents in urine of wolf (*Canis lupus*) and their dependence on reproductive hormones. *J. Chem. Ecol.* 12, 297–314. doi: 10.1007/BF01045612

Rogers, L. L., Mech, L. D., Dawson, D., Peek, J. M., and Korb, M. (1980). Deer distribution in relation to wolf pack territory edges. *J. Wildl. Manage.* 44, 253–258. doi: 10.2307/3808381

Severud, W. J., Belant, J. L., Bruggink, J. G., and Windels, S. K. (2011). Predator cues reduce American beaver use of foraging trails. *Human Wildl. Interact.* 5, 296–305.

Sharma, K., Vander-Meer, R. K., and Fadamiro, H. Y. (2011). Phorid fly, *Pseudacteon tricuspis*, response to alkylpyrazine analogs of a fire ant, *Solenopsis invicta*, alarm pheromone. *J. Insect Physiol.* 57, 939–944. doi: 10.1016/j.jinsphys.2011.04.007

Stankowich, T., and Coss, R. G. (2006). Effects of predator behavior and proximity on risk assessment by Columbian black-tailed deer. *Behav. Ecol.* 17, 246–254. doi: 10.1093/beheco/arj020

Staples, L. G., McGregor, I. S., Apfelbach, R., and Hunt, G. E. (2008). Cat odor, but not trimethylthiazoline (fox odor), activates accessory olfactory and defense-related brain regions in rats. *Neuroscience* 151, 937–947. doi: 10.1016/j.neuroscience.2007.11.039

Sullivan, T. P., and Crump, D. R. (1986). Feeding responses of snowshoe hares (*Lepus americanus*) to volatile constituents of red fox (*Vulpes vulpes*) urine. *J. Chem. Ecol.* 12, 729–739. doi: 10.1007/BF01012105

Sullivan, T. P., Crump, D. R., and Sullivan, D. S. (1988a). Use of predator odors as repellents to reduce feeding damage by herbivores: III. Montane and meadow voles (*Microtus montanus* and *Microtus pennsylvanicus*). *J. Chem. Ecol.* 14, 363–377.

Sullivan, T. P., Crump, D. R., and Sullivan, D. S. (1988b). Use of predator odors as repellents to reduce feeding damage by herbivores: IV. Northern pocket gophers (*Thomomys talpoides*). *J. Chem. Ecol.* 14, 379–389.

Sullivan, T. P., Nordstrom, L. O., and Sullivan, D. S. (1985a). Use of predator odors as repellents to reduce feeding damage by herbivores: I. Snow shoe Hares(*Lepus americanus*). *J. Chem. Ecol.* 11, 903–919.

Sullivan, T. P., Nordstrom, L. O., and Sullivan, D. S. (1985b). Use of predator odors as repellents to reduce feeding damage by herbivores: II. Block Tailed Dear(*Odocoileus hemionus columbianus*). *J. Chem. Ecol.* 11, 921–935.

Swihart, R. K., Pignatello, J. J., and Mattina, M. J. I. (1991). Aversive responses of white-tailed deer, *odocoileus viginianus*, to predator urines. *J. Chem. Ecol.* 17, 767–777. doi: 10.1007/BF00994199

Takahashi, L. K. (2014). Olfactory systems and neural circuits that modulate predator odor fear. *Front. Behav. Neurosci.* 8:72. doi: 10.3389/fnbeh.2014.00072

Takahashi, L. K., Chan, M. M., and Pilar, M. L. (2008). Predator odor fear conditioning: current perspectives and new directions. *Neurosci. Biobehav. Rev.* 32, 1218–1227. doi: 10.1016/j.neubiorev.2008.06.001

Takahashi, L. K., Hubbard, D. T., Lee, I., Dar, Y., and Sipes, S. M. (2007). Predator odor-induced conditioned fear involves the basolateral and medial amygdala. *Behav. Neurosci.* 121, 100–110. doi: 10.1037/0735-7044.121.1.100

Takahashi, L. K., Nakashima, B. R., Hong, H., and Watanabe, K. (2005). The smell of danger: a behavioral and neural analysis of predator odor-induced fear. *Neurosci. Biobehav. Rev.* 29, 1157–1167. doi: 10.1016/j.neubiorev.2005.04.008

Tentschert, J., Bestmann, H. J., Hölldobler, B., and Heinze, J. (2000). 2,3-dimethyl-5-(2-methylpropyl)pyrazine, a trail pheromone component of *Eutetramorium mocquerysi* Emery (1899) (*Hymenoptera: Formicidae*). *Naturwissenschaften* 87, 377–380. doi: 10.1007/s001140050745

Trdan, S., and Vidrih, M. (2008). Quantifying the damage of red deer (*Cervus elaphus*) grazing on grassland production in southeastern Slovenia. *Euro. J. Wildl. Res.* 54, 138–141. doi: 10.1007/s10344-007-0106-2

Trinh, K., and Storm, D. R. (2003). Vomeronasal organ detects odorants in absence of signaling through main olfactory epithelium. *Nat. Neurosci.* 6, 519–525. doi: 10.1038/nn1039

Tsantili-Kakoulidou, A., and Kier, L. B. (1992). A quantitative structure-activity relationship (QSAR) study of alkylpyrazine odor modalities. *Pharm Res.* 9, 1321–1323. doi: 10.1023/A:1015865603263

Vernet-Maury, E. (1980). Trimethyl-thiazoline in fox feces: a natural alarming substance for the rat. *Olfaction Taste* 7, 407.

Vernet-Maury, E., Polak, E. H., and Demael, A. (1984). Structure-activity relationship of stress-inducing odorants in the rat. *J. Chem. Ecol.* 10, 1007–1018. doi: 10.1007/BF00987509

Wallace, K. J., and Rosen, J. B. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behav. Neurosci.* 114, 912–922. doi: 10.1037/0735-7044.114.5.912

Walker, B. L. (2005). *The Lost Wolves of Japan*. Seattle, WA: University of Washington Press. doi: 10.1017/S002191180700040X

Wernecke, K. E., Vincenz, D., Storsberg, S., D'Hanis, W., Goldschmidt, J., and Fendt, M. (2015). Fox urine exposure induces avoidance behavior in rats and activates the amygdalar olfactory cortex. *Behav. Brain Res.* 279,76–81. doi: 10.1016/j.bbr.2014.11.020

Wilson, S. R., Carmack, M., Novotny, M., Jorgenson, J. W., and Whitten, W. K. (1978). Δ^3-isopentenyl methyl sulfide. A new terpenoid in the scent mark of the Red Fox. *J. Org. Chem.* 43, 4675–4676. doi: 10.1021/jo00418a038

Woolfson, A., and Rothschild, M. (1990). Speculating about pyrazines. *Proc. Biol. Sci.* 242, 113–119. doi: 10.1098/rspb.1990.0113

Zager, P., and Beecham, J. (2006). The role of American black bears and brown bears as predators on ungulates in North America. *Ursus* 17, 95–108. doi: 10.2192/1537-6176(2006)17[95:TROABB]2.0.CO;2

Zhang, J. X., Soini, H. A., Bruce, K. E., Wiesler, D., Woodley, S. K., Baum, M. J., et al. (2005). Putative chemosignals of the ferret (*Mustela furo*) associated with individual and gender recognition. *Chem. Senses* 30, 727–737. doi: 10.1093/chemse/bji065

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 28 July 2015
doi: 10.3389/fnins.2015.00253

Identification of pyridine analogs as new predator-derived kairomones

Julien Brechbühl[1], *Fabian Moine*[1], *Monique Nenniger Tosato*[1], *Frank Sporkert*[2] *and Marie-Christine Broillet*[1]*

[1] *Department of Pharmacology and Toxicology, Faculty of Biology and Medicine, University of Lausanne, Lausanne, Switzerland,* [2] *University Center of Legal Medicine, Lausanne-Geneva, Lausanne, Switzerland*

In the wild, animals have developed survival strategies relying on their senses. The individual ability to identify threatening situations is crucial and leads to increase in the overall fitness of the species. Rodents, for example have developed in their nasal cavities specialized olfactory neurons implicated in the detection of volatile cues encoding for impending danger such as predator scents or alarm pheromones. In particular, the neurons of the Grueneberg ganglion (GG), an olfactory subsystem, are implicated in the detection of danger cues sharing a similar chemical signature, a heterocyclic sulfur- or nitrogen-containing motif. Here we used a "*from the wild to the lab*" approach to identify new molecules that are involuntarily emitted by predators and that initiate fear-related responses in the recipient animal, the putative prey. We collected urines from carnivores as sources of predator scents and first verified their impact on the blood pressure of the mice. With this approach, the urine of the mountain lion emerged as the most potent source of chemical stress. We then identified in this biological fluid, new volatile cues with characteristic GG-related fingerprints, in particular the methylated pyridine structures, 2,4-lutidine and its analogs. We finally verified their encoded danger quality and demonstrated their ability to mimic the effects of the predator urine on GG neurons, on mice blood pressure and in behavioral experiments. In summary, we were able to identify here, with the use of an integrative approach, new relevant molecules, the pyridine analogs, implicated in interspecies danger communication.

Keywords: olfaction, Grueneberg ganglion, predator scents, blood pressure, HS-SPME, GC-MS analysis, calcium imaging, behavior

OPEN ACCESS

Edited by:
Thomas Endres, Otto-von-Guericke University, Germany

Reviewed by:
Kazumi Osada, Health Sciences University of Hokkaido, Japan
Xavier Grosmaitre, Centre National de la Recherche Scientifique, France

***Correspondence:**
Marie-Christine Broillet, Department of Pharmacology and Toxicology, Faculty of Biology and Medicine, University of Lausanne, Bugnon 27, CH-1005 Lausanne, Switzerland
mbroille@unil.ch

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *07 May 2015*
Accepted: *09 July 2015*
Published: *28 July 2015*

Citation:
Brechbühl J, Moine F, Nenniger Tosato M, Sporkert F and Broillet M-C (2015) Identification of pyridine analogs as new predator-derived kairomones. Front. Neurosci. 9:253. doi: 10.3389/fnins.2015.00253

Introduction

Predators and preys interact actively and continuously using their senses to find food or to avoid being eaten (Kavaliers and Choleris, 2001). In addition to their visual and auditory appreciation of their threatening neighborhood, preys have, for example, developed elaborated olfactory abilities to detect predator scents. Indeed by eating meat, carnivores involuntary influence their own scents that will be interpreted by the prey as the presence of an impending danger (Nolte et al., 1994; Hui, 2012). Profound behavioral alterations as well as modulation of essential physiological conditions such as the increase of stress-related hormones or the elevation of the blood pressure of the preys have been, for example, observed in the presence of these predator scents (Dielenberg and McGregor, 2001; Horii et al., 2010; Takahashi, 2014). These scents are a cocktail of chemical molecules with diverse physicochemical properties and structures, which

act as interspecies communicating cues and are therefore named kairomones (Brown et al., 1970). They are present in the biological secretions of predators from their anal glands, feces, or urines (Apfelbach et al., 2005; Wyatt, 2014). They are deciphered by different olfactory neurons segregated in subsystems present in the nasal cavities of the preys (Takahashi, 2014). Large arrays of predator-derived proteins acting as non-volatile kairomones can be detected by specific neurons found in the vomeronasal organ (VNO) of the prey (Papes et al., 2010; Isogai et al., 2011). On the other hand, small volatile kairomones are mainly detected by sensory neurons found in the main olfactory epithelium (MOE) or in the Grueneberg ganglion (GG) subsystems. Kairomones may have their physiological effects in preys as single chemical cues. For example, the 2-phenylethylamine (PEA) that is found at higher level in carnivore's urine than in rodent's urine (>300 μM in lion vs. <1 μM in rat) is specifically detected by amine-sensitive neurons in the MOE (Ferrero et al., 2011; Dewan et al., 2013; Zhang et al., 2013). The 2,4,5-trimethylthiazoline (TMT) found in red fox feces (Fendt et al., 2005) is also a robust single volatile kairomone that is detected concomitantly by the dorsal MOE (Kobayakawa et al., 2007) as well as by the GG (Brechbühl et al., 2013b) to further initiate important behavioral effects (Perez-Gomez et al., 2015).

The particularity of the GG olfactory subsystem resides in its specifically tuned chemical detection abilities. Indeed, this rostral olfactory subsystem found in rodents (Grüneberg, 1973; Tachibana et al., 1990; Fuss et al., 2005; Koos and Fraser, 2005; Fleischer et al., 2006a; Roppolo et al., 2006; Storan and Key, 2006; Fleischer and Breer, 2010; Brechbühl et al., 2014) is dedicated to the detection of volatile danger molecules from both intra and interspecies origins such as alarm pheromones (Brechbühl et al., 2008; Debiec and Sullivan, 2014) and kairomones (Brechbühl et al., 2013b) which share heterocyclic sulfur- or nitrogen-containing structures (Mamasuew et al., 2011; Brechbühl et al., 2013a,b; Hanke et al., 2013). These chemical cues initiate, in the recipient animal, stress reactions (Brechbühl et al., 2013b; Matsuo et al., 2015; Perez-Gomez et al., 2015). Molecules such as the 2-*sec*-butyl-4,5-dihydrothiazole (SBT, a recently identified mouse alarm pheromone, Brechbühl et al., 2013b), the 2,6-dimethylpyrazine [2,6-DMP, found for example in the wolf (Osada et al., 2013) and the bobcat (Mattina et al., 1991)] or the 2,3-dimethylpyridine (2,3-lutidine, Mamasuew et al., 2011; Hanke et al., 2013) share this same signature and were found to initiate GG-evoked neuronal responses.

The identification of chemical molecules that encode for innate fear reactions throughout the initiation of hard-wire neuronal circuitries is a challenging aspect of neuroscience and is fundamental for studying the interspecies communication as well as the pharmacological aspect of the neuronal olfactory pathways. The difficulty in the search of new encoding danger molecules resides principally in the screening of large repertoires of chemicals found in the wild (predator scents or alerting conspecifics) as well as to the vast possibility of detecting neurons found in the nose of the prey. Indeed, after classical chemical identification, each candidate molecule needs to be evaluated individually on the animal for its potential fear-evoked reactions before testing it on olfactory neurons, which could be time and animal-consuming (Brechbühl et al., 2013b; Kiyokawa et al., 2013; Osada et al., 2013). Moreover, encoding danger cues could have diverse chemical structures and small structural variations might also provoke distinctive sensations and neurophysiological responses in the recipient animal (Araneda et al., 2000; Nara et al., 2011; Peterlin et al., 2014). Taking advantage of the chemical structural fingerprint of known GG ligands as baits (Brechbühl et al., 2013b), we here used a "*from the wild to the lab*" approach to identify new predator-derived kairomones with the hypothesis that they will initiate GG responses and generate fear-reactions in mice (Brechbühl et al., 2008, 2013b; Debiec and Sullivan, 2014; Perez-Gomez et al., 2015). We first collected urines from different carnivores and we evaluated further by a non-invasive tail-cuff approach (measurement of blood pressure) the stress responses they generated in mice. The most potent urine, the mountain lion urine, was then analyzed by HS-SPME/GC-MS. We identified in this biological fluid, new putative kairomone candidates. We focused our attention on novel small volatile molecules, in particular the 2,4-lutidine (2,4-dimethylpyridine) and its analogs, as they shared the structural GG-fingerprint and indeed generated GG-evoked neuronal responses. We finally verified their encoded danger quality in mice using both blood pressure measurements and behavioral analysis. In summary, we used an integrative approach to identify new relevant volatile molecules implicated in predator-prey communication and identified pyridine analogs as new potent kairomones.

Materials and Methods

Animals

C57BL/6 (*Mus musculus*; Janvier Labs) and OMP-GFP mice (Potter et al., 2001) were used from pups to adult ages. The OMP-GFP is a particular gene-targeted mouse strain that expresses the green fluorescent protein (GFP) as a histological reporter under the control of the olfactory marker protein (OMP) promoter (Mombaerts et al., 1996; Potter et al., 2001) that is specifically expressed in mature olfactory sensory neurons (Margolis, 1972). Mice were killed by cervical dislocation or by CO_2 inhalation. The experimental procedures were in accordance with the Swiss legislation and approved by the EXPANIM commitee of the Lemanique Animal Facility Network and the veterinary authority of the Canton de Vaud (SCAV).

Urine Collection and Conditioning

Urine samples were directly collected at the Servion Zoo (Servion, CH) or purchased (PredatorPee, Inc., USA). They could originate from one or more than one animal. Carnivore urines were from the mountain lion (*Puma concolor*), snow leopard (*Panthera uncia*), Siberian tiger (*Panthera tigris altaica*), bobcat (*Lynx rufus*), gray wolf (*Canis lupus*), red fox (*Vulpes vulpes*), and coyote (*Canis latrans*). Attempts of urine collection were also performed from the serval (*Leptailurus serval*), artic fox (*Alopex lagopus*), artic wolf (*Canis lupus arctos*), and ferret (*Mustela putorius furo*), but the amounts of urine collected were not sufficient for further analysis. As internal control, urine of a non-carnivore, the American bison (*Bison bison*) was used. Urine

samples were rapidly filtered (0.22 μm), aliquoted in sterile tubes and kept at −80°C until used.

Measurement of the Mice Blood Pressure by the Tail-cuff Approach

The indirect and non-invasive computerized tail-cuff method (Krege et al., 1995; Fox, 2007) was used to monitor simultaneously the blood pressure of 5–6 adult male C57BL/6 mice (10.6 ± 1.9 weeks). Mice were trained for the procedure during five continuous days with the affiliated investigator (Sorge et al., 2014) and the equipment (BP-2000; Visitech) in a behavioral room (23°C, normal light cycle) to limit experimental-related stress such as balloon inflation (Zhao et al., 2011). The system was composed of tree principal parts: the platform, where mice were in experimentation, the control unit, which comprised the power supply and the air pump and a computer to generate experimental protocols and to record individual waveform signals. After calibrating the tail-cuff apparatus, mice were placed on the platform in the magnetic restrainers, their tails were inserted through the cuff/pulse sensor and immobilized with adhesive tape. The platform was heated at 37–39°C to increase the detection of the oscillation waveforms generated by the blood flow rate in the tails. Each session was composed of 10 successive measurements of the systolic and diastolic pressure after the automatic determination of the pulse rate. The system used a photoplethysmographic signal waveform analysis (Shelley and Shelley, 2001; Alian and Shelley, 2014). Briefly, a red light-emitting diode (LED) illuminated the tails of the mice and changes in light absorption due to vessel dilation were detected across time and displayed as oscillating waveform signals on the computer. Diastolic and systolic pressures were perceived by monitoring the vessel dilation during the occlusion cuffs inflation (balloon inflation). The diastolic pressure was defined as the cuff pressure necessary to observe the decrease of the waveform amplitude. The systolic blood pressure was defined as the cuff inflation pressure necessary to fall below 10% of its original stable amplitude (Krege et al., 1995). The mean pressure was calculated as the mean of the measured diastolic and systolic pressure. Measurements obtained in the presence of excessive animal movements were discarded. For each automatized session, mean individual pressures (diastolic, systolic and mean pressure) were obtained as the average of the 10 attempted measurements.

Screening for Volatile Bioreactive Samples by the Tail-cuff Approach

Measurements of blood pressure were performed to evaluate the natural stressful stimuli (Dielenberg et al., 2001) in mice. Tail-cuff recording sessions were done in the morning (9.00–11.00 a.m.) and in the afternoon (1.30–3.00 p.m.). To examine the impact of the tested volatile cues on mice blood pressure, pieces of blotting papers (1 × 1 cm) were placed in front of each tested mouse without any animal contact. 100 μl of the tested substances were deposited on them. The general evaluation procedure was performed with one conditioning measuring session (blotting papers alone) followed by three continuous control sessions (Ctrl; blotting papers with water) and three continuous sessions with the tested substances (Test; blotting papers with pure urine or 1% of synthetic cues in water, Hacquemand et al., 2013; Osada et al., 2013). Average of the three Ctrl sessions and the three test sessions were used to obtain the individual Ctrl and Test pressures. Each urine was evaluated once per animal to avoid any learning process (Kass et al., 2013).

HS-SPME/GC-MS Analysis

Headspace coupled to solid phase microextraction (HS-SPME) followed by gas chromatography coupled to mass spectrometry (GC-MS) was used to analyze volatile compounds released from the selected urine samples (Sporkert and Pragst, 2000; Ouyang and Pawliszyn, 2006; Brechbühl et al., 2013b; Osada et al., 2013). Briefly, for volatile analysis of the urines, a triplicate experiment (three different aliquots originating from the same urine sample) was performed with a polydimethylsiloxane-divinylbenzene portable fiber of 60 μm film thickness (PDMS-DVB, Supelco) inserted into the headspace of airtight 20 ml vials (# 8010, Agilent) containing 500 μl of the urine saturated with sodium chloride and placed on a heater (at 40°C) under constant magnetic agitation for 30 min. The same procedure was done with water to identify volatile contaminants present in the experimental procedure. Qualitative analyses of the extracted components present on the fibers were performed using a GC (6890 Plus, Agilent) coupled with a selective MS (HP 5973N, Agilent) with the analyzing software (MSD ChemStation, Agilent). The SPME fiber extracts were desorbed in the injector in splitless mode for 5 min at 250°C. The oven was set at an initial temperature of 40°C and ran for 20 min until 250°C. The MS was operated in the full-scan mode between 10 and 300 amu. The column used (DB-XLB, Agilent) measured 30 m length with a 0.25 mm I.D. and 0.25 μm film thickness with helium 5.0 as carrier gas. WileyN7 and NIST14 Mass Spectral libraries were used for chemical identification. Characteristic m/z values for each volatile of interest were obtained from reference spectra and used to verify the presence of the corresponding compound. For precise chemical identification, indicated by dots (•), similar procedures with pure reference standards (1:100,000), were used to confirm the presence of the identified cues. The specific ion (m/z = 107) at a retention time 8.285 min ($RT_{8.285}$) was used, for example, to identify the 2,4-lutidine.

Tissue Preparation and Calcium Imaging

Noses from OMP-GFP mice (P1-7) of both sexes were dissected in ice-cold artificial cerebrospinal fluid (ACSF), containing 118 mM NaCl, 25 mM $NaHCO_3$, 10 mM D-glucose, 2 mM KCl, 2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, and 2 mM $CaCl_2$ (pH 7.4) saturated with oxycarbon gas [95% O_2: 5% CO_2; (vol/vol)] under a fluorescence-equipped dissecting microscope (M165 FC; Leica). For calcium imaging experiments, acute tissue slice preparations of the GG were performed (Brechbühl et al., 2008, 2014). Briefly, the tip of the nose was included in a block of low melting 4–5% agar. Coronal slices of 70 μm were generated on ice with a vibroslicer (VT1200S, Leica). Slices were selected under a fluorescent stereomicroscope (M165 FC, Leica) in accordance with their general morphology and their GFP expression. Fura-2 acetoxymethyl ester (AM) (5 μM; TEFLabs) was used as a

ratiometric calcium dye. Slices were incubated with adjunction of pluronic acid (0.1%; Pluronic F-127, Invitrogen) for 60 min (37°C, 5% CO_2) and were immobilized with a slice anchor in a bath chamber (RC-26, Warner Instruments). A bipolar temperature controller (SC-20/CL-100, Warner instruments) was used to maintain the bath temperature between 23 and 25°C. Visualizations were made under an inverted fluorescence microscope (Axio Observer.A1, Zeiss) with a 40x objective and a sensitive camera (Cool SNAP-HQ^2, Photometrics). The software MetaFluor (MetaFluor, Visitron Systems) was used to monitor intracellular calcium variations and to acquire images (Brechbühl et al., 2011).

Chemostimulation

Urine and synthetic cues (ordered from Sigma-Aldrich, Alfa Aesar, or Contech) were used as chemostimulants for calcium imaging experiments and were prepared fresh before each experiment directly diluted in ACSF with osmolarities situated between 285 and 290 Osm/L. The final dilution of the tested urine was at (1:1000) and the final concentration of synthetic cues was at 100 μM (Spehr et al., 2002; Brechbühl et al., 2013a). A short perfusion of extracellular potassium (KCl; 25 mM) was used as a cellular viability test. During the perfusion of ACSF, the spontaneous (Liu et al., 2012) intracellular calcium changes were considered as baseline activity. An increase twice larger than this baseline (corresponding to near 10% of the KCl response) was considered as a neuronal evoked-response (Brechbühl et al., 2013b).

Behavioral Analysis

An open field exploration test (Bailey and Crawley, 2009; Brechbühl et al., 2013b) was used to challenge the anxiety and stress-related behaviors of seven adult male C57BL/6 mice (10.6 ± 0.8 weeks). Briefly, 7 days before the beginning of the test, each mouse was isolated with food and water *ad libitum*. Room temperature was maintained at 23°C in a 12:12 h light/dark inverted cycle. Animals were trained to handling, familiar with the test arena context (a closed Plexiglas box of 45 × 25 × 19 cm) and to the presence of a piece of blotting paper (3 × 3 cm) to minimize environmental-related stress. Behavioral experiments were performed by the affiliated investigator (Sorge et al., 2014) during the nocturnal phase. They were recorded for at least 5 min from the top of the arena covered by a Plexiglas plate by an IR-sensitive HD camera under nightshot vision (infra red illumination) and subsequently analyzed offline in simple blind conditions with a video tracking system (ANY-maze, Stoelting). To assess the stress-related behaviors displayed by mice in the presence of 1% synthetic predator-derived kairomones, neutral cue (Water) or pure predator urines (200 μl on blotting paper), the number of visits in the central zone of the arena (corresponding to the half of the total arena surface Bailey and Crawley, 2009), the total walking distance, the defecation (number of fecal pellets), the number of risk assessment episodes and the total freezing duration were quantified (Vernet-Maury et al., 1984; Blanchard et al., 1990; Fendt et al., 2005; Staples et al., 2008; Brechbühl et al., 2013b; Hacquemand et al., 2013). Each tested substance sessions were compared to a control session (Ctrl) performed before with only blotting paper and used to calculate the index of each evaluated behaviors (as a percent for the central zone, the walking distance and the freezing or as a score for the defecation and the risk assessment). The automatic detection of the center of the animal was used as reference point by the video tracking system. The freezing on/off (30/40) thresholds with a minimum freezing period threshold of 500 ms were used. To minimize animal habituation, only one test session and its Ctrl session were performed per day and each tested substance was used only once per mouse (Kass et al., 2013).

Statistics

The open source statistical package R version 3.1.2 was used. Normality and homogeneity were evaluated by the Shapiro test. Accordingly, comparisons between Ctrl and tested sessions were performed with the one-tailed paired Student's *t*-test or Wilcoxon *w*-test. Values are expressed as mean ± SEM. Significance levels are indicated as follows: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns for non-significant.

Results

Measurements of the Mice Blood Pressure Revealed Bioreactivity of Urine Samples

Predator urines act as natural stressors in rodents due to their kairomone content (Apfelbach et al., 2005; Fendt et al., 2005; Kobayakawa et al., 2007; Ferrero et al., 2011). Indeed, after olfactory detection, typical alterations of behaviors or increases of stress-related hormones and blood pressure have been previously observed in the presence of predator urines (Takahashi, 2014). However, significant differences between urine samples were reported depending, for example, on the period of collection (Osada et al., 2013), the origin of the species (emitter or recipient; Apfelbach et al., 2005) or the preceding diet of the predator itself (Nolte et al., 1994). Based on these reports, we took advantage of a rapid and non-invasive experimental strategy to screen bioreactive predator urines (**Figure 1**) by measuring the elevation of mice blood pressure with the tail-cuff approach (**Figure 1A**) (Krege et al., 1995). We first challenged this method by measuring the variation of the blood pressure of mice exposed to 1% TMT, a potent red fox-derived kairomone known to elicit fear in rodents (Fendt et al., 2005; Horii et al., 2010, 2013; Hacquemand et al., 2013; Takahashi, 2014). As expected significant increases in blood pressure were observed in mice between the TMT exposure and its control level (**Figure 1B**). We thus next measured the mice blood pressure in the presence of the volatile fraction of the different predator urines. Systolic, diastolic, and mean blood pressures were systematically measured according to the signal waveform displayed by the tail-cuff approach (**Figures 1C,D**) while mice were exposed to the collected carnivore urines from *Felidae* such as the mountain lion (Mt. Lion; *Puma concolor*), snow leopard (Snow Leopard; *Panthera uncia*), Siberian tiger (Tiger; *Panthera tigris altaica*) and the bobcat (Bobcat; *Lynx rufus*) (**Figures 1E–H**) as well as urine from *Canidae* such as the gray wolf (Wolf; *Canis lupus*), red fox (Red Fox; *Vulpes vulpes*) and coyote (Coyote; *Canis latrans*) (**Figures 1I–K**) used here as mice predators. We observed and confirmed by this approach

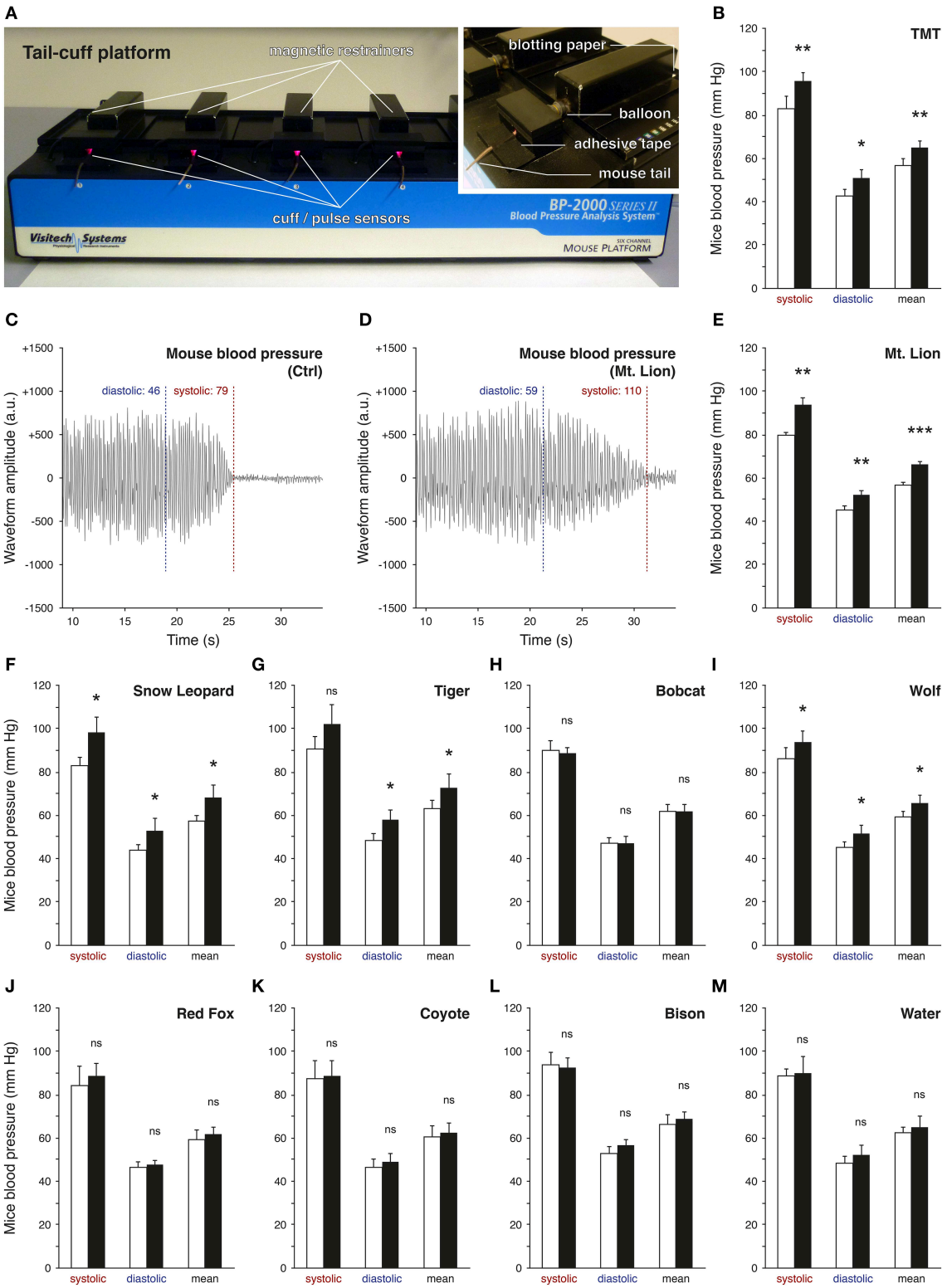


FIGURE 1 | Screening of predator urines via tail-cuff analysis. The blood pressure of mice is analyzed by tail-cuff measurements. **(A)** Mice are placed on the tail-cuff platform, which is connected to a control unit and a computer. They are maintained in magnetic restrainers and their tails are placed in the cuff/pulse optical sensor. Details of the experimental procedure are shown; the blotting paper (tested cues), the inflatable balloon (tail pressure control) and the fixation of the tail with adhesive tape. **(B)** The system is tested here with 1% TMT (synthetic red fox kairomone), which significantly increases the mice blood pressure. **(C,D)** Examples of one

(Continued)

FIGURE 1 | Continued
measurement for the diastolic and the systolic pressures performed on the same mouse under control conditions (**C**, Ctrl) or test conditions, here exposed to the pure urine of the mountain lion (**D**, Mt. Lion). Oscillating waveforms are obtained according to the automatic analysis of the photoplethysmographic signal detected by the pulse sensors [red LED in **(A)**]. Waveform amplitudes are indicated by arbitrary units (a.u.). The mean pressure is calculated as the mean between the diastolic and the systolic pressures. **(E–M)** Tests of different carnivore urines (**E–H**, *Felidae*; **I–K**, *Canidae*), non-carnivore urine (**L**, Bison) or only water (**M**, Water). Exposure to the urine of the Mt. Lion induced the most significant increase in mice blood pressure **(E)**. Control conditions (white bars) and test sessions (black bars) are shown **(B–M)**. Five adult male mice were used **(B,E–M)**. Values are expressed as mean ± SEM; one-tailed paired *t*-test or *w*-test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

that the tested predator urines were not equally efficient to induce an elevation in mice blood pressure, an early physiological parameter (Dielenberg et al., 2001). Furthermore, and consistent with previous reports, urine samples originating from the same predator species (snow leopard in our case) but collected at different moments were indeed not equivalent (data not shown; Osada et al., 2013). Nevertheless, the mountain lion emerged (**Figure 1E**) as the most potent source of volatile kairomones across the tested carnivore urines as its presence significantly increased mice blood pressures nearly by 15% compared to the control level for the systolic ($Ctrl_{systolic}$: 79.6 ± 1.4 mm Hg; Mt. $Lion_{systolic}$: 93.7 ± 3.4 mm Hg; *t*-test: **), diastolic ($Ctrl_{diastolic}$: 45.1 ± 1.7 mm Hg; Mt. $Lion_{diastolic}$: 52.4 ± 1.7 mm Hg; *t*-test: **) and mean blood pressure ($Ctrl_{mean}$: 56.7 ± 1.4 mm Hg; Mt. $Lion_{mean}$: 66.4 ± 1.2 mm Hg; *t*-test: ***). As non-carnivore sample and internal procedure controls (Fendt, 2006), urine from a *Bovidae* (Bison; *Bison bison*) (**Figure 1L**) as well as water (**Figure 1M**) were respectively used and were indeed not able to increase blood pressure in mice. The tail-cuff method, in addition to the blood pressure measurements, also allowed the evaluation of the animal heart rates (HR) by using the frequencies of the waveform signals (**Figures 1C,D**). Interestingly and consistent with previous observations (Dielenberg et al., 2001), we saw here for the TMT and for the fear-inducing predator urines (Mt. Lion, Snow Leopard, Tiger and Wolf), a systematic negative drift of the HR (−8.8 ± 6.3%) between the control and the tested situations. This physiological criterion was not affected by predator urines that did not induce an elevation of blood pressure nor by water (+1.6 ± 0.9%). With this first technical approach, we thus screened bioreactive urines and selected the one issued from the mountain lion as the most potent source of volatile kairomones for further investigations.

Identification of Putative Volatile Kairomones by HS-SPME/GC-MS

We used a HS-SPME/GC-MS approach to extract and analyze volatile chemicals present in the urine of the mountain lion (**Table 1** and **Figure 2**). In our experimental conditions, we identified 98 volatile candidates, 61 of them were referenced in the chemical databases. Among them, numerous pyrazine and thiazolic analog structures were detected (**Figure 2A**). A couple of compounds were previously found in predator urines and/or shown to act as kairomones in mice (Zhang et al., 2005; Brechbühl et al., 2013b; Osada et al., 2013). To identify new putative kairomones, we hypothesized that potential new GG ligands would encode for innate fear-reactions (Brechbühl et al., 2008, 2013b; Debiec and Sullivan, 2014; Perez-Gomez et al., 2015). We thus focused on volatile candidates that also share the heterocyclic sulfur- and/or nitrogen-containing structure related to the detection ability of this olfactory subsystem (Mamasuew et al., 2011; Brechbühl et al., 2013b). We were particularly interested by the pyridine analogs as they share this chemical signature and they were, for another derivative, previously found to evoke neuronal responses in the GG (Mamasuew et al., 2011; Hanke et al., 2013). Interestingly, the 2,4-lutidine (2,4-Lu; also known as 2,4-dimethylpyridine) identified according to its retention time ($RT_{8.285}$; **Figures 2A,B**) and its corresponding mass spectra (MS; **Figures 2C–E**) had a relative abundance of ionized product similar to the one observed for the pyrazine structures (**Figure 2A**). We evaluated its concentration in the original urine of the mountain lion to be in the range of 60–120 μM by comparison with the HS-SPME/GC-MS detection of its synthetic cue (2,4-Lu Sy.; **Figure 2B**). This value corresponds to concentrations previously observed for other kairomones in predator urines (Ferrero et al., 2011). Thus, from the molecular structures detected in the urine of the mountain lion and the chemical identification process we selected pyridine analogs as new putative predator-derived kairomones.

Pyridine Analogs Mimicked the Systemic Effects of Predator Urine in Mice

The 2,4-lutidine and two other pyridine analogs, the 3,4-lutidine (3,4-Lu; also known as 3,4-dimethylpyridine) and the 4-picoline (4-Pi; also known as 4-methylpyridine) (**Table 1**) were selected for further evaluation of their potential fear-like reactivity in mice (**Figure 3**). In a first set of experiments, we performed calcium imaging on GFP-expressing GG neurons from mouse coronal slices previously incubated in Fura-2AM, a ratiometric calcium sensitive dye (Brechbühl et al., 2008, 2013b) (**Figure 3A**). GG slices were continuously perfused with oxycarbonated artificial cerebrospinal fluid (ACSF) at room temperature. Chemical stimulations were performed with a perfusion system and by direct dilution of the tested cues in ACSF. Fura-loaded GG neurons were identified by their intrinsic green fluorescence and by their specific morphology (Brechbühl et al., 2014). GG-evoked responses were observed and graphed by the variation of the Fura-2 ratio fluorescence (Fura-2 ratio) and brief stimulation of KCl was used to evaluate the GG neuronal viability (**Figures 3B–E**). In a total of six individual experiments (*n* total of recorded and viable GG neurons = 60), we found that successive stimulations with the selected pyridines initiated reproducible and reversible responses in GG neurons at a concentration of 100 μM (Brechbühl et al., 2013b), a concentration similar to the one previously estimated in the tested urine of the mountain

TABLE 1 | HS-SPME/GC-MS analysis of the urine of the mountain lion.

RT	ID	Molecule	Usual name
2.141	1	1-Propanol	Propyl alcohol
4.254	2	3-Buten-1-ol, 3-methyl-	Isoprenol
4.678	3	1,2-Propanediol	Propylene glycol
4.775	4	1H-Pyrrole	–
5.314	5	2-Buten-1-ol, 3-methyl-	Prenol
5.870	–	Unidentified compound	–
6.304	6•	Pyridine, 4-methyl-	4-Picoline
6.450	7	Pyrazine, 2-methyl-	–
6.617	8	2-Pentenal, 2-methyl-	–
6.728	9	Ethanol, 2-(methylthio)-	–
6.989	10	Cyclopentanone, 3-methyl-	–
7.069	11	Thiazole	–
7.149	12	1-Hexanol	Hexyl alcohol
7.409	–	Unidentified compound	–
7.489	13•	Pyridine, 2,6-dimethyl-	2,6-Lutidine
7.531	14	3-Heptanone	Butyl ethyl ketone
7.590	15	Methane, bis(methylthio)-	2,4-Dithiapentane
7.743	16	Oxime-, methoxy-phenyl-	–
8.084	17•	Pyrazine, 2,6-dimethyl-	–
8.195	18	1H-Pyrrole, 2,5-dimethyl-	–
8.285	19•	Pyridine, 2,4-dimethyl-	2,4-Lutidine
8.525	20	1-Butanamine, N-butyl-	Dibutylamine
8.674	21	Pyridine, 2-ethyl-6-methyl	2-Picoline, 6-ethyl-
8.754	22•	Pyridine, 3,4-dimethyl-	3,4-Lutidine
8.845	23	Cyclopentanone, 3-ethyl-	–
9.144	24	2-Octanone	–
8.942	25	Phenol	–
9.171	26	1H-Pyrrole, 2,3,5-trimethyl-	–
9.266	27	Pyrazine, 2-ethyl-6-methyl-	–
9.338	28	Pyrazine, 2-ethyl-5-methyl-	–
9.415	29•	Pyrazine, 2,3,5-trimethyl -	–
9.627	–	Unidentified compound	–
9.651	30	Thiazole, 2-acetyl-	
9.707	31	Ethane, 1,2-bis(methylthio)-	2,5-Dithiahexane
9.821	32	Pyrazine, 2-isopropyl-5-methyl-	–
9.856	33	Furan, 2-Acetyl-5-methyl-	–
10.009	34	Phenol, 2-methyl-	*o*-Cresol
10.100	–	Unidentified compound	–
10.190	–	Unidentified compound	–
10.273	35	Phenol, 4-methyl-	*p*-Cresol
10.284	36•	Pyrazine, 3-ethyl-2,5-dimethyl-	–
10.371	37•	Pyrazine, 2-ethyl-3,5-dimethyl-	–
10.419	38	Pyrazine, 2-methyl-3-propyl-	–
10.489	39	Pyrazine, 2-methyl-5-propyl-	–
10.694	–	Unidentified compound	–
10.795	40	Thiazole, 2-acetyl-4-methyl-	–
10.909	41	Pyrazine, 2-methyl-6-propyl-	–
10.965	42	2-Propanone, 1-phenyl-	Phenylacetone
10.986	43	1H-Pyrrole-2-carboxaldehyde, 3,4-dimethyl-	–
11.014	44	2-Cyclohexen-1-one, 3,5,5-trimethyl-	Isophorone
11.104	45	Pyrazine, 2,3-diethyl-5-methyl-	–
11.239	46	Pyrazine, 2,5-dimethyl-3-propyl-	–
11.333	47	Pyrazine, 3,5-dimethyl-2-propyl-	–
11.451	–	Unidentified compound	–
11.507	–	Unidentified compound	–
11.538	48	Pyrazine, 2-acetyl-3,5-dimethyl-	–
11.834	–	Unidentified compound	–
11.875	49	Pyrazine, 2,5-diethyl-3,6-dimethyl-	–
11.921	–	Unidentified compound	–
11.976	–	Unidentified compound	–
12.022	50	Pyrazine, 2,3,5-trimethyl-6-propyl-	–
12.077	51	Pyrazine, 2,5-dimethyl-3-(1-propenyl)-, (Z)-	–
12.119	-	Unidentified compound	–
12.136	52	Benzothiazole	–
12.160	–	Unidentified compound	–
12.220	53	Pyrazine, 2-Isopropenyl-3,6-dimethyl-	–
12.296	–	Unidentified compound	–
12.345	–	Unidentified compound	–
12.522	54	Pyrazine, 2-methyl-3-propyl-	–
12.657	–	Unidentified compound	–
12.800	55	1H-Indole	–
13.015	56	3,3-dimethyl-4,5-dithiahexan-1-ol	–
13.081	–	Unidentified compound	–
13.148	–	Unidentified compound	–
13.353	57	Pyrazine, 3,6-dipropyl-2,5-dimethyl-	–
13.429	58	Pyrazine, 3-isopentyl-2,5-dimethyl-	–
13.592	59	Pyrazine, 2,6-dimethyl-3-(2-methyl-1-butyl)-	–
13.634	–	Unidentified compound	–
13.707	–	Unidentified compound	–
13.822	60	1H-Benzimidazole, 2,5-dimethyl-	–
13.864	–	Unidentified compound	–
13.923	–	Unidentified compound	–
13.978	61	5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	Geranyl acetone
14.142	–	Unidentified compound	–
14.194	–	Unidentified compound	–
14.246	–	Unidentified compound	–
14.284	–	Unidentified compound	–
14.406	–	Unidentified compound	–
14.548	–	Unidentified compound	–
14.736	–	Unidentified compound	–
14.931	–	Unidentified compound	–
15.031	–	Unidentified compound	–
15.424	–	Unidentified compound	–
15.978	–	Unidentified compound	–
16.046	–	Unidentified compound	–
16.070	–	Unidentified compound	–
16.547	–	Unidentified compound	–
18.907	–	Unidentified compound	–

Volatiles from the urine of the mountain lion are listed according to their retention time (RT) expressed in minutes. Sixty-one referenced volatiles were detected in our experimental conditions. The identification number (ID), the chemical nomenclature (Molecule) and the usual name, if existing, of the identified molecules are mentioned. Detected compounds with no attributed name found in the Wiley7N or the NIST14 libraries are listed as "Unidentified compound." Precise chemical identifications have been performed for the volatiles marked with dots (•), otherwise they have been tentatively identified from the libraries.

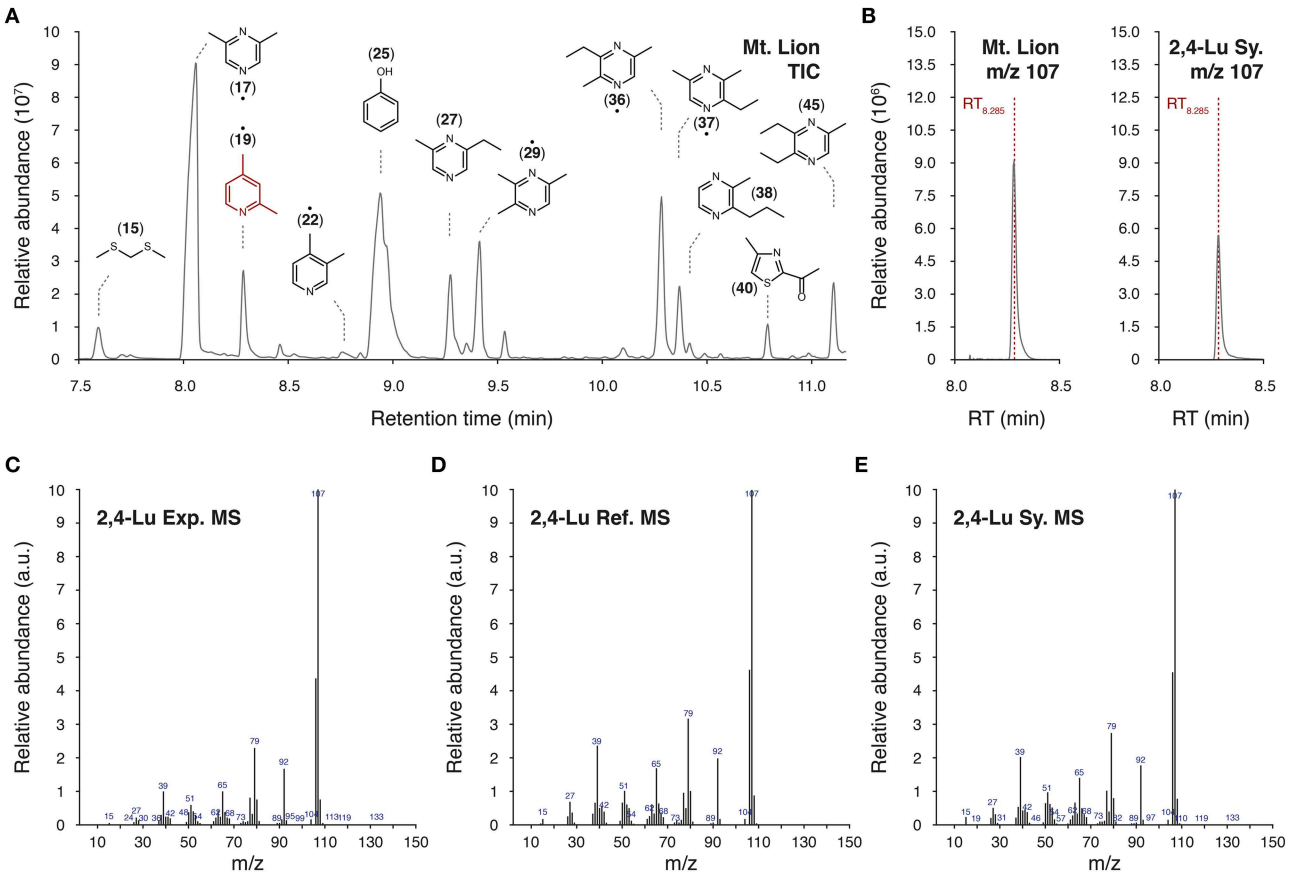


FIGURE 2 | Identification of pyridine analogs as putative kairomones. **(A)** Representative part of a total ion chromatogram (TIC) of volatiles emitted from the urine of the mountain lion obtained by HS-SPME/GC-MS. Chemical formulas of a selection of volatiles are indicated above peaks with their affiliated ID number. When not mentioned as precise chemical identification (•), molecules have been tentatively identified by WileyN7/NIST14 mass spectral libraries. The 2,4-lutidine (2,4-Lu; ID19) is highlighted in red. **(B–E)** Detailed GC-MS analysis of the HS-SPME fiber extract. Here, the identification of the 2,4-lutidine is shown as an example. The mass charge ratio (m/z) value of 107 was used for the precise identification of the 2,4-lutidine (2,4-Lu) thanks to its retention time of 8.285 min ($RT_{8.285}$) in the urine of the mountain lion (**B**, Mt. Lion) and for the synthetic 2,4-lutidine (**B**, Sy.). Experimental m/z spectrum (**C**, Exp. MS) is compared with the reference mass spectrum (**D**, Ref. MS) found in the WileyN7/NIST14 mass spectral libraries as well as with the corresponding mass spectrum of the synthetic cue (**E**, Sy. MS).

lion. As a control, we found that the pyridine-sensitive GG neurons were also able to respond to the biological source of these pyridines, the urine of the mountain lion (Mt. Lion, 1:1000) as well as to pyrazines with a chemical structure related to the ones detected by the GG such as the 2,6-dimethylpyrazine (2,6-DMP; Mamasuew et al., 2011; Brechbühl et al., 2013b; Osada et al., 2013), the 2-ethyl-3,5-dimethylpyrazine (2-EDMP) or the 2,3,5-trimethylpyrazine (2,3,5-TMP; Mamasuew et al., 2011; Osada et al., 2013) (**Figure 3B**). Interestingly, the tested pyridines were not equally efficient in evoking neuronal responses in GG neurons (**Figures 3C–F**). For example, we recorded GG neurons responding partially (**Figure 3C**), not responding (**Figure 3D**) or fully responding (**Figure 3E**) to the three different pyridines. Among all the observations, the 2,4-lutidine emerged as the most potent single pyridine in terms of the number of GG-evoked responses ($n = 28/35$; **Figure 3F**). The perfusion of a mix of the three pyridines (Pyridine mix; $n = 24/26$) was equally efficient to a mix composed by the three tested pyrazines (Pyrazine mix; $n = 23/24$) or to the urine of the mountain lion ($n = 58/60$; **Figure 3F**). In summary, this first experimental approach showed that pyridine analogs act as GG stimuli.

The Grueneberg ganglion subsystem is implicated in olfactory danger communication (Brechbühl et al., 2008, 2013b; Debiec and Sullivan, 2014; Perez-Gomez et al., 2015). These pyridine-evoked GG responses should therefore induce fear-like reactions in the recipient animal. We thus challenged mice with these pyridine analogs. Mice blood pressures were measured in the presence of 1% pyrazine mix (**Figure 3G**) or 1% pyridine mix (**Figure 3H**). We observed, with this approach, that pyrazine analogs as well as pyridine analogs were sufficient to mimic the fear-like reaction observed with the pure source of predator kairomones, the urine of the mountain lion (**Figure 1E**). Furthermore, at the single chemical level, the 2,4-lutidine alone (1% 2,4-lutidine) emerged as potent fear-inducer as it was sufficient to significantly increase mice blood pressure (**Figure 3I**). Interestingly, we also saw with the tail-cuff approach

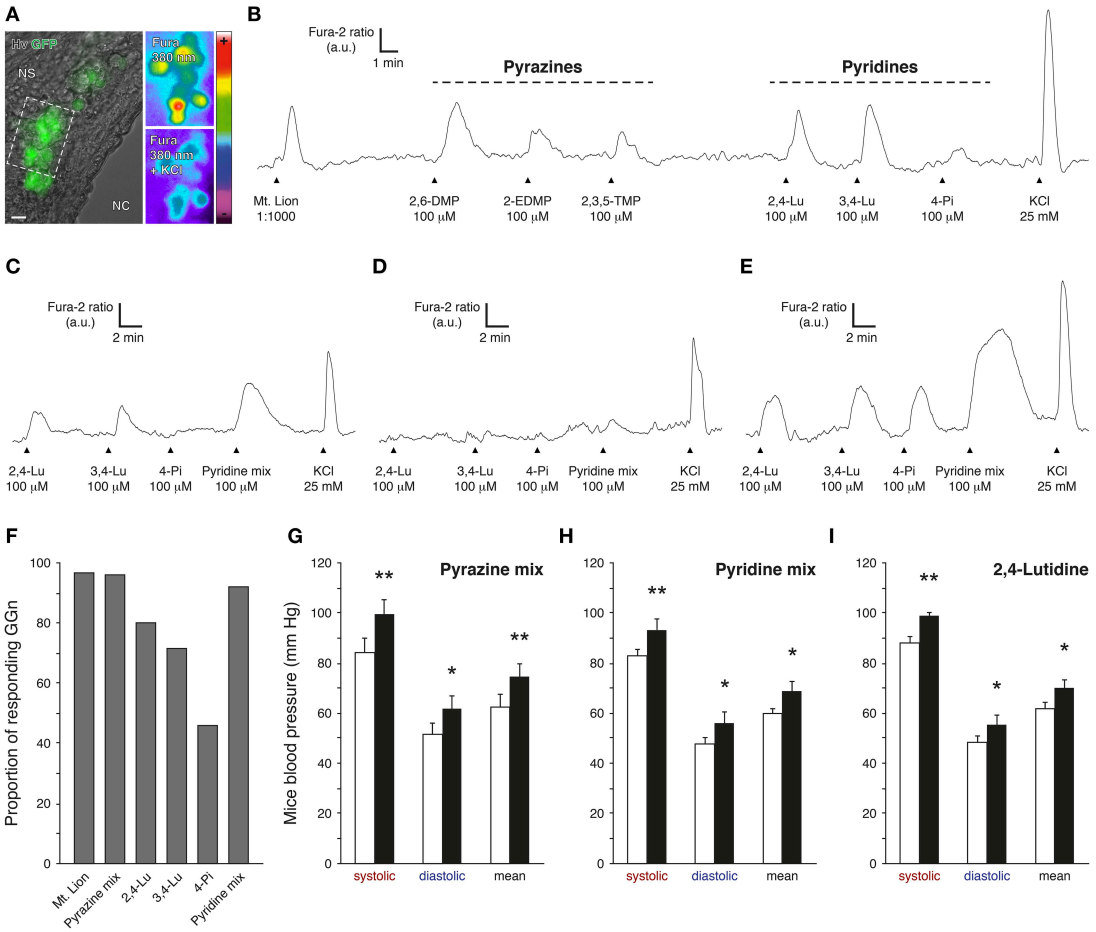


FIGURE 3 | Pyridine analogs are sufficient to mimic the systemic effects of predator urine in mice. (A) Coronal slice from the Grueneberg ganglion (GG) of an OMP-GFP mouse loaded with Fura-2AM. GG neurons (GGn) are localized between the nasal cavity (NC) and the nasal septum (NS). According to their intrinsic GFP fluorescence, GGn can be selected and observed either under Hoffman modulation view (Hv) or 380 nm in color-encoded map for unbound Fura. Here, a typical intracellular calcium increase induced by a control pulse of KCl (25 mM) observed before and at the peak in selected GGn (dashed rectangle). Scale bar, 10 μm in **(A)**. **(B)** Representative continuous recording of a GGn responding to diluted urine of the mountain lion (Mt. Lion; 1:1000), to a set of pyrazines (2,6-dimethylpyrazine, 2,6-DMP; 2-ethyl-3,5-dimethylpyrazine, 2-EDMP; 2,3,5-trimethylpyrazine, 2,3,5-TMP; 100 μM) and to a set of pyridines (2,4-lutidine, 2,4-Lu; 3,4-lutidine, 3,4-Lu; 4-picoline, 4-Pi; 100 μM). **(C–E)** Examples of GGn with differential pattern of pyridine-evoked responses. **(F)** Proportion of responding GGn to the different tested cues. The Pyrazine mix (2,6-DMP, 2-EDMP, 2,3,5-TMP; 100 μM) and the Pyridine mix (2,4-Lu, 3,4-Lu, 4-Pi; 100 μM) were both able to initiate similar numbers of GGn responses comparable to the one observed with the diluted urine of the mountain lion (1:1000). A total of 60 viable GGn isolated from 6 mice (P1-7) were used **(A–F)**. Fluorescence intensity Fura-2 ratio = F340/F380 is indicated by arbitrary units (a.u.); times are indicated by horizontal bars in **(B–E)**. **(G–I)** Mice blood pressure analyzes by tail-cuff measurements for the Pyrazine mix **(G)**, the Pyridine mix **(H)** or the 2,4-lutidine **(I)** at a dilution of 1%. Control conditions (white bars) and test sessions (black bars) are shown **(G–I)**. Six adult male C57BL/6 mice were used **(G–I)**. Values are expressed as mean ± SEM; one-tailed paired *t*-test or *w*-test, $^{*}P < 0.05$; $^{**}P < 0.01$; ns, not significant.

that these synthetic cues generated a similar systematic negative drift of the HR (−9.7 ± 2.0%) as the one observed for the mountain lion urine (−5.5 ± 2.0%). Thus, we showed here that pyridine analogs not only activated GG neurons at physiological concentrations but that they were able and sufficient to mimic the systemic responses observed with the predator urine.

Pyridine Analogs Evoked Innate Fear Reactions in Mice

Physiological alterations such as blood pressure increases and GG neuronal activations are consistent evidences of fear-like encoding cues, but to verify the ability of pyridine analogs to evoke innate fear in mice, we challenged them in an open field behavioral test (**Figure 4**). Mice were placed in an arena in the presence of the different tested substances deposited on a blotting paper (**Figure 4A**). They were observed in the presence of a neutral cue (Water) (Dewan et al., 2013; Perez-Gomez et al., 2015) or in the presence of mountain lion urine (Mt. Lion) (Dewan et al., 2013) or 1% pyrazine mix (Pyrazine mix) (Osada et al., 2013) used as internal non-aversive and stress-inducing references to evaluate the potency of the candidate kairomones 1% pyridine mix (Pyridine mix) and 1% 2,4-lutidine

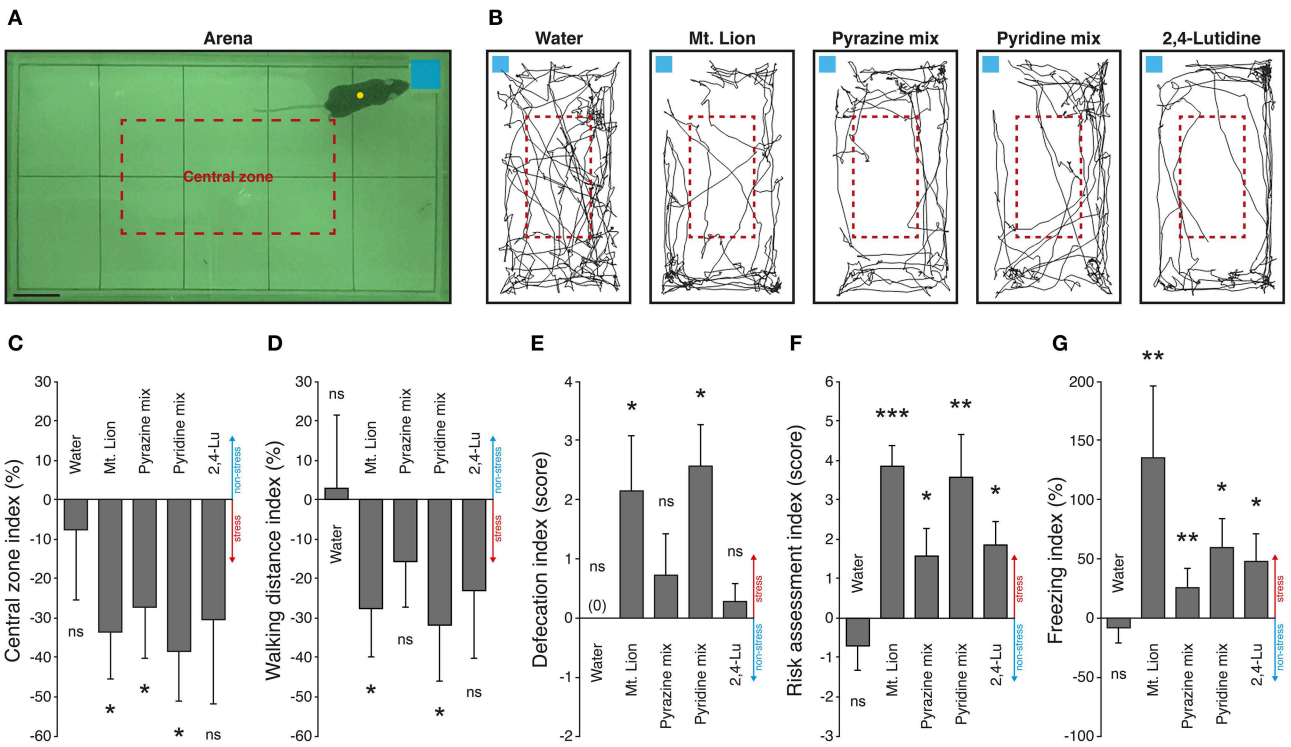


FIGURE 4 | Pyridine analogs generate stress-related behaviors in mice. An open field exploration test is used to evaluate the innate fear reactions of mice. **(A)** The behavioral arena observed during nocturnal phase. The limits of the arena zone (black rectangle) and the central zone (red dashed rectangle) are shown. Mice are automatically tracked (yellow circle) in the presence of different tested cues deposited on a blotting paper (blue square). Scale bar, 5 cm in **(A)**. **(B)** Representative tracking distances covered by the same mouse in the presence of a neutral cue (Water), pure mountain lion urine (Mt. Lion), a mix of pyrazines (Pyrazine mix; 2,6-DMP, 2-EDMP, 2,3,5-TMP; 1%), a mix of pyridines (Pyridine mix; 2,4-Lu, 3,4-Lu, 4-Pi; 1%) or 2,4-lutidine (2,4-Lu; 1%). **(C–G)** Stress-related behaviors are quantified according to the control session and displayed as indexes. Stress (red) or non-stress (blue) tendencies are displayed on graphics. The number of visits in the central zone of the arena **(C)**, the total walking distance recorded during 5 min **(D)**, the defecation (number of fecal pellets) **(E)**, the risk assessment episodes **(F)** and the freezing behaviors **(G)** were quantified. Innate fear reactions were observed for the mountain lion urine, the mixes of pyrazines and pyridines and for the 2,4-lutidine. Seven adult male mice were used **(C–G)**. Values are expressed as mean ± SEM; one-tailed paired *t*-test or *w*-test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

(2,4-Lu) to generate fear. We first tracked the movements of the mice during sessions of 5 min under control and test situations (**Figure 4B**). We observed a decrease of the exploratory behavior performed in the central zone arena (**Figure 4C**), as well as a decrease of the general walking activity previously described as anxiety traits (Vernet-Maury et al., 1984; Bailey and Crawley, 2009; Brechbühl et al., 2013b) (**Figure 4D**). Additional important stress-related criteria such as increases of defecation (**Figure 4E**), risk assessment episodes (**Figure 4F**) and freezing behaviors (**Figure 4G**) were also displayed (Blanchard et al., 1990; Apfelbach et al., 2005; Fendt et al., 2005; Brechbühl et al., 2008; Staples et al., 2008; Hacquemand et al., 2013). In summary, the neutral cue used here was not able to generate any stress-related reactions in mice. On the other hand, strong stress responses were observed for the mountain lion urine and, for both mixes of synthetic cues, the pyrazines, and the pyridines. Consistent with our previous observations done on GG slices or with blood pressure measurements, the 2,4-lutidine alone was also able to generate stress reactions in mice especially for the risk assessment and for the freezing behaviors (**Figures 4F,G**). Thus, as for pyrazine analogs (Osada et al., 2013), pyridine analogs could therefore be considered as new predator-derived kairomones as they initiate innate fear reactions in mice.

Discussion

Odorants present in the environment provide relevant information about the context in which the animals live. Preys, for example, detect the presence of their predators and avoid fatal encounters through the deciphering of specific predator scents (Isogai et al., 2011). This olfactory ability is crucial for their survival and is mediated by parallel and/or complementary olfactory neuronal pathways (Ma, 2010; Takahashi, 2014). After screening the effectiveness of fear-inducing predator urines, we focused our attention on volatiles that could be detected by the Grueneberg ganglion based on their structural fingerprint (Brechbühl et al., 2013b). We found pyridine analogs that shared this feature as new putative predator-derived kairomones and showed that these candidate molecules were indeed detectable by mice GG neurons. Nevertheless, they could also activate

additional neuronal pathways as they have, for humans, a strong and aversive smell (Brechbühl et al., 2013b). Olfactory neurons in the VNO (Osada et al., 2013) or in the MOE (Matsuo et al., 2015) could therefore be implicated alongside with the GG neurons in the deciphering of these molecules and allow, for example, fine behavioral adjustment in the recipient animal (Perez-Gomez et al., 2015). The use of mouse models with targeted invalidation of olfactory subsystems could clarify their specific involvement in the pyridine analog recognition. The analysis of stress-evoked effects recorded in the presence of pyridine analogs in mice with a genetic deletion of the essential MOE component, the cyclic nucleotide-gated channel A2 (CNGA2) (Brunet et al., 1996), or the key VNO-signaling element, the transient receptor potential channel type 2 (TRPC2) (Stowers et al., 2002; Papes et al., 2010) as well as in mice with surgical ablation of the GG (Brechbühl et al., 2008) would be of particular interest. Nevertheless, we found that pyridine analogs were able and sufficient to initiate blood pressure increase, a known early physiological response to predator scents (Dielenberg et al., 2001) and to evoke fear-reactions in the recipient mice. Pyridine analogs could therefore be considered as new chemical actors in the predator-prey communication.

The identification of single kairomones encoding for innate fear reactions in prey is a fundamental ethological challenge. For our HS-SPME/GC-MS experiments, we chose a PDMS-DVB fiber according to its physical characteristics and ability to trap numerous volatiles of interest (Soso et al., 2014). Supplementary analyses performed either with other SPME fibers or alternative chemical detection systems would probably allow identification of additional putative kairomones. Indeed, not all molecules can be adsorbed by our method of identification, consistent here with our inability to detect known kairomones present in the urine of the mountain lion such as the PEA (Ferrero et al., 2011). Yet based on the chemical structures of the detected molecules (Hui, 2012), the present study proposes multiple GG-activating kairomone candidates such as numerous pyrazine or pyrrole analogs. These compounds as well as the ones not referenced in the used chemical libraries deserve additional investigations. Confirmation of the presence of pyridine analogs in other predator urines and their encoding danger quality for other rodentia preys would also reveal the ubiquity of these molecules in the predator-prey communication context (Brechbühl et al., 2014).

Predator scents are complex cocktails of molecules with different physicochemical properties. Here we first used the non-invasive tail-cuff approach to demonstrate the physiological effects of fear-like responses in mice exposed to volatile sources. We used this approach to screen fear-inducing urines and we identified the urine of the mountain lion as the most potent source of volatile kairomones as it generated in mice the most significant increase in blood pressure. These observations were also correlated with the GG neuronal responses evoked by the different predator urines in calcium imaging experiments (data not shown). Nevertheless, predator urines that did not induce fear reactions in mice, as observed by this technical approach, could also be the source of non-volatile kairomones (Papes et al., 2010; Isogai et al., 2011) and could induce strong aversive effects in classical behavioral experiments in mice. Compared to other non-invasive analysis of fear reactions such as the behavioral analysis (e.g., freezing, risk assessment) (Kobayakawa et al., 2007; Brechbühl et al., 2013b), the tail-cuff method allows the collection of rapid and still robust results, and thus is an interesting alternative strategy for screening large arrays of fear-inducing cues such as predator urines or kairomone candidates. Nevertheless, pleasant and unpleasant odorants such as lavender oil or the non-predatory odor butyric acid could induce decreases or only extremely weak increases of blood pressure respectively (Major and Silver, 1999; Tanida et al., 2006; Horii et al., 2010). Additional methods could therefore be used to confirm the innate fear-inducing effects of the candidate cues screened by the tail-cuff method. Here we chose the behavioral analysis, but alternative methods could also be performed such as telemetry measurements (Dielenberg et al., 2004), that could provide additional and long-lasting physiological values (e.g., general animal activity, animal temperature) and/or invasive approaches such as the analysis of the animal corticosterone level (Thomas et al., 2006; Ferrero et al., 2011; Brechbühl et al., 2013b).

In addition to the blood pressure measurements, the tail-cuff method also allows the evaluation of the animal heart rates. This physiological value was reported to be less relevant for fear-induced situations (Dielenberg and McGregor, 2001; Takahashi, 2014) and thus was not primarily used in this study as a criterion for screening and evaluating fear-inducing urines. Interestingly and consistently with previous observations (Dielenberg et al., 2001), we saw, as a whole, for the fear-inducing predator urines and the synthetic cues, a systematic drift of the HR ($-9.1 \pm 3.8\%$) between the control and the tested situations ($Ctrl_{meanHR}$: 458.4 ± 33.4 bpm; $Test_{meanHR}$: 411.7 ± 27.1 bpm; *t*-test: *). This physiological trait deserves further investigations and could be, for example, a contextual adaptation of the animal to hiding strategies (Kavaliers and Choleris, 2001; Barber and Conner, 2007; Pereira et al., 2012).

Predator-derived kairomones such as the pyrazine (Osada et al., 2013) or the pyridine analogs appear to be important contributors to the predator-prey chemical communication. For the prey, the Grueneberg ganglion acts as a specialized olfactory subsystem implicated in the detection of these meat-derived cues (Brechbühl et al., 2013a,b). The biochemical pathways of these kairomones are taking place in the predator guts. They are unknown but mostly depend of the meat-metabolism produced by the digestion and the species-dependent commensal microflora (Brown, 1979; Schellinck and Brown, 2000; Apfelbach et al., 2005). Their chemical formations could use different amino acids as origins to generate convergent structures (Suyama and Adachi, 1980; Yu and Zhang, 2010; Hui, 2012). We may here speculate that danger cues derivating from similar biochemical pathways would have analogous chemical structures and thus could be deciphered by related GG neuronal pathways (Brechbühl et al., 2013a). Here we show that pyridine and pyrazine analogs that are chemically similar (nitrogen containing molecules) were detected by the same GG neurons. They could derive from conserved biochemical pathways. On the other hand, danger molecules that also evoked GG responses and innate fear reactions such as the mouse alarm pheromone SBT,

the red fox kairomone TMT (nitrogen and sulfur containing molecules) or the stoat kairomone 2-PT (2-propylthietane; sulfur containing molecule) have distinctive chemical structures and could therefore use alternative GG signaling (Brechbühl et al., 2008, 2013a,b; Perez-Gomez et al., 2015). Indeed, more than one receptor or signaling pathway are expressed in GG neurons (Fleischer et al., 2006b, 2007, 2009; Liu et al., 2009, 2012; Brechbühl et al., 2013a, 2014) and could therefore be used to detect the large array of danger cues in a single GG neuron differently from the single recognition cascades that take place in the neurons of the MOE or VNO (Touhara and Vosshall, 2009; Ma, 2012). Consistent with previous reports (Mamasuew et al., 2011; Hanke et al., 2013), this characteristic is reinforced in the present study by the differential patterns of pyridine-evoked responses observed in GG neurons. Calcium imaging experiments revealed the 2,4-lutidine as the most potent tested pyridine analog. Nevertheless, using a mix of pyridine analogs as a source of GG stimulus, we found that both the proportion of GG-evoked responses as well as the cellular intensity of the response itself were increased. These cumulative neuronal effects are consistent with the potential expression of multiple signaling pathways in a single neuron (Yu and Zhang, 2014; Yu et al., 2014) and they could be an evolutionary conserved feature to increase the coding complexity and broadening of the neuronal tuning profile (Spehr and Leinders-Zufall, 2005; Brechbühl et al., 2013a).

Our study gives new insights into how organisms interact and communicate chemically. Indeed, physiological impacts could be observed in the prey after the olfactory detection of chemical danger cues occurred. This prey's ability to detect kairomones, such as the pyridine analogs, present in the biological fluids of the predator increases the overall fitness of the species and could thus be considered as an evolutionary strategy for survival (Apfelbach et al., 2005).

Author Contributions

JB, FS, and MCB designed research; JB, FM, MNT, and MCB performed research; JB and FM analyzed data; and JB and MCB wrote the paper.

Acknowledgments

We thank I. Rodriguez for the OMP-GFP mice; C. Berthonneche and A. Sarre from the cardiovascular assessment facility of the University of Lausanne and its head T. Pedrazzini for the tail-cuff apparatus and advices; M. Clerc from the Servion zoo and its head R. Bulliard as well as M. Klaey, C. Verdumo, and E. Stoop for their help during the collection of urines. N. Steiner and J.-P. Hornung for the ANY-maze tracking software resource. C. Giroud and M. Beaumann for their help during pilot experiments; R. Stoop and O. Alijevic for fruitful discussions on the manuscript. This work was supported by the Department of Pharmacology and Toxicology, University of Lausanne, and by the Swiss National Foundation Grant FNS 3100A0-125192 (to MCB).

References

Alian, A. A., and Shelley, K. H. (2014). Photoplethysmography. *Best Pract. Res. Clin. Anaesthesiol.* 28, 395–406. doi: 10.1016/j.bpa.2014.08.006

Apfelbach, R., Blanchard, C. D., Blanchard, R. J., Hayes, R. A., and McGregor, I. S. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neurosci. Biobehav. Rev.* 29, 1123–1144. doi: 10.1016/j.neubiorev.2005.05.005

Araneda, R. C., Kini, A. D., and Firestein, S. (2000). The molecular receptive range of an odorant receptor. *Nat. Neurosci.* 3, 1248–1255. doi: 10.1038/81774

Bailey, K. R., and Crawley, J. N. (2009). "Anxiety-related behaviors in mice," in *Methods of Behavior Analysis in Neuroscience*, *2nd Edn.*, Chapter 5, ed J. J. Buccafusco (Boca Raton, FL: CRC Press).

Barber, J. R., and Conner, W. E. (2007). Acoustic mimicry in a predator-prey interaction. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9331–9334. doi: 10.1073/pnas.0703627104

Blanchard, R. J., Blanchard, D. C., Rodgers, J., and Weiss, S. M. (1990). The characterization and modelling of antipredator defensive behavior. *Neurosci. Biobehav. Rev.* 14, 463–472

Brechbühl, J., Klaey, M., and Broillet, M. C. (2008). Grueneberg ganglion cells mediate alarm pheromone detection in mice. *Science* 321, 1092–1095. doi: 10.1126/science.1160770

Brechbühl, J., Klaey, M., Moine, F., Bovay, E., Hurni, N., Nenniger Tosato, M., et al. (2014). Morphological and physiological species-dependent characteristics of the rodent Grueneberg ganglion. *Front Neuroanat.* 8:87. doi: 10.3389/fnana.2014.00087

Brechbühl, J., Luyet, G., Moine, F., Rodriguez, I., and Broillet, M. C. (2011). Imaging pheromone sensing in a mouse vomeronasal acute tissue slice preparation. *J. Vis. Exp.* 58:e3311. doi: 10.3791/3311

Brechbühl, J., Moine, F., and Broillet, M. C. (2013a). Mouse Grueneberg ganglion neurons share molecular and functional features with *C. elegans* amphid neurons. *Front. Behav. Neurosci.* 7:193. doi: 10.3389/fnbeh.2013.00193

Brechbühl, J., Moine, F., Klaey, M., Nenniger-Tosato, M., Hurni, N., Sporkert, F., et al. (2013b). Mouse alarm pheromone shares structural similarity with predator scents. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4762–4767. doi: 10.1073/pnas.1214249110

Brown, R. E. (1979). "Mammalian social odors: a critical review," in *Advances in the Study of Behavior*, eds J. S. Rosenblatt, R. A. Hinde, C. Beer, and M.-C. Busnel (New York, NY: Academic Press), 103–162.

Brown, W. L., Eisner, T., and Whittaker, R. H. (1970). Allomones and kairomones: transspecific chemical messengers. *Bioscience* 20, 21. doi: 10.2307/1294753

Brunet, L. J., Gold, G. H., and Ngai, J. (1996). General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. *Neuron* 17, 681–693.

Debiec, J., and Sullivan, R. M. (2014). Intergenerational transmission of emotional trauma through amygdala-dependent mother-to-infant transfer of specific fear. *Proc. Natl. Acad. Sci. U.S.A.* 111, 12222–12227. doi: 10.1073/pnas.1316740111

Dewan, A., Pacifico, R., Zhan, R., Rinberg, D., and Bozza, T. (2013). Non-redundant coding of aversive odours in the main olfactory pathway. *Nature* 497, 486–489. doi: 10.1038/nature12114

Dielenberg, R. A., Carrive, P., and McGregor, I. S. (2001). The cardiovascular and behavioral response to cat odor in rats: unconditioned and conditioned effects. *Brain Res.* 897, 228–237. doi: 10.1016/S0006-8993(01)02227-2

Dielenberg, R. A., Leman, S., and Carrive, P. (2004). Effect of dorsal periaqueductal gray lesions on cardiovascular and behavioral responses to cat odor exposure in rats. *Behav. Brain Res.* 153, 487–496. doi: 10.1016/j.bbr.2004.01.015

Dielenberg, R. A., and McGregor, I. S. (2001). Defensive behavior in rats towards predatory odors: a review. *Neurosci. Biobehav. Rev.* 25, 597–609. doi: 10.1016/S0149-7634(01)00044-6

Fendt, M. (2006). Exposure to urine of canids and felids, but not of herbivores, induces defensive behavior in laboratory rats. *J. Chem. Ecol.* 32, 2617–2627. doi: 10.1007/s10886-006-9186-9

Fendt, M., Endres, T., Lowry, C. A., Apfelbach, R., and McGregor, I. S. (2005). TMT-induced autonomic and behavioral changes and the neural

basis of its processing. *Neurosci. Biobehav. Rev.* 29, 1145–1156. doi: 10.1016/j.neubiorev.2005.04.018

Ferrero, D. M., Lemon, J. K., Fluegge, D., Pashkovski, S. L., Korzan, W. J., Datta, S. R., et al. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11235–11240. doi: 10.1073/pnas.1103317108

Fleischer, J., and Breer, H. (2010). The Grueneberg ganglion: a novel sensory system in the nose. *Histol. Histopathol.* 25, 909–915.

Fleischer, J., Hass, N., Schwarzenbacher, K., Besser, S., and Breer, H. (2006a). A novel population of neuronal cells expressing the olfactory marker protein (OMP) in the anterior/dorsal region of the nasal cavity. *Histochem. Cell Biol.* 125, 337–349. doi: 10.1007/s00418-005-0077-x

Fleischer, J., Mamasuew, K., and Breer, H. (2009). Expression of cGMP signaling elements in the Grueneberg ganglion. *Histochem. Cell Biol.* 131, 75–88. doi: 10.1007/s00418-008-0514-8

Fleischer, J., Schwarzenbacher, K., Besser, S., Hass, N., and Breer, H. (2006b). Olfactory receptors and signalling elements in the Grueneberg ganglion. *J. Neurochem.* 98, 543–554. doi: 10.1111/j.1471-4159.2006.03894.x

Fleischer, J., Schwarzenbacher, K., and Breer, H. (2007). Expression of trace amine-associated receptors in the Grueneberg ganglion. *Chem. Senses* 32, 623–631. doi: 10.1093/chemse/bjm032

Fox, J. G. (2007). *The Mouse in Biomedical Research: Normative Biology, Husbandry, and Models*. New York, NY: Elsevier.

Fuss, S. H., Omura, M., and Mombaerts, P. (2005). The Grueneberg ganglion of the mouse projects axons to glomeruli in the olfactory bulb. *Eur. J. Neurosci.* 22, 2649–2654. doi: 10.1111/j.1460-9568.2005.04468.x

Grüneberg, H. (1973). A ganglion probably belonging to the N. terminalis system in the nasal mucosa of the mouse. *Z. Anat. Entwicklungsgesch.* 140, 39–52. doi: 10.1007/BF00520716

Hacquemand, R., Choffat, N., Jacquot, L., and Brand, G. (2013). Comparison between low doses of TMT and cat odor exposure in anxiety- and fear-related behaviors in mice. *Behav. Brain Res.* 238, 227–231. doi: 10.1016/j.bbr.2012.10.014

Hanke, W., Mamasuew, K., Biel, M., Yang, R. B., and Fleischer, J. (2013). Odorant-evoked electrical responses in Grueneberg ganglion neurons rely on cGMP-associated signaling proteins. *Neurosci. Lett.* 539, 38–42. doi: 10.1016/j.neulet.2013.01.032

Horii, Y., Nagai, K., and Nakashima, T. (2013). Order of exposure to pleasant and unpleasant odors affects autonomic nervous system response. *Behav. Brain Res.* 243, 109–117. doi: 10.1016/j.bbr.2012.12.042

Horii, Y., Nikaido, Y., Nagai, K., and Nakashima, T. (2010). Exposure to TMT odor affects adrenal sympathetic nerve activity and behavioral consequences in rats. *Behav. Brain Res.* 214, 317–322. doi: 10.1016/j.bbr.2010.05.047

Hui, Y. H. (2012). *Handbook of Meat and Meat Processing, 2nd Edn*. Florida: Taylor & Francis.

Isogai, Y., Si, S., Pont-Lezica, L., Tan, T., Kapoor, V., Murthy, V. N., et al. (2011). Molecular organization of vomeronasal chemoreception. *Nature* 478, 241–245. doi: 10.1038/nature10437

Kass, M. D., Rosenthal, M. C., Pottackal, J., and McGann, J. P. (2013). Fear learning enhances neural responses to threat-predictive sensory stimuli. *Science* 342, 1389–1392. doi: 10.1126/science.1244916

Kavaliers, M., and Choleris, E. (2001). Antipredator responses and defensive behavior: ecological and ethological approaches for the neurosciences. *Neurosci. Biobehav. Rev.* 25, 577–586. doi: 10.1016/S0149-7634(01)00042-2

Kiyokawa, Y., Kodama, Y., Kubota, T., Takeuchi, Y., and Mori, Y. (2013). Alarm pheromone is detected by the vomeronasal organ in male rats. *Chem. Senses* 38, 661–668. doi: 10.1093/chemse/bjt030

Kobayakawa, K., Kobayakawa, R., Matsumoto, H., Oka, Y., Imai, T., Ikawa, M., et al. (2007). Innate versus learned odour processing in the mouse olfactory bulb. *Nature* 450, 503–508. doi: 10.1038/nature06281

Koos, D. S., and Fraser, S. E. (2005). The Grueneberg ganglion projects to the olfactory bulb. *Neuroreport* 16, 1929–1932. doi: 10.1111/j.1460-9568.2005.04468.x

Krege, J. H., Hodgin, J. B., Hagaman, J. R., and Smithies, O. (1995). A noninvasive computerized tail-cuff system for measuring blood pressure in mice. *Hypertension* 25, 1111–1115.

Liu, C. Y., Fraser, S. E., and Koos, D. S. (2009). Grueneberg ganglion olfactory subsystem employs a cGMP signaling pathway. *J. Comp. Neurol.* 516, 36–48. doi: 10.1002/cne.22096

Liu, C. Y., Xiao, C., Fraser, S. E., Lester, H. A., and Koos, D. S. (2012). Electrophysiological characterization of Grueneberg ganglion olfactory neurons: spontaneous firing, sodium conductance, and hyperpolarization-activated currents. *J. Neurophysiol.* 108, 1318–1334. doi: 10.1152/jn.00907.2011

Ma, M. (2010). "Multiple olfactory subsystems convey various sensory signals," in *The Neurobiology of Olfaction*, ed A. Menini (Boca Raton, FL: Taylor and Francis Group, LLC), 225–240.

Ma, M. (2012). Odor and pheromone sensing via chemoreceptors. *Adv. Exp. Med. Biol.* 739, 93–106. doi: 10.1007/978-1-4614-1704-0_6

Major, D. A., and Silver, W. L. (1999). Odorants presented to the rat nasal cavity increase cortical blood flow. *Chem. Senses* 24, 665–669.

Mamasuew, K., Hofmann, N., Breer, H., and Fleischer, J. (2011). Grueneberg ganglion neurons are activated by a defined set of odorants. *Chem. Senses* 36, 271–282. doi: 10.1093/chemse/bjq124

Margolis, F. L. (1972). A brain protein unique to the olfactory bulb. *Proc. Natl. Acad. Sci. U.S.A.* 69, 1221–1224. doi: 10.1073/pnas.69.5.1221

Matsuo, T., Hattori, T., Asaba, A., Inoue, N., Kanomata, N., Kikusui, T., et al. (2015). Genetic dissection of pheromone processing reveals main olfactory system-mediated social behaviors in mice. *Proc. Natl. Acad. Sci. U.S.A.* 112, E311–E320. doi: 10.1073/pnas.1416723112

Mattina, M. J., Pignatello, J. J., and Swihart, R. K. (1991). Identification of volatile components of bobcat (*Lynx rufus*) urine. *J. Chem. Ecol.* 17, 451–462. doi: 10.1007/BF00994344

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., et al. (1996). Visualizing an olfactory sensory map. *Cell* 87, 675–686. doi: 10.1016/S0092-8674(00)81387-2

Nara, K., Saraiva, L. R., Ye, X., and Buck, L. B. (2011). A large-scale analysis of odor coding in the olfactory epithelium. *J. Neurosci.* 31, 9179–9191. doi: 10.1523/JNEUROSCI.1282-11.2011

Nolte, D. L., Mason, J. R., Epple, G., Aronov, E., and Campbell, D. L. (1994). Why are predator urines aversive to prey? *J. Chem. Ecol.* 20, 1505–1516. doi: 10.1007/BF02059876

Osada, K., Kurihara, K., Izumi, H., and Kashiwayanagi, M. (2013). Pyrazine analogues are active components of wolf urine that induce avoidance and freezing behaviours in mice. *PLoS ONE* 8:e61753. doi: 10.1371/journal.pone.0061753

Ouyang, G., and Pawliszyn, J. (2006). SPME in environmental analysis. *Anal. Bioanal. Chem.* 386, 1059–1073. doi: 10.1007/s00216-006-0460-z

Papes, F., Logan, D. W., and Stowers, L. (2010). The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. *Cell* 141, 692–703. doi: 10.1016/j.cell.2010.03.037

Pereira, A. G., Cruz, A., Lima, S. Q., and Moita, M. A. (2012). Silence resulting from the cessation of movement signals danger. *Curr. Biol.* 22, R627–R628. doi: 10.1016/j.cub.2012.06.015

Perez-Gomez, A., Bleymehl, K., Stein, B., Pyrski, M., Birnbaumer, L., Munger, S. D., et al. (2015). Innate predator odor aversion driven by parallel olfactory subsystems that converge in the ventromedial hypothalamus. *Curr. Biol.* 25, 1340–1346. doi: 10.1016/j.cub.2015.03.026

Peterlin, Z., Firestein, S., and Rogers, M. E. (2014). The state of the art of odorant receptor deorphanization: a report from the orphanage. *J. Gen. Physiol.* 143, 527–542. doi: 10.1085/jgp.201311151

Potter, S. M., Zheng, C., Koos, D. S., Feinstein, P., Fraser, S. E., and Mombaerts, P. (2001). Structure and emergence of specific olfactory glomeruli in the mouse. *J. Neurosci.* 21, 9713–9723.

Roppolo, D., Ribaud, V., Jungo, V. P., Luscher, C., and Rodriguez, I. (2006). Projection of the Grüneberg ganglion to the mouse olfactory bulb. *Eur. J. Neurosci.* 23, 2887–2894. doi: 10.1111/j.1460-9568.2006.04818.x

Schellinck, H. M., and Brown, R. E. (2000). Selective depletion of bacteria alters but does not eliminate odors of individuality in Rattus norvegicus. *Physiol. Behav.* 70, 261–270. doi: 10.1016/S0031-9384(00)00277-8

Shelley, K. H., and Shelley, S. (2001). "Pulse oximeter waveform: photoelectric plethysmography," in *Clinical Monitoring: Practical Applications for Anesthesia and Critical Care*, ed W. B. Saunders (Philadelphia, PA: University of Michigan), 420–423.

Sorge, R. E., Martin, L. J., Isbester, K. A., Sotocinal, S. G., Rosen, S., Tuttle, A. H., et al. (2014). Olfactory exposure to males, including men, causes

stress and related analgesia in rodents. *Nat. Methods* 11, 629–632. doi: 10.1038/nmeth.2935

Soso, S. B., Koziel, J. A., Johnson, A., Lee, Y. J., and Fairbanks, W. S. (2014). Analytical methods for chemical and sensory characterization of scent-markings in large wild mammals: a review. *Sensors (Basel)* 14, 4428–4465. doi: 10.3390/s140304428

Spehr, M., Hatt, H., and Wetzel, C. H. (2002). Arachidonic acid plays a role in rat vomeronasal signal transduction. *J. Neurosci.* 22, 8429–8437.

Spehr, M., and Leinders-Zufall, T. (2005). One neuron-multiple receptors: increased complexity in olfactory coding? *Sci. STKE* 2005:pe25. doi: 10.1126/stke.2852005pe25

Sporkert, F., and Pragst, F. (2000). Use of headspace solid-phase microextraction (HS-SPME) in hair analysis for organic compounds. *Forensic Sci. Int.* 107, 129–148. doi: 10.1016/S0379-0738(99)00158-9

Staples, L. G., McGregor, I. S., Apfelbach, R., and Hunt, G. E. (2008). Cat odor, but not trimethylthiazoline (fox odor), activates accessory olfactory and defense-related brain regions in rats. *Neuroscience* 151, 937–947. doi: 10.1016/j.neuroscience.2007.11.039

Storan, M. J., and Key, B. (2006). Septal organ of Grüneberg is part of the olfactory system. *J. Comp. Neurol.* 494, 834–844. doi: 10.1002/cne.20858

Stowers, L., Holy, T. E., Meister, M., Dulac, C., and Koentges, G. (2002). Loss of sex discrimination and male-male aggression in mice deficient for TRP2. *Science* 295, 1493–1500. doi: 10.1126/science.1069259

Suyama, K., and Adachi, S. (1980). Origin of alkyl-substituted pyridines in food flavor: formation of the pyridines from the reaction of alkanals with amino acids. *J. Agric. Food Chem.* 28, 546–549. doi: 10.1021/jf60229a048

Tachibana, T., Fujiwara, N., and Nawa, T. (1990). The ultrastructure of the ganglionated nerve plexus in the nasal vestibular mucosa of the musk shrew (*Suncus murinus*, insectivora). *Arch. Histol. Cytol.* 53, 147–156. doi: 10.1679/aohc.53.147

Takahashi, L. K. (2014). Olfactory systems and neural circuits that modulate predator odor fear. *Front. Behav. Neurosci.* 8:72. doi: 10.3389/fnbeh.2014.00072

Tanida, M., Niijima, A., Shen, J., Nakamura, T., and Nagai, K. (2006). Olfactory stimulation with scent of lavender oil affects autonomic neurotransmission and blood pressure in rats. *Neurosci. Lett.* 398, 155–160. doi: 10.1016/j.neulet.2005.12.076

Thomas, R. M., Urban, J. H., and Peterson, D. A. (2006). Acute exposure to predator odor elicits a robust increase in corticosterone and a decrease in activity without altering proliferation in the adult rat hippocampus. *Exp. Neurol.* 201, 308–315. doi: 10.1016/j.expneurol.2006.04.010

Touhara, K., and Vosshall, L. B. (2009). Sensing odorants and pheromones with chemosensory receptors. *Annu. Rev. Physiol.* 71, 307–332. doi: 10.1146/annurev.physiol.010908.163209

Vernet-Maury, E., Polak, E. H., and Demael, A. (1984). Structure-activity relationship of stress-inducing odorants in the rat. *J. Chem. Ecol.* 10, 1007–1018. doi: 10.1007/BF00987509

Wyatt, T. D. (2014). *Pheromones and Animal Behavior*: *Chemical Signals and Signatures*. New York, NY: Cambridge University Press.

Yu, A.-N., and Zhang, A.-D. (2010). Aroma compounds generated from thermal reaction of l-ascorbic acid with l-cysteine. *Food Chem.* 121, 1060–1065. doi: 10.1016/j.foodchem.2010.01.049

Yu, Y., Boyer, N. P., and Zhang, C. (2014). Three structurally similar odorants trigger distinct signaling pathways in a mouse olfactory neuron. *Neuroscience* 275, 194–210. doi: 10.1016/j.neuroscience.2014.05.063

Yu, Y., and Zhang, C. (2014). Purinergic signaling negatively regulates activity of an olfactory receptor in an odorant-dependent manner. *Neuroscience* 275, 89–101. doi: 10.1016/j.neuroscience.2014.05.055

Zhang, J., Pacifico, R., Cawley, D., Feinstein, P., and Bozza, T. (2013). Ultrasensitive detection of amines by a trace amine-associated receptor. *J. Neurosci.* 33, 3228–3239. doi: 10.1523/JNEUROSCI.4299-12.2013

Zhang, J. X., Soini, H. A., Bruce, K. E., Wiesler, D., Woodley, S. K., Baum, M. J., et al. (2005). Putative chemosignals of the ferret (*Mustela furo*) associated with individual and gender recognition. *Chem. Senses* 30, 727–737. doi: 10.1093/chemse/bji065

Zhao, X., Ho, D., Gao, S., Hong, C., Vatner, D. E., and Vatner, S. F. (2011). Arterial Pressure Monitoring in Mice. *Curr. Protoc. Mouse Biol.* 1, 105–122. doi: 10.1002/9780470942390.mo100149

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 09 September 2015
doi: 10.3389/fnins.2015.00321

Temporary inactivation of the anterior part of the bed nucleus of the stria terminalis blocks alarm pheromone-induced defensive behavior in rats

*Tino Breitfeld[1], Johann E. A. Bruning[1], Hideaki Inagaki[2,3], Yukari Takeuchi[2], Yasushi Kiyokawa[2] and Markus Fendt[1,4]**

[1] Institute for Pharmacology and Toxicology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany, [2] Laboratory of Veterinary Ethology, The University of Tokyo, Tokyo, Japan, [3] Center for Animal Research and Education, Nagoya University, Nagoya, Japan, [4] Center of Behavioral Brain Sciences, Otto-von-Guericke University Magdeburg, Magdeburg, Germany

OPEN ACCESS

Edited by:
Kathleen Dave,
St. Luke's University Health Network, USA

Reviewed by:
Carmen Agustín-Pavón,
Universidad Jaume I, Spain
John Wemmie,
University of Iowa, USA

***Correspondence:**
Markus Fendt,
Institute for Pharmacology and Toxicology, Otto-von-Guericke University Magdeburg, Leipziger Strasse 44, D-39120 Magdeburg, Germany
markus.fendt@med.ovgu.de

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: 24 June 2015
Accepted: 27 August 2015
Published: 09 September 2015

Citation:
Breitfeld T, Bruning JEA, Inagaki H, Takeuchi Y, Kiyokawa Y and Fendt M (2015) Temporary inactivation of the anterior part of the bed nucleus of the stria terminalis blocks alarm pheromone-induced defensive behavior in rats. Front. Neurosci. 9:321. doi: 10.3389/fnins.2015.00321

Rats emit an alarm pheromone in threatening situations. Exposure of rats to this alarm pheromone induces defensive behaviors, such as head out behavior, and increases c-Fos expression in brain areas involved in the mediation of defensive behaviors. One of these brain areas is the anterior bed nucleus of the stria terminalis (aBNST). The goal of the present study was to investigate if pharmacological inactivation of the aBNST by local microinjections of the $GABA_A$ receptor-agonist muscimol modulates alarm pheromone-induced defensive behaviors. We first established the behavioral paradigm of alarm pheromone-induced defensive behaviors in Sprague-Dawley rats in our laboratory. In a second experiment, we inactivated the aBNST, then exposed rats to one of four different odors (neck odor, female urine, alarm pheromone, fox urine) and tested the effects of the aBNST inactivation on the behavior in response to these odors. Our data show that temporary inactivation of the aBNST blocked head out behavior in response to the alarm pheromone. This indicates that the aBNST plays an important role in the mediation of the alarm pheromone-induced defensive behavior in rats.

Keywords: anxiety, alarm pheromone, BNST, fear, muscimol, odor-induced anxiety, rats, risk assessment behavior

Introduction

Pheromones are olfactory signals that are used for intraspecific communication (Karlson and Luscher, 1959). They can transmit different information, e.g., sex, age, and reproduction status, about the releaser (Beny and Kimchi, 2014). Additionally, pheromones have different ecological functions including marking a trail or territory, attracting potential mating partners, inducing aggregation or dispersion of conspecifics or warning conspecifics to potential danger (Hauser et al., 2011). Pheromones with the latter function are called alarm pheromones (Inagaki et al., 2014).

Alarm pheromones have been described in different mammalians including rats (Kiyokawa et al., 2006), mice (Brechbühl et al., 2013), deer (Müller-Schwarze et al., 1984), cattle (Boissy et al., 1998), pigs (Vieuille-Thomas and Signoret, 1992), and humans (Radulescu and Mujica-Parodi, 2013). For the alarm pheromone of rats, it is known that it is emitted from the perianal region (Kiyokawa et al., 2004), consists of at least two active ingredients, 4-methylpentanal and hexanal

(Inagaki et al., 2014), and provokes in conspecifics a wide range of behavioral changes related to anxiety. For example, it aggravates stress-induced hyperthermia (Kikusui et al., 2001), enhances the acoustic startle reflex (Inagaki et al., 2009), and deteriorates male sexual behavior (Kobayashi et al., 2011). Alarm pheromone effects on defensive behavior can be tested in a modified open-field test paradigm where rats have a choice to stay in an open arena or escape into a safe hiding box. In this paradigm, exposure to alarm pheromone increases the time spent in the hiding box and induces typical "head out" behavior from the hiding box while the time spent in the open arena and for grooming is decreased (Kiyokawa et al., 2006).

In parallel with these behavioral analyses, the neural mechanisms underlying the alarm pheromone effects were also analyzed. The vomeronasal system was found to be involved in the detection of the alarm pheromone. Removal of the vomeronasal organ blocked the pheromone effects on stress-induced hyperthermia (Kiyokawa et al., 2007), acoustic startle reflex (Kiyokawa et al., 2013), and defensive behaviors in the modified open-field test (Kiyokawa et al., 2013). Mapping c-Fos expression throughout the brain in response to the alarm pheromone provided insights into the brain regions involved in pheromone effects (Kiyokawa et al., 2005b; Kobayashi et al., 2013, 2015). However, causal relationships between the alarm pheromone effects and any brain regions have not yet been demonstrated.

The bed nucleus of the stria terminalis (BNST) has been known as an important brain structure for the responses mediated by sustained fear or anxiety (Walker and Davis, 1997; Davis and Shi, 1999; Fendt et al., 2003; Takahashi et al., 2005; Poulin et al., 2009; Bota et al., 2012; Crestani et al., 2013). In addition, the BNST is one of the brain regions that compose the vomeronasal system (Brennan and Kendrick, 2006) and receives direct projection from the accessory olfactory bulb (AOB) (von Campenhausen and Mori, 2000), but not from the main olfactory bulb (Igarashi et al., 2012). Therefore, the BNST appears to be an excellent candidate for a brain relay structure connecting the vomeronasal system and alarm pheromone-induced behavioral changes. Indeed, increased c-Fos expression in response to the alarm pheromone or to its active ingredients has been repeatedly observed in the anterior part of the BNST (Kiyokawa et al., 2005b; Kobayashi et al., 2013, 2015; Inagaki et al., 2014).

The aim of the present study was to test the hypothesis that the BNST is involved in the mediation of alarm pheromone-induced behavioral changes. First, we established the modified open-field test paradigm (Kiyokawa et al., 2006) in our laboratory. In a second experiment, we assessed the role of the BNST, especially its anterior part (aBNST), in alarm pheromone-induced defensive behavior by temporally inactivating the aBNST by local injections of muscimol.

Methods and Materials

Animals

All experiments were performed with naive male Sprague-Dawley rats (8–11 weeks at the start of the study). The animals were housed in groups of 4 to 6 in standard laboratory cages (standard conditions: 20–22°C; L 06:00; LD 12:12; humidity 50–65%). Food and water were available *ad libitum*. Some of the rats (15 male cagemates and 5 female littermates) were only used as donor animals for odor samples [alarm pheromone, neck odor, urine (urine from female rats)] but not for behavioral tests. All experiments were performed during the light phase. All experiments were performed according to international guidelines for ethical contact in the care and use of animals (2010/63/EU) and were approved by the local authorities (Landesverwaltungsamt Sachsen-Anhalt, Az. 42502-2-1238 UniMD).

Preparation of Odor Samples

Alarm Pheromone

We prepared a water solution containing alarm pheromone according to an established method that has been previously described in detail (Kiyokawa et al., 2004, 2005a). After anesthetizing the donor animal with pentobarbital sodium (50 mg/kg; i.p.), the anal region was cleaned and two intradermal needles (27G) were placed at the edge of both sides of the anal canal. The rat was put into an acrylic box (20 × 20 × 10 cm), without touching the walls. This box was previously washed with cleanser (7X, MP Biomedicals, Santa Ana, CA, USA) and the walls and the ceiling were sprayed with approximately 5 ml of purified water. Then, the box was closed and the needles were connected with a pulse generator (Model 2100, A-M Systems, Sequim, USA).

Afterwards, 15 electrical stimuli (10 mA, 1 s duration, 20 s inter-stimulus intervals) were applied to stimulate the perianal region of the donor rat. Subsequently, we waited for one more minute in order to let the released alarm pheromone dissolve in water droplets. The donor rat was then removed and the water droplets were collected and stored in a refrigerator until use (1–2 h later).

Water Sample

Purified water was prepared before the experiment and used as a control water sample.

Fox Urine

We used commercially available fox urine (Main Odor Solutions, Maine, USA).

Neck Odor

For gaining the neck odor, which was used as a neutral odor stimulus, we used the same procedure as for gaining alarm pheromone. However, the two intradermal needles were placed in the neck.

Female Rat Urine

Female rats were placed in a metabolic cage (Tecniplast, Hohenpeißenberg, Germany) for 30 min and the urine delivered by these animals was collected. We collected several times from each animal and put all urine samples together, i.e., we had a mixture of urine from all different phases of the estrous cycles in the end.

Fox urine was used as an example odor from another species that is able to induce defensive behavior (Funk and Amir, 2000;

Fendt, 2006; Wernecke et al., 2015). Neck odor and female urine were used as additional odors originating from the same species. Both odors should be neutral with respect to defensive behavior (Kiyokawa et al., 2004, 2005b). Female urine can also induces appetitive behaviors, however, this was not expected in the present study since sexually naive male rats and not freshly collected female urine from different estrous phases were used (cf. Lydell and Doty, 1972).

Surgery

The animals were anesthetized with an isoflurane/oxygen mixture (5% isoflurane for induction, then 2.0–2.5%) and fixed into a stereotaxic apparatus. The skull was exposed and stainless steel guide cannulas (custom-made; diameter: 0.7 mm, length: 8.0 mm) were bilaterally implanted aiming at the aBNST: 0.1 mm caudal, ± 3.9 mm lateral, and 6.8 mm ventral to bregma at a 20° angle to avoid penetration of the ventricles. Cannulas were fixed with dental cement and anchoring screws. After the surgery, there was a recovery period of 5–8 days.

Microinjections and Drugs

For microinjections (Experiment 2), injection cannulas connected via tubes to two microliter syringes (10 µl, Hamilton, Switzerland) were used. Injection speed and volume were controlled by a microinjection pump (CMA 100, Schmidlein Labor + Service AG, Neuheim, CH). For the injection, the injection cannulas were put into the implanted guide cannulas and 0.3 µl of the saline or muscimol (0.15 nmol) solution was injected over 30 s. The injection cannulas remained one more minute in the brain in order to allow better diffusion. After the injections, the animal was put back into its home cage for about 15 min before it was submitted to the behavioral experiment.

Local microinjections of the $GABA_A$ receptor agonist muscimol are a widely used method to induce a temporary inactivation of a brain area without affecting fibers of passage (Moser and Moser, 1998; Wilensky et al., 1999; Fendt et al., 2003). Such injections effectively block neural activity as shown by electrophysiological recordings (Krupa et al., 1999; Edeline et al., 2002; van Duuren et al., 2007; Larson et al., 2013).

Apparatus and Behavioral Procedure

We used the modified open-field test which was developed to measure defensive behavior in response to alarm pheromone (Kiyokawa et al., 2006). All behavioral experiments were conducted in a rectangular arena ($70 \times 47 \times 50\,cm^3$). In one of the four edges of the arena there was a removable small hiding box ($24.5 \times 17.5 \times 12.5\,cm^3$) with an entrance hole (diameter 10 cm). The arena was located in a dimly lit room (center of the open field: ca 68 lx; background noise: 47 dB SPL).

On the first 5 days, rats were handled daily and then acclimatized to the arena (10 min). Notably, the hiding box was not placed into the arena during these acclimation sessions, and was put in the home cages for 24 h on the last day of the acclimation days.

During the experimental sessions, the animals were put into the arena which only contained a small glass vial (4.0 cm diameter; with 1 ml of the odor sample) in one corner. The rats were allowed to explore the arena with the odor for 5 min (acclimation period). Then, for the next 10 min (test period), the hiding box was put into the corner diagonally opposite of the odor sample. After the test, the tested animal was transferred into a separate cage to not transfer any odors to the yet non-tested cage mates. The arena was cleaned thoroughly with hot water and was exhausted with fresh air for about 5 min. Only after all animals from a cage were tested, the rats were again put together in the original home cage.

In Experiment 1, 13 rats were tested once with the alarm pheromone and once with a water sample (purified water). The two tests were performed on consecutive days in a balanced order.

In Experiment 2, saline ($n = 10$), or muscimol ($n = 9$) was injected into the aBNST. Then, the animals were exposed to the four odors (neck odor, female urine, alarm pheromone and fox urine). Each animal was tested with all odor samples in a balanced order on four consecutive days (Latin square design), with injections of saline or muscimol before each test.

The behavior of the animals was videotaped by a camera (Panasonic WV-CL930) fixed 30 cm above the box. For tracking the animals and further analysis of the behavior, a video tracking software was used (EthoVision XL, Noldus Information Technology, Version 8, Wageningen, NL). Head out behavior, stretched attend behavior and grooming behavior were manually scored by two experienced blinded observers (inter-observer reliability: $r^2 = 0.99$, $p < 0.0001$). We used the following definitions (Kiyokawa et al., 2004, 2006): "Head out" is if the rat is in the hiding box and pokes the head or head and shoulders out of the entrance hole with their hind paws remaining inside the hiding box. "Conceal" is defined as the rat being entirely inside the hiding box. We defined the zone "near the stimulus" as an area of $10\,cm^2$ in the edge of the stimuli. "Outside" is defined as time the rats spent in the open field.

Histology

In Experiment 2, the brains of the rats were removed after the experiments and fixed with 4%-formaldehyde-10%-sucrose solution. On the following 2 days, sucrose concentration was increased daily by 10%. Then, 60 µm slices were cut with a cryostat (Leica CM 3050) at −22°C and Nissl-stained (1% cresylviolet). Lastly, the localization of the injection sites and brain integrity were checked with a microscope (Leica MZ 125). The injection sites were put into schematic drawings adapted from Paxinos and Watson (2014).

Descriptive and Analytical Statistics

All data are expressed as means ± SEM. For statistical analysis, data were first checked for Gaussian distribution (D'Agostino and Pearson omnibus normality test). Non-Gaussian distributed data were either analyzed with non-parametric tests (Wilcoxon matched-pairs signed rank test, Mann-Whitney test). Parametric statistical tests were used if log-transformation led to Gaussian distribution. Normally-distributed data were then analyzed by *t*-test or analysis of variance (ANOVA). If appropriate, a within-subject design (repeated measure) was used. The significance level was set at $p < 0.05$ for all statistical tests.

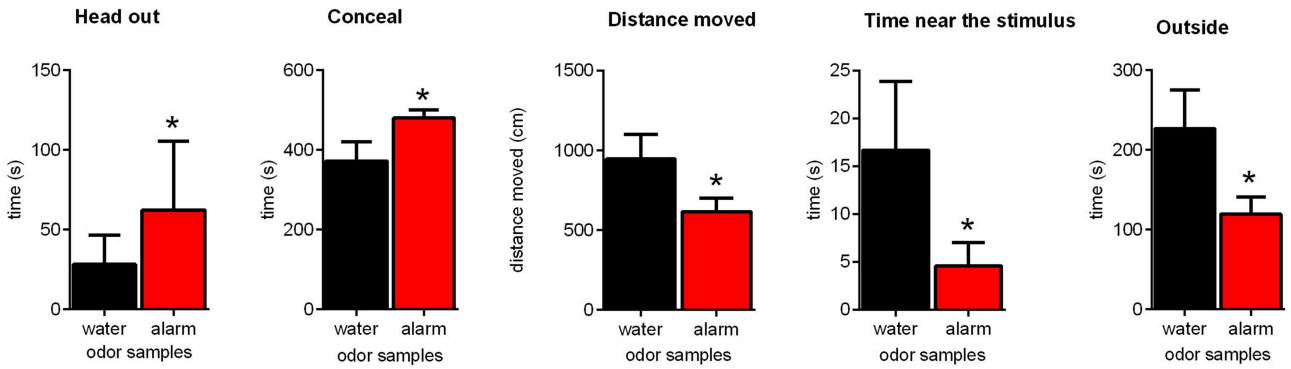


FIGURE 1 | Effects of the alarm pheromone on the behavior of Sprague-Dawley rats. Rats were exposed to either the alarm pheromone (alarm) or water. Data are expressed as means ± SEMs. *$p < 0.05$, paired t-tests.

Results

Experiment 1: Establishment of the Modified Open-field Test with Sprague-dawley Rats

Aim of this experiment was to establish the paradigm published by Kiyokawa et al. (2006) in Sprague-Dawley rats and to replicate these findings. Exposing the rats to the alarm pheromone in an arena induced several behavioral changes (**Figure 1**). Alarm pheromone exposure increased the time of head out behavior [paired t-test: $t_{(12)} = 2.53$, $p = 0.03$] and of conceal [$t_{(12)} = 2.44$, $p = 0.03$]. Furthermore, the time spent near the stimulus ($W_{12} = -51.00$, $p = 0.04$), the time spent outside the hiding box [$t_{(12)} = 2.44$, $p = 0.03$] and the distance moved [$t_{(12)} = 2.29$, $p = 0.04$] decreased. Stretched attend behavior and grooming behavior were only seen very occasionally and therefore excluded from further analysis.

Experiment 2: The Role of the aBNST in the Alarm Pheromone Effects

In our second experiment, we injected either saline or muscimol into the aBNST to investigate the role of the aBNST in alarm pheromone-induced defensive behavior. In addition to the alarm pheromone, we also exposed the rats to neck odor, female rat urine and fox urine.

Histological analysis of the injections sites revealed that 19 rats received bilateral injections into the aBNST (saline: $n = 10$; muscimol: $n = 9$), consisting of the anterior, dorsal and lateral divisions of the BNST (see **Figure 2**). Some animals had to be excluded from the analysis because of misplaced injections ($n = 12$) (lateral ventricle, medial preoptic area, caudate putamen, nucleus accumbens, lateral preoptic area, parastrial nucleus, intermediate lateral septal nucleus, medial preoptic nucleus, ventrolateral preoptic nucleus), lesions in the injection area ($n = 5$), or abnormal behavior after muscimol injections (rotation behavior; $n = 6$).

First, we analyzed the distance moved in the acclimation period to check for potential effects of intra-aBNST muscimol injections on locomotor activity (**Figure 3**). This was clearly not the case [ANOVA: $F_{(1, 68)} = 0.08$, $p = 0.78$]. We further

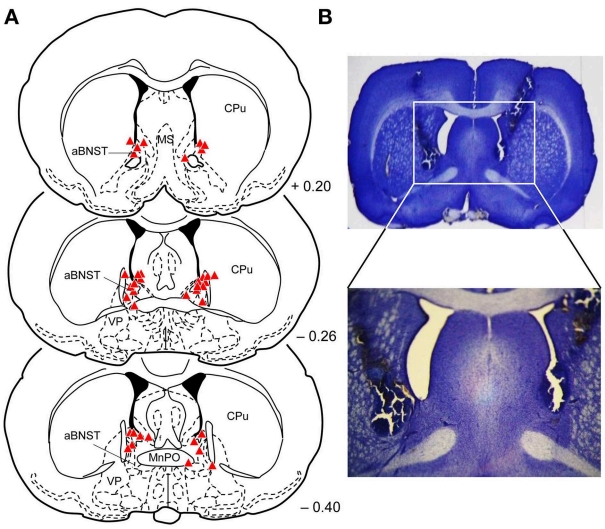


FIGURE 2 | Injection sites into the aBNST. (A) Reconstruction of the different injection sites of saline or muscimol into the aBNST. The coronal sections were taken from the atlas of Paxinos and Watson (2014). Numbers indicate distance from bregma in mm. aBNST, anterior bed nucleus of the stria terminalis; MnPo, median preoptic nucleus; MS, medial septal nucleus; CPu, caudate putamen; LPO, lateral preoptic area; VP, ventral pallidum; f, fornix. **(B)** Photomicrographs with a representative example of aBNST injection sites.

confirmed that locomotor activity in the acclimation phase was not affected by the type of odor [$F_{(3, 68)} = 2.27$, $p = 0.09$] and that there was no interaction between treatment and odor [$F_{(3, 68)} = 0.19$, $p = 0.89$].

During the testing period, head out behavior was the only behavior that was significantly affected by odors [**Figure 4A**; ANOVA: factor odor: $F_{(3, 65)} = 2.87$; $p = 0.04$]. Particularly, the alarm pheromone increased head out behavior (paired t-tests: $t = 3.23$, $p < 0.01$ and $t = 2.54$, $p < 0.03$; comparison with neck odor and female urine, respectively). Notable, despite statistical analysis revealed no significant effect, the time spent in the center of the arena (as percentage of time spent outside) was slightly decreased by fox urine [**Figure 4B**; ANOVA: factor odor:

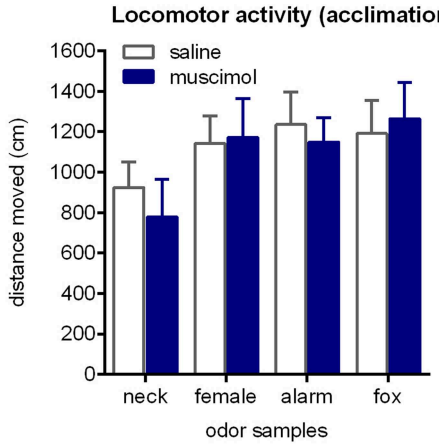


FIGURE 3 | Locomotor activity during the acclimation phase. Rats were exposed to neck odor (neck), female urine (female), alarm pheromone (alarm), or fox urine (fox). Neither intra-aBNST injections of muscimol nor the type of odor samples affected distance traveled. Data are expressed as means ± SEMs.

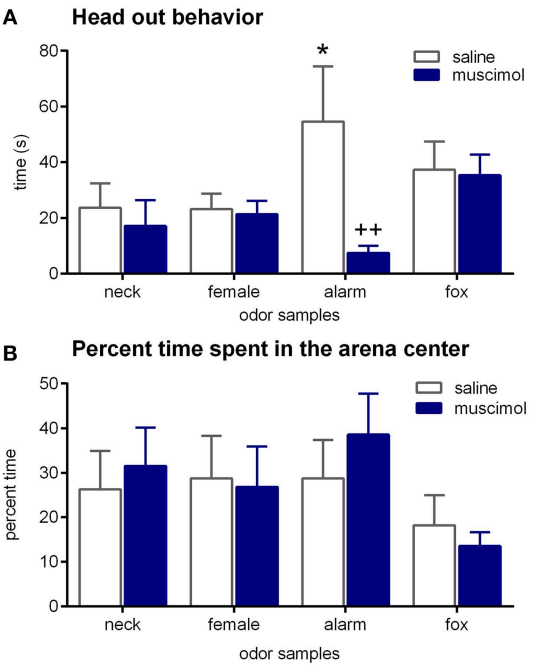


FIGURE 4 | Effects of odors and aBNST inactivation on defensive behavior. Rats were exposed to neck odor (neck), female urine (female), alarm pheromone (alarm), or fox urine (fox). **(A)** Head out behavior, **(B)** time spent in the center, in percentage of the time spent outside. Data are expressed as means ± SEMs. $^{*}p < 0.05$, $^{++}p < 0.01$, comparison with saline/alarm pheromone.

$F_{(3,\ 68)} = 1.46$, $p = 0.23$]. All other behaviors, such as conceal, distance moved, time near the stimulus, and outside, were not significantly affected by odors, especially alarm pheromone, in this experiment (**Table 1**; ANOVAs: factor odor: $Fs < 0.61$, $ps > 0.61$).

Muscimol injections into the aBNST specifically reduced head out behavior in response to the alarm pheromone (**Figure 4A**). An ANOVA using treatment as between-subject factor and odor as within-subject factor revealed a significant main effect of odor [$F_{(3,\ 65)} = 2.87$, $p = 0.04$] and a significant interaction between treatment and odor [$F_{(3,\ 65)} = 3.22$, $p = 0.03$], whereas there was only a tendency for a main effect of treatment [$F_{(1,\ 65)} = 3.35$, $p = 0.07$]. Notably, only head out behavior during exposure to the alarm pheromone was significantly decreased by intra-aBNST muscimol injections (*post-hoc* Dunnett test: $t = 3.41$, $p = 0.005$), whereas it was not affected by muscimol injections during the exposure to other odors ($ts < 1.04$, $ps > 0.76$). It should be mentioned that bilaterally misplaced muscimol injections apparently had no effect on alarm pheromone-induced head out behavior (saline/neck: 17.0 ± 3.1 s; saline/alarm pheromone: 32.9 ± 15.3 s; muscimol/neck: 17.7 ± 3.7 s; muscimol/alarm pheromone: 28.3 ± 14.3 s). However, variability was very high in these animals and group size ($n = 5$; most misplaced injections were unilateral or into the ventricle) too small for statistical analyses.

Discussion

In the present study, we assessed the hypothesis that the aBNST plays a crucial role in alarm pheromone-induced defensive behavior in rats. In Experiment 1, Sprague-Dawley rats showed increased head out behavior, as well as increased conceal behavior and decreased time spent outside, time spent near the stimulus, and the distance moved in response to the alarm pheromone. These results suggest that we successfully established the behavioral paradigm developed by Kiyokawa et al. (2006) in our laboratory with Sprague-Dawley rats. In Experiment 2, we showed that local muscimol injections into the aBNST lead to a blockade of head out behavior in response to the alarm pheromone. Based on these findings, we suggest that the aBNST is an important brain region for alarm pheromone-induced defensive behavior in rats.

In Experiment 1, we provided first evidence that Sprague-Dawley rats emit an alarm pheromone that induces several defensive behavior, as it was previously shown for Wistar rats (Kiyokawa et al., 2006). The present results show that the behavioral changes in response to the alarm pheromone are very similar in Sprague-Dawley and Wistar rats. Therefore, it would be plausible that Sprague-Dawley and Wistar rats share 4-methylpentanal and hexanal as active ingredients of their alarm pheromones. However, there were also some differences. In Sprague-Dawley rats, there was a clear avoidance of the alarm pheromone (**Figure 1**; time near stimulus), whereas Wistar rats did not avoid their alarm pheromone (Kiyokawa et al., 2006). One possible explanation might be that Sprague-Dawley rats are more sensitive to their alarm pheromone than Wistar rats. This hypothesis is supported by the findings from the forced swimming test paradigm. When rats were forced to swim in water, they released an "alarm substance" in water that decreased immobility of subsequent swimming rats (Abel and Bilitzke, 1990). The effects of this alarm substance were greater in Sprague-Dawley rats than in Wistar rats (Abel, 1992).

TABLE 1 | Behaviors observed in the modified open-field test.

Behavior	Treatment	Odor sample				ANOVA results		
		Neck	Female	Alarm	Fox	Odor	Treatment	Interaction
Conceal [s]	Saline	463 ± 64	470 ± 61	484 ± 41	537 ± 14	$p = 0.79$	$p = 0.23$	$p = 0.59$
	Muscimol	559 ± 9	534 ± 16	488 ± 70	526 ± 20			
Distance moved [cm]	Saline	423 ± 164	277 ± 77	424 ± 159	241 ± 41	$p = 0.95$	$p = 0.39$	$p = 0.37$
	Muscimol	172 ± 31	343 ± 91	263 ± 56	314 ± 141			
Time near stimulus [s]	Saline	2.7 ± 1.8	2.4 ± 1.9	3.4 ± 2.3	0.9 ± 0.4	$p = 0.69$	$p = 0.08$	$p = 0.91$
	Muscimol	0.2 ± 0.2	1.2 ± 0.8	1.1 ± 0.6	0.2 ± 0.2			
Outside [s]	Saline	100 ± 60	98 ± 64	49 ± 21	19 ± 4	$p = 0.61$	$p = 0.21$	$p = 0.57$
	Muscimol	13 ± 7	42 ± 23	56 ± 32	23 ± 11			
Fecal boli	Saline	0.8 ± 0.5	1.6 ± 0.6	1.8 ± 0.4	0.7 ± 0.3	$p = 0.63$	$p = 0.41$	$p = 0.51$
	Muscimol	0.8 ± 0.4	1.1 ± 0.8	0.8 ± 0.3	1.0 ± 0.5			

Therefore, it is possible that a difference in sensitivity to the alarm pheromone resulted in contrasting avoidance behavior of Sprague-Dawley and Wistar rats in the modified open-field test. This is supported by studies demonstrating a greater sensitivity of Sprague-Dawley rats to other odors that can induce defensive behavior (e.g., Rosen et al., 2006; Fendt and Endres, 2008). However, it should also be noted that both Sprague-Dawley and Wistar rats were much less sensitive to the alarm substance of the other strain in the forced swimming test (Abel, 1992). Therefore, it has to be addressed by future studies if this is also the case for the alarm pheromone.

Using the modified open-field test, we assessed the role of the aBNST in defensive behavior to the alarm pheromone in our second experiment. However, in contrast to the Experiment 1, head out behavior was the only behavior that was robustly modulated by the alarm pheromone in saline-injected rats (**Figure 4**). Besides the difference in the repeated number of test rats underwent, one significant difference was that rats received local injections into the aBNST shortly before the behavioral tests in the Experiment 2. Although rats were acclimatized for such a procedure several times, they might still be distressed by the manipulations from the injection *per se*. Indeed, time spent outside was strongly decreased in Experiment 2 as compared to Experiment 1 (general means: 60 ± 19 s vs. 227 ± 48 s, respectively), i.e., rats tended to be in the hiding box more and spent only a short time in the open arena. This in turn means that defensive behaviors expressed in the hiding box (such as head out behavior) are more likely to be affected by exposure to alarm pheromone than behaviors expressed outside the hiding box (such as distance moved or time near the stimulus).

In Experiment 2, inactivation of the aBNST clearly decreased head out behavior in response to the alarm pheromone. These results suggest that the aBNST plays an important role in defensive behavior to the alarm pheromone and support previous studies showing an increased c-Fos expression in the aBNST when animals were exposed to alarm pheromone (Kiyokawa et al., 2005b; Kobayashi et al., 2013, 2015; Inagaki et al., 2014). The question is how the aBNST is embedded in the neural circuitry mediating defensive responses to the alarm pheromone. It was previously demonstrated that removal of the vomeronasal organ blocks the autonomic and behavioral effects of the alarm pheromone (Kiyokawa et al., 2007, 2013) indicating that the vomeronasal organ is required to detect the alarm pheromone. Then, after being transmitted to the AOB, alarm pheromone information should be transmitted to the aBNST in order to evoke defensive behaviors. Although the BNST is a part of the vomeronasal system, anatomical analyses revealed that the AOB sends its projection to the posterior part of the BNST (pBNST), rather than the aBNST (von Campenhausen and Mori, 2000). This means that the aBNST most probably receive alarm pheromone information from the vomeronasal organ via the posterior part of the BNST (pBNST). Given that the pBNST sends dense projections to the aBNST (Dong and Swanson, 2004), we hypothesize that the alarm pheromone activates the aBNST via intra-BNST connections from the pBNST. Alternatively, the medial amygdala (MeA) might be an additional candidate linkage site between the AOB and aBNST. It is known that the MeA receives direct projections from the AOB (von Campenhausen and Mori, 2000) and that the MeA sends projections to the BNST (Meurisse et al., 2009). Therefore, this anatomical evidence proposes the MeA as an additional candidate for linking between the AOB and the BNST. From the aBNST, there are several projections to the midbrain and brainstem mediating autonomic or behavioral changes. Autonomic changes are most probably mediated by projections via the paraventricular nucleus of the thalamus (Kobayashi et al., this issue) whereas behavioral changes may be mediated by direct and indirect projections to the medial hypothalamic defense system (Canteras, 2002; Canteras et al., this issue).

In contrast to the alarm pheromone, there was no robust effect of fox urine in this study. Nonetheless, we believe that the aBNST play an important role in defensive behavior to predator odor. A recent study described a significant decrease in freezing behavior in rats exposed to cat urine samples after muscimol injections into the BNST (Xu et al., 2012). In addition, it was demonstrated that the BNST is important for defensive behavior induced by exposure to trimethylthiazoline (TMT), a component of the fox anal secretion (Fendt et al., 2003, 2005).

In Experiment 2, fox urine did not increase head out behavior and only slightly decrease the time spent in the center of the open field (in percentage of time spent outside) in the saline-injected rats (**Figure 4**), which makes it impossible to evaluate the effects of fox urine, as well as the role of the aBNST in defensive behavior to fox urine.

Taken together, we first established the modified open-field test paradigm using Sprague-Dawley rats. Second, we demonstrated that temporary inactivation of the aBNST blocks alarm pheromone-induced head out behavior, indicating that the aBNST is a crucial part of the neural circuitry involved in the defensive behavior to the alarm pheromone. Further analyses focusing on the role of the aBNST will clarify the neural mechanisms of the alarm pheromone effects.

Acknowledgments

The authors thank Kathrin Freke for animal care and Timothy French for language editing.

References

Abel, E. L. (1992). Response to alarm substance in different rat strains. *Physiol. Behav.* 51, 345–347. doi: 10.1016/0031-9384(92)90151-Q

Abel, E. L., and Bilitzke, P. J. (1990). A possible alarm substance in the forced swimming test. *Physiol. Behav.* 48, 233–239. doi: 10.1016/0031-9384(90)90306-O

Beny, Y., and Kimchi, T. (2014). Innate and learned aspects of pheromone-mediated social behaviours. *Anim. Behav.* 97, 301–311. doi: 10.1016/j.anbehav.2014.09.014

Boissy, A., Terlouw, C., and Le Neindre, P. (1998). Presence of cues from stressed conspecifics increases reactivity to aversive events in cattle: evidence for the existence of alarm substances in urine. *Physiol. Behav.* 63, 489–495. doi: 10.1016/S0031-9384(97)00466-6

Bota, M., Sporns, O., and Swanson, L. W. (2012). Neuroinformatics analysis of molecular expression patterns and neuron populations in gray matter regions: the rat BST as a rich exemplar. *Brain Res.* 1450, 174–193. doi: 10.1016/j.brainres.2012.02.034

Brechbühl, J., Moine, F., Klaey, M., Nenniger-Tosato, M., Hurni, N., Sporkert, F., et al. (2013). Mouse alarm pheromone shares structural similarity with predator scents. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4762–4767. doi: 10.1073/pnas.1214249110

Brennan, P. A., and Kendrick, K. M. (2006). Mammalian social odours: attraction and individual recognition. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361, 2061–2078. doi: 10.1098/rstb.2006.1931

Canteras, N. S. (2002). The medial hypothalamic defensive system: hodological organization and functional implications. *Pharmacol. Biochem. Behav.* 71, 481–491. doi: 10.1016/S0091-3057(01)00685-2

Crestani, C. C., Alves, F. H., Gomes, F. V., Resstel, L. B., Correa, F. M., and Herman, J. P. (2013). Mechanisms in the bed nucleus of the stria terminalis involved in control of autonomic and neuroendocrine functions: a review. *Curr. Neuropharmacol.* 11, 141–159. doi: 10.2174/1570159X11311020002

Davis, M., and Shi, C. (1999). The extended amygdala: are the central nucleus of the amygdala and the bed nucleus of the stria terminalis differentially involved in fear versus anxiety? *Ann. N.Y. Acad. Sci.* 877, 281–291. doi: 10.1111/j.1749-6632.1999.tb09273.x

Dong, H. W., and Swanson, L. W. (2004). Projections from bed nuclei of the stria terminalis, posterior division: implications for cerebral hemisphere regulation of defensive and reproductive behaviors. *J. Comp. Neurol.* 471, 396–433. doi: 10.1002/cne.20002

Edeline, J. M., Hars, B., Hennevin, E., and Cotillon, N. (2002). Muscimol diffusion after intracerebral microinjections: a reevaluation based on electrophysiological and autoradiographic quantifications. *Neurobiol. Learn. Mem.* 78, 100–124. doi: 10.1006/nlme.2001.4035

Fendt, M. (2006). Exposure to urine of canids and felids, but not of herbivores, induces defensive behavior in laboratory rats. *J. Chem. Ecol.* 32, 2617–2627. doi: 10.1007/s10886-006-9186-9

Fendt, M., and Endres, T. (2008). 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox odor - just repugnant or really fear-inducing? *Neurosci. Biobehav. Rev.* 32, 1259–1266. doi: 10.1016/j.neubiorev.2008.05.010

Fendt, M., Endres, T., and Apfelbach, R. (2003). Temporary inactivation of the bed nucleus of the stria terminalis but not of the amygdala blocks freezing induced by trimethylthiazoline, a component of fox feces. *J. Neurosci.* 23, 23–28.

Fendt, M., Endres, T., Lowry, C. A., Apfelbach, R., and McGregor, I. S. (2005). TMT-induced autonomic and behavioral changes and the neural basis of its processing. *Neurosci. Biobehav. Rev.* 29, 1145–1156. doi: 10.1016/j.neubiorev.2005.04.018

Funk, D., and Amir, S. (2000). Circadian modulation of fos responses to odor of the red fox, a rodent predator, in the rat olfactory system. *Brain Res.* 866, 262–267. doi: 10.1016/S0006-8993(00)02249-6

Hauser, R., Wiergowski, M., Kaliszan, M., Gos, T., Kernbach-Wighton, G., Studniarek, M., et al. (2011). Olfactory and tissue markers of fear in mammals including humans. *Med. Hypotheses* 77, 1062–1067. doi: 10.1016/j.mehy.2011.09.003

Igarashi, K. M., Ieki, N., An, M., Yamaguchi, Y., Nagayama, S., Kobayakawa, K., et al. (2012). Parallel mitral and tufted cell pathways route distinct odor information to different targets in the olfactory cortex. *J. Neurosci.* 32, 7970–7985. doi: 10.1523/JNEUROSCI.0154-12.2012

Inagaki, H., Kiyokawa, Y., Tamogami, S., Watanabe, H., Takeuchi, Y., and Mori, Y. (2014). Identification of a pheromone that increases anxiety in rats. *Proc. Natl. Acad. Sci. U.S.A.* 111, 18751–18756. doi: 10.1073/pnas.1414710112

Inagaki, H., Nakamura, K., Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2009). The volatility of an alarm pheromone in male rats. *Physiol. Behav.* 96, 749–752. doi: 10.1016/j.physbeh.2008.12.006

Karlson, P., and Luscher, M. (1959). Pheromones': a new term for a class of biologically active substances. *Nature* 183, 55–56. doi: 10.1038/183055a0

Kikusui, T., Takigami, S., Takeuchi, Y., and Mori, Y. (2001). Alarm pheromone enhances stress-induced hyperthermia in rats. *Physiol. Behav.* 72, 45–50. doi: 10.1016/S0031-9384(00)00370-X

Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2004). Alarm pheromones with different functions are released from different regions of the body surface of male rats. *Chem. Senses* 29, 35–40. doi: 10.1093/chemse/bjh004

Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2005a). Alarm pheromone that aggravates stress-induced hyperthermia is soluble in water. *Chem. Senses* 30, 513–519. doi: 10.1093/chemse/bji044

Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2005b). Mapping the neural circuit activated by alarm pheromone perception by c-Fos immunohistochemistry. *Brain Res.* 1043, 145–154. doi: 10.1016/j.brainres.2005.02.061

Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2007). Removal of the vomeronasal organ blocks the stress-induced hyperthermia response to alarm pheromone in male rats. *Chem. Senses* 32, 57–64. doi: 10.1093/chemse/bjl036

Kiyokawa, Y., Kodama, Y., Kubota, T., Takeuchi, Y., and Mori, Y. (2013). Alarm pheromone is detected by the vomeronasal organ in male rats. *Chem. Senses* 38, 661–668. doi: 10.1093/chemse/bjt030

Kiyokawa, Y., Shimozuru, M., Kikusui, T., Takeuchi, Y., and Mori, Y. (2006). Alarm pheromone increases defensive and risk assessment behaviors in male rats. *Physiol. Behav.* 87, 383–387. doi: 10.1016/j.physbeh.2005.11.003

Kobayashi, T., Kiyokawa, Y., Arata, S., Takeuchi, Y., and Mori, Y. (2013). c-Fos expression during the modulation of sexual behavior by an alarm pheromone. *Behav. Brain Res.* 237, 230–237. doi: 10.1016/j.bbr.2012.09.042

Kobayashi, T., Kiyokawa, Y., Takeuchi, Y., and Mori, Y. (2011). Neural pathway for alarm-pheromone induced suppression of male sexual behavior in rats. *Neurosci. Res.* 71, E263. doi: 10.1016/j.neures.2011.07.1147

Kobayashi, T., Kiyokawa, Y., Takeuchi, Y., and Mori, Y. (2015). Neural correlates underlying naloxone-induced amelioration of sexual behavior

deterioration due to an alarm pheromone. *Front. Neurosci.* 9:52. doi: 10.3389/fnins.2015.00052

Krupa, D. J., Ghazanfar, A. A., and Nicolelis, M. A. (1999). Immediate thalamic sensory plasticity depends on corticothalamic feedback. *Proc. Natl. Acad. Sci. U.S.A.* 96, 8200–8205. doi: 10.1073/pnas.96.14.8200

Larson, T. A., Wang, T. W., Gale, S. D., Miller, K. E., Thatra, N. M., Caras, M. L., et al. (2013). Postsynaptic neural activity regulates neuronal addition in the adult avian song control system. *Proc. Natl. Acad. Sci. U.S.A.* 110, 16640–16644. doi: 10.1073/pnas.1310237110

Lydell, K., and Doty, R. L. (1972). Male rat odor preferences for female urine as a function of sexual experience, urine age, and urine source. *Horm. Behav.* 3, 205–212. doi: 10.1016/0018-506X(72)90033-5

Meurisse, M., Chaillou, E., and Lévy, F. (2009). Afferent and efferent connections of the cortical and medial nuclei of the amygdala in sheep. *J. Chem. Neuroanat.* 37, 87–97. doi: 10.1016/j.jchemneu.2008.09.001

Moser, M. B., and Moser, E. I. (1998). Distributed encoding and retrieval of spatial memory in the hippocampus. *J. Neurosci.* 18, 7535–7542.

Müller-Schwarze, D., Altieri, R., and Porter, N. (1984). Alert odor from skin gland in deer. *J. Chem. Ecol.* 10, 1707–1729. doi: 10.1007/BF00987357

Paxinos, G., and Watson, C. (2014). *The Rat Brain in Stereotaxic Coordinates*. San Diego, CA: Academic Press.

Poulin, J. F., Arbour, D., Laforest, S., and Drolet, G. (2009). Neuroanatomical characterization of endogenous opioids in the bed nucleus of the stria terminalis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 33, 1356–1365. doi: 10.1016/j.pnpbp.2009.06.021

Radulescu, A. R., and Mujica-Parodi, L. R. (2013). Human gender differences in the perception of conspecific alarm chemosensory cues. *PLoS ONE* 8:e68485. doi: 10.1371/journal.pone.0068485

Rosen, J. B., West, E. A., and Donley, M. P. (2006). Not all rat strains are equal: differential unconditioned fear responses to the synthetic fox odor 2,4,5-trimethylthiazoline in three outbred rat strains. *Behav. Neurosci.* 20, 290–297. doi: 10.1037/0735-7044.120.2.290

Takahashi, L. K., Nakashima, B. R., Hong, H., and Watanabe, K. (2005). The smell of danger: a behavioral and neural analysis of predator odor-induced fear. *Neurosci. Biobehav. Rev.* 29, 1157–1167. doi: 10.1016/j.neubiorev.2005.04.008

van Duuren, E., van der Plasse, G., van der Blom, R., Joosten, R. N. J. M., Mulder, A. B., Pennartz, C. M., et al. (2007). Pharmacological manipulation of neuronal ensemble activity by reverse microdialysis in freely moving rats: a comparative study of the effects of tetrodotoxin, lidocaine, and muscimol. *J. Pharmacol. Exp. Ther.* 323, 61–69. doi: 10.1124/jpet.107.124784

Vieuille-Thomas, C., and Signoret, J. P. (1992). Pheromonal transmission of an aversive experience in domestic pig. *J. Chem. Ecol.* 18, 1551–1557. doi: 10.1007/BF00993228

von Campenhausen, H., and Mori, K. (2000). Convergence of segregated pheromonal pathways from the accessory olfactory bulb to the cortex in the mouse. *Eur. J. Neurosci.* 12, 33–46. doi: 10.1046/j.1460-9568.2000.00879.x

Walker, D. L., and Davis, M. (1997). Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. *J. Neurosci.* 17, 9375–9383.

Wernecke, K. E., Vincenz, D., Storsberg, S., D'Hanis, W., Goldschmidt, J., and Fendt, M. (2015). Fox urine exposure induces avoidance behavior in rats and activates the amygdalar olfactory cortex. *Behav. Brain Res.* 279, 76–81. doi: 10.1016/j.bbr.2014.11.020

Wilensky, A. E., Schafe, G. E., and Ledoux, J. E. (1999). Functional inactivation of the amygdala before but not after auditory fear conditioning prevents memory formation. *J. Neurosci.* 19, RC48.

Xu, H. Y., Liu, Y. J., Xu, M. Y., Zhang, Y. H., Zhang, J. X., and Wu, Y. J. (2012). Inactivation of the bed nucleus of the stria terminalis suppresses the innate fear responses of rats induced by the odor of cat urine. *Neuroscience* 221, 21–27. doi: 10.1016/j.neuroscience.2012.06.056

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 21 July 2015
doi: 10.3389/fnins.2015.00239

ASIC1A in the bed nucleus of the stria terminalis mediates TMT-evoked freezing

Rebecca J. Taugher[1,2], *Ali Ghobbeh*[1,2], *Levi P. Sowers*[2,3], *Rong Fan*[1,2] and *John A. Wemmie*[1,2,3]*

[1] Department of Psychiatry, University of Iowa, Iowa City, IA, USA, [2] Department of Veterans Affairs Medical Center, Iowa City, IA, USA, [3] Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA, USA

Mice display an unconditioned freezing response to TMT, a predator odor isolated from fox feces. Here we found that in addition to freezing, TMT caused mice to decrease breathing rate, perhaps because of the aversive smell. Consistent with this possibility, olfactory bulb lesions attenuated this effect of TMT, as well as freezing. Interestingly, butyric acid, another foul odor, also caused mice to reduce breathing rate. However, unlike TMT, butyric acid did not induce freezing. Thus, although these aversive odors may affect breathing, the unpleasant smell and suppression of breathing by themselves are insufficient to cause freezing. Because the acid-sensing ion channel-1A (ASIC1A) has been previously implicated in TMT-evoked freezing, we tested whether *Asic1a* disruption also altered breathing. We found that TMT reduced breathing rate in both *Asic1a*$^{+/+}$ and *Asic1a*$^{-/-}$ mice, suggesting that ASIC1A is not required for TMT to inhibit breathing and that the absence of TMT-evoked freezing in the *Asic1a*$^{-/-}$ mice is not due to an inability to detect TMT. These observations further indicate that ASIC1A must affect TMT freezing in another way. Because the bed nucleus of the stria terminalis (BNST) has been critically implicated in TMT-evoked freezing and robustly expresses ASIC1A, we tested whether ASIC1A in the BNST plays a role in TMT-evoked freezing. We disrupted ASIC1A in the BNST of *Asic1a*$^{loxP/loxP}$ mice by delivering Cre recombinase to the BNST with an adeno-associated virus (AAV) vector. We found that disrupting ASIC1A in the BNST reduced TMT-evoked freezing relative to control mice in which a virus expressing eGFP was injected. To test whether ASIC1A in the BNST was sufficient to increase TMT-evoked freezing, we used another AAV vector to express ASIC1A in the BNST of *Asic1a*$^{-/-}$ mice. We found region-restricted expression of ASIC1A in the BNST increased TMT-elicited freezing. Together, these data suggest that the BNST is a key site of ASIC1A action in TMT-evoked freezing.

Keywords: TMT, bed nucleus of the stria terminalis, ASIC1A, respiratory rate, predator odor

OPEN ACCESS

Edited by:
Yasushi Kiyokawa, The University of Tokyo, Japan

Reviewed by:
Jeffrey B. Rosen, University of Delaware, USA
Markus Fendt, Otto-von-Guericke University Magdeburg, Germany

***Correspondence:**
John A. Wemmie, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, 1312 PBDB, 200 Hawkins Dr., Iowa City, IA 52242, USA
john-wemmie@uiowa.edu

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *21 May 2015*
Accepted: *23 June 2015*
Published: *21 July 2015*

Citation:
Taugher RJ, Ghobbeh A, Sowers LP, Fan R and Wemmie JA (2015) ASIC1A in the bed nucleus of the stria terminalis mediates TMT-evoked freezing. Front. Neurosci. 9:239. doi: 10.3389/fnins.2015.00239

Introduction

Exposure to odors from predators, such as foxes and cats, can elicit unconditioned freezing and avoidance responses in rodents (Takahashi et al., 2005). Because of the lack of a clear conditioning event, these predator odor-evoked responses are thought to be analogous to specific phobias in humans (Rosen et al., 2008).

One widely used odor is trimethylthiazoline (TMT), a synthetic compound originally isolated from fox feces (Fendt et al., 2005). TMT evokes freezing and avoidance responses in rodents (Vernet-Maury et al., 1984; Fendt et al., 2003). Because this freezing is seen even in naïve animals raised in the laboratory setting, TMT is thought to be an unconditioned fear-evoking stimulus. Though the neurocircuitry of TMT-evoked freezing has not been completely delineated, growing evidence suggests that it relies on different circuitry than conditioned fear behaviors. Lesion studies indicate a critical role for the basolateral, but not the medial amygdala in fear conditioning (Ledoux et al., 1990; Nader et al., 2001); conversely, lesion studies point to the medial amygdala playing a critical role in TMT-evoked freezing and suggest that the basolateral amygdala plays a smaller, modulatory role (Wallace and Rosen, 2001; Fendt et al., 2003; Muller and Fendt, 2006). The bed nucleus of the stria terminalis (BNST) has also been firmly implicated in TMT-evoked freezing. Exposure to TMT induces robust c-Fos activity in the BNST (Day et al., 2004; Asok et al., 2013), and inactivation of the BNST blocks TMT-evoked freezing (Fendt et al., 2003).

Our recent studies suggest that the BNST, in addition to its role in TMT-evoked freezing, plays a key role in unconditioned freezing and avoidance responses to carbon dioxide (CO_2) (Taugher et al., 2014). In addition, we found that a receptor in the BNST that is activated by extracellular acidosis, the acid-sensing ion channel-1a (ASIC1A), was both necessary and sufficient for CO_2-evoked freezing (Taugher et al., 2014). Curiously, this same receptor has also been implicated in TMT-evoked freezing: global disruption of *Asic1a* attenuated freezing to TMT as well as freezing to CO_2 (Coryell et al., 2007, 2008; Ziemann et al., 2009; Price et al., 2014; Taugher et al., 2014). Thus, we hypothesized that the BNST might be a critical site of ASIC1A action in TMT-evoked freezing.

Materials and Methods

Mice

Asic1a$^{-/-}$ and *Asic1a*$^{loxP/+}$ mice were generated as previously described (Wemmie et al., 2002; Kreple et al., 2014; Taugher et al., 2014). *Asic1a*$^{-/-}$ mice were maintained on a C57BL/6J background and *Asic1a*$^{loxP/loxP}$ mice were maintained on a C57BL/6T background. Male and female mice 12–18 weeks of age were used in the behavioral experiments and experimental groups were sex-matched and age-matched. All mice were kept on a 12 h light-dark cycle, with all experiments being performed during the light cycle. Mice were fed standard chow and water *ad libitum*. Animal care met the standards set by the National Institutes of Health and all experiments were approved by the University of Iowa Animal Care and Use Committee.

Olfactory Bulb Lesions

Mice were anesthetized with a mixture of ketamine and xylazine. A burr hole was drilled above each olfactory bulb, and a glass pipette attached to vacuum suction was inserted into the burr hole to aspirate the olfactory bulb. Mice were sutured, and allowed to recover for at least 5 days before undergoing further experimentation. Sham surgeries, were performed as described above, but without insertion of the glass pipette. Ablation of the olfactory bulb was visually confirmed post-mortem.

TMT-Evoked Freezing

Mice were placed in a clear, airtight plexiglas behavior chamber (20 × 21 × 17 cm, width × depth × height) with a beaker containing tissue paper carrying 6 μL undiluted trimethylthiazoline (TMT) (Contech enterprises) or 21 μL undiluted butyric acid (Sigma) applied with a micropipette. TMT volume sufficient to cause robust freezing in wild-type mice was empirically determined and butyric acid dose was adjusted for its difference in volatility as previously described (Hotsenpiller and Williams, 1997). Mice were videotaped, and freezing over a 10 min period was scored by a trained observer blinded to experimental conditions. Freezing was defined as an absence of motion other than respiration.

Plethysmography

The rate of breathing was measured using standard plethysmographic techniques similar to those that have been previously described (Taugher et al., 2014). Mice were placed in a plethysmography chamber with continuous gas flow (700 ml/min). The protocol consisted of >10 min of baseline in compressed air, followed by an exposure to 3 μL TMT or 10.5 μL butyric acid. Volumes of TMT and butyric acid were proportionally reduced due to the smaller volume of the plethsmography chamber. All data were acquired using custom-written Matlab software. All data segments ≥5 s in duration that did not contain sighs, sniffing, or movement artifacts were selected for analysis. At least 30 s of data were analyzed for each condition. Breath volume and thus minute ventilation (rate × volume) could not be accurately measured because of the inability to adequately clean the temperature and humidity sensors after predator odor exposure, thus only respiratory rate was quantified. Data are reported as the percentage change in respiratory rate, relative to respiratory rate in compressed air.

Viral Injections

Adeno-associated viruses (AAV) were injected into the BNST and targeting was confirmed as previously described (Taugher et al., 2014). Briefly, AAV2/1 viruses expressing ASIC1A, Cre recombinase, or eGFP under control of a CMV (University of Iowa Gene Transfer Vector Core) were injected into the BNST bilaterally. AAV-ASIC1A and AAV-Cre were coinjected with AAV-eGFP to aid with localization. Coordinates (relative to bregma) were: anteroposterior +0.4 mm, lateral ± 1.0 mm, ventral 4.3 mm from the pial surface. Behavioral testing was done at least 3 weeks after virus injection. After behavioral testing, injection sites were located. Hits were defined as having bilaterally transduced the anterior BNST both dorsal and ventral to the anterior commissure. Mice in which the injections did not hit the BNST bilaterally were excluded including five *Asic1a*$^{loxP/loxP}$ mice and three *Asic1a*$^{-/-}$ mice.

ASIC1A Immunohistochemistry

ASIC1A immunohistochemistry was performed as previously described (Taugher et al., 2014). Briefly, 18 μm cryosections

were postfixed in 4% paraformaldehyde and 4% sucrose in PBS, then permeablized with 0.25% Triton X-100, then blocked with 5% goat serum, then immunostained with primary antibody [polyclonal anti-ASIC1 antiserum (MTY19) diluted 1:500 (Wemmie et al., 2003)], followed by immunostaining with secondary antibody [goat-anti rabbit IgG coupled to Alexa-Fluor-568 diluted 1:500 (Invitrogen)]. Sections were imaged with a confocal microscope (Zeiss 710).

Statistical Analysis

All values are reported as mean ± SEM. A Student's *t*-test was used to assess significance between two groups. Welch's correction was applied when comparing two groups of significantly different variance. An ANOVA was used to assess significance between more than two groups. Planned contrast testing (Student's *t*-test) was performed within the context of the ANOVA, to test for differences between two groups. $p < 0.05$ was considered significant for all tests. All statistical analyses were performed using Prism software (GraphPad).

Results

TMT Reduces Breathing and Evokes Freezing

While observing mice during TMT exposure, we noticed that they appeared to breathe less frequently than during exposure to compressed air alone. Thus, we used whole-body plethysmography to quantify the effects of TMT on breathing rate, and indeed TMT exposure dramatically reduced rate of breathing (**Figures 1A,B**). In air, mice continuously took quick, rhythmic breaths, whereas in TMT, mice exhaled more slowly and there were distinct pauses between breaths (**Figure 1A**). Volume measurements were not possible with our plethysmographic set-up. Thus, it is not clear whether the reduction in breathing rate was accompanied by volume changes, although for each mouse it appeared that the amplitude of individual breaths did not change very much. This suggests an overall decrease in minute ventilation (rate × volume). Because a recent study in rats found that olfactory bulb lesions eliminated TMT-evoked freezing (Ayers et al., 2013), we wondered whether TMT effects on breathing rate might also depend on the olfactory bulb. Therefore, we lesioned the olfactory bulb bilaterally and tested breathing rate. We found that olfactory bulb lesions significantly attenuated effects of TMT on breathing rate (**Figure 1B**), as well as freezing (**Figure 1C**). Together, these observations are consistent with previous studies suggesting that effects of TMT depend on olfaction and extend those observations to another effect of TMT, reduction in breathing rate.

Butyric Acid Reduces Breathing Rate but does not Evoke Freezing

We next sought to determine if the reduction in breathing rate observed in response to TMT might also be caused by other foul odors. Therefore, we tested the effects of butyric acid, another pungent odor, which, like TMT, is aversive and induces avoidance behaviors in rats and mice (Endres and Fendt, 2009). Similar to TMT, butyric acid dramatically reduced breathing rate (**Figure 2A**), suggesting the effect on breathing rate generalizes to at least two aversive odors. However, in contrast to TMT, butyric acid failed to induce freezing (**Figure 2B**), which is consistent with previous assertions that butyric acid differs from TMT in this regard (Coryell et al., 2007; Endres and Fendt, 2009). Thus, although both aversive odors caused mice to slow their breathing rate by a similar degree, the reduction in breathing rate by itself is probably not sufficient to cause the freezing behavior seen with TMT.

In previous studies, we found that acid sensing ion channels (ASICs) contribute to TMT-evoked freezing (Coryell et al., 2007, 2008; Price et al., 2014), and studies herein replicated the effect; in response to TMT, *Asic1a*$^{-/-}$ mice froze significantly less than *Asic1a*$^{+/+}$ controls (**Figure 2B**). Therefore, we wondered whether the breathing response to TMT might also be affected by ASIC1A. We repeated whole-body plethysmography with the *Asic1a*$^{-/-}$ mice and found that they were equally sensitive to the breathing rate reduction induced by TMT as *Asic1a*$^{+/+}$ controls. These results suggest that the *Asic1a*$^{-/-}$ mice were able to detect

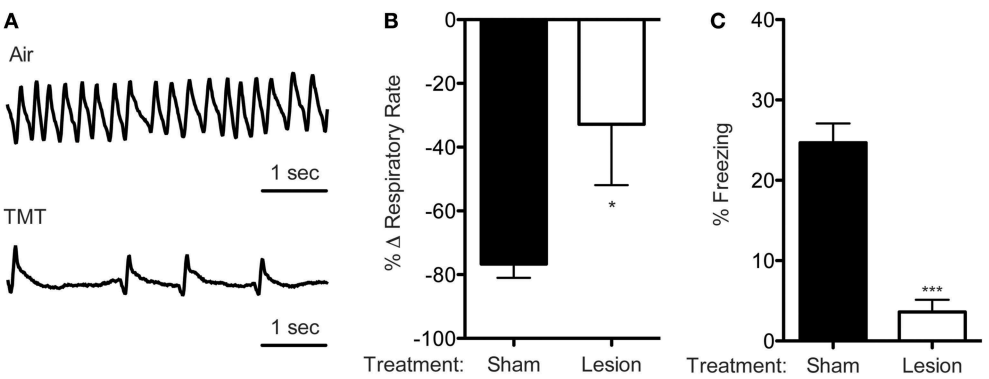


FIGURE 1 | TMT reduces breathing and evokes freezing. (A) Representative plethysmography traces from a sham mouse in air and TMT. **(B)** TMT exposure dramatically reduces respiratory rate. This reduction in breathing rate is attenuated by olfactory bulb lesions [One-tailed *t*-test, $t_{(4)} = 2.239$, $^{*}p = 0.0443$, $n = 5$ per group]. **(C)** TMT also evoked freezing which was largely abolished by olfactory bulb lesions [$t_{(9)} = 7.069$, $^{***}p < 0.0001$, sham $n = 6$, lesion $n = 5$].

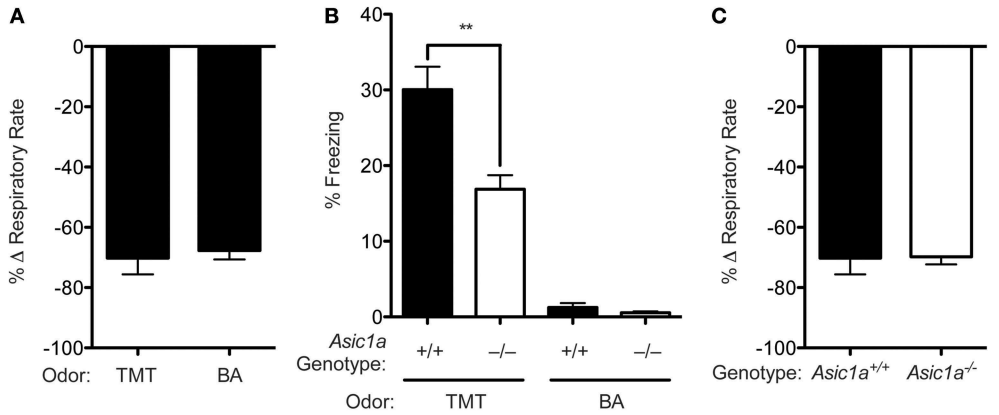


FIGURE 2 | Butyric acid reduces breathing rate but does not evoke freezing. (A) Exposure to either TMT or butyric acid elicits a similar reduction in breathing rate in wild-type mice [$t_{(5)} = 0.2768$, $p = 0.7930$, TMT $n = 5$, butyric acid $n = 2$]. **(B)** Exposure to TMT, but not butyric acid (BA) elicits robust freezing behavior and *Asic1a* disruption attenuated TMT-evoked freezing. A Two-Way ANOVA revealed an effect of genotype [$F_{(1,\ 34)} = 13.39$, $p = 0.0009$, from left to right $n = 10$, 10, 9, 9, respectively], an effect of odor [$F_{(1,\ 34)} = 141.7$, $p < 0.0001$] and a genotype by odor interaction [$F_{(1,\ 34)} = 10.78$, $p = 0.0024$]. Planned contrast testing demonstrated that $Asic1a^{-/-}$ mice had a reduction in freezing to TMT (**$p = 0.0016$), but not butyric acid (BA) ($p = 0.2673$). **(C)** *Asic1a* disruption did not alter the effect of TMT on breathing rate [$t_{(8)} = 0.08014$, $p = 0.9381$, $n = 5$ per group].

TMT normally. In addition, because TMT-induced breathing effects depended on olfaction, these results further suggest that the ability to smell TMT was intact in the $Asic1a^{-/-}$ mice (Coryell et al., 2007) (**Figure 2C**). Finally, these results suggest that the freezing deficit in the $Asic1a^{-/-}$ mice must be due to some other mechanism besides a deficit in olfaction.

ASIC1A in the BNST is Necessary for Normal TMT-Evoked Freezing

ASIC1A is relatively abundant in many of the fear circuit structures that have been previously implicated in TMT-evoked freezing, including the BNST, PAG, and medial and basolateral amygdala (Wemmie et al., 2003; Coryell et al., 2007; Price et al., 2014). Because the BNST has been critically implicated in TMT-evoked freezing (Fendt et al., 2003), and because the BNST is a key site of ASIC1A action in freezing to another unconditioned stimulus, CO_2 (Taugher et al., 2014), we hypothesized that the BNST might be a key site of ASIC1A action in TMT-evoked freezing. To test this hypothesis, we used AAV-Cre to selectively disrupt ASIC1A in the BNST of $Asic1a^{loxP/loxP}$ mice. Consistent with our previous observations (Taugher et al., 2014), injection of AAV-Cre into the BNST of $Asic1a^{loxP/loxP}$ mice effectively reduced ASIC1A expression the BNST, including both dorsal and ventral portions of the BNST and potentially in closely adjacent structures (**Figures 3A,B**). Next, we tested the behavioral effects of this manipulation on TMT-evoked freezing behavior. Disruption of ASIC1A in the BNST significantly reduced TMT-evoked freezing (**Figure 3C**), suggesting that the BNST is a key site of ASIC1A action in TMT-evoked freezing.

ASIC1A in the BNST is Sufficient to Rescue TMT-Evoked Freezing Deficits in Asic1a$^{-/-}$ Mice

Because the previous data suggest that ASIC1A in the BNST is required for intact TMT-evoked freezing, we sought to test whether it might also be sufficient to increase TMT-evoked freezing in $Asic1a^{-/-}$ mice. To test this, we used an AAV to specifically express ASIC1A in the BNST of $Asic1a^{-/-}$ mice. Consistent with our previous observations (Taugher et al., 2014), we found that AAV-ASIC1A produced ASIC1A expression in both dorsal and ventral portions of the BNST (**Figure 4A**). Moreover, AAV-ASIC injected $Asic1a^{-/-}$ mice froze more to TMT than their AAV-eGFP injected counterparts (**Figure 4B**), suggesting that region-restricted expression of ASIC1A in the BNST is sufficient to augment TMT-evoked freezing.

Discussion

During these studies, we observed that TMT can exert a striking reduction in breathing rate. TMT exposure caused mice to reduce breathing rate by up to 70% compared to compressed air. This likely translated to a reduction in minute ventilation, although mice remained awake and active for the duration of the TMT exposure, suggesting that ventilation was sufficient to maintain consciousness. In addition, if minute ventilation fell sufficiently, then one might expect a rise in systemic CO_2 concentration and, in turn, an increase in breathing rate and/or volume through the hypercapnic ventilatory response (Hodges et al., 2008). However, for the duration of the TMT exposure, up to 10 min, we never observed a compensatory increase in breathing rate. However, there were periods of locomotor activity that interfered with the plethysmographic traces, during which time mice may have increased breathing rate and/or volume to compensate for the recorded bradypnea.

Why might mice slow their breathing in response to odors such as TMT and butyric acid? Interestingly, a previous study investigating anxiety-associated respiratory dysfunction in a mouse model of Rett syndrome reported respiratory irregularities during stressful stimuli which were attenuated by antalarmin,

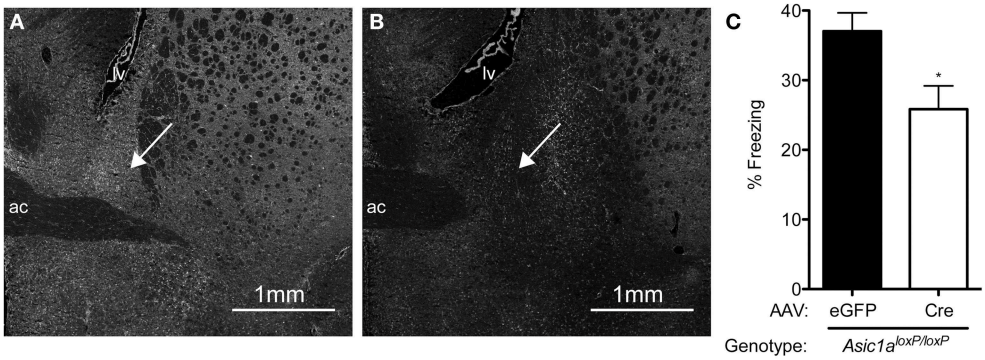


FIGURE 3 | ASIC1A in the BNST is necessary for normal TMT-evoked freezing. (A,B) Examples of ASIC1A immunohistochemistry in the BNST of *Asic1a*$^{loxP/loxP}$ mice injected with AAV-eGFP or AAV-Cre, showing a decrease in ASIC1A expression in the BNST (arrow) in the AAV-Cre injected mouse. **(C)** Cre-mediated disruption of *Asic1a* in the BNST reduced TMT-evoked freezing [$t_{(30)} = 2.643$, $^*p = 0.0129$, AAV-eGFP $n = 19$, AAV-Cre $n = 13$].

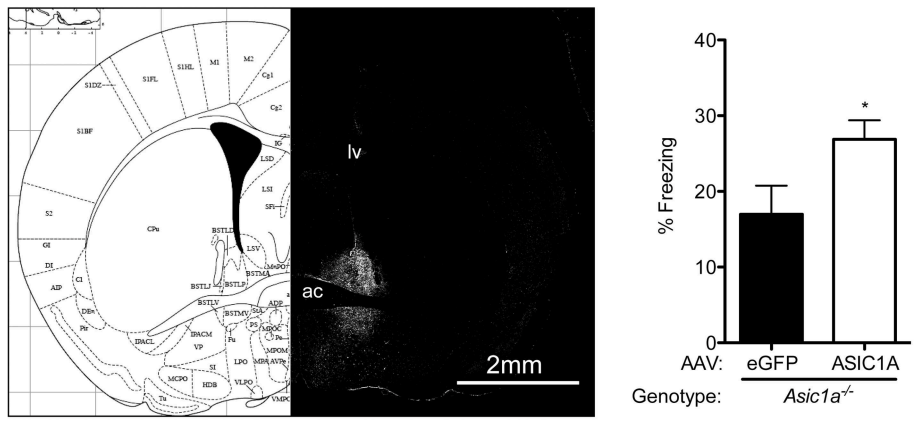


FIGURE 4 | Expression of ASIC1A in the BNST increases TMT-evoked freezing in *Asic1a*$^{-/-}$ mice. (A) ASIC1A immunohistochemistry showing localized expression of ASIC1A in the BNST of *Asic1a*$^{-/-}$ mice injected with AAV-ASIC1A. For reference, the lateral ventricle (lv) and anterior commissure (ac) are labeled and a mirror image atlas schematic of the corresponding coronal section (Paxinos and Franklin, 2001) is shown. **(B)** Expression of AAV-ASIC1A in the BNST of *Asic1a*$^{-/-}$ mice increased TMT-evoked freezing [$t_{(12)} = 2.249$, $^*p = 0.0441$, AAV-eGFP $n = 5$, AAV-ASIC1A $n = 9$].

an antagonist of the corticotropin releasing hormone receptor 1 (Ren et al., 2012). In that study, TMT was one of the stressors tested and it was reported to induce apneic episodes, which were particularly striking in mice lacking MeCP2. Thus, one possible explanation is psychological stress, which conceivably might be induced by odors that are unpleasant or fear evoking. Alternatively, perhaps the breathing response is an effort to avoid the unpleasant smell or sense of disgust (Boiten, 1998), which might be volitional or avolitional. Acrid odors can often accompany dangerous substances; detecting and avoiding these odors can be critical for survival (Santos et al., 2004). Thus, it could be adaptive for an animal to reduce its rate of breathing until the smell has dissipated or the threat has passed.

In this study, olfactory bulb lesions attenuated the effect of TMT on breathing, suggesting that a large part of the TMT response is mediated through olfaction. However, some effect on breathing rate remained in the olfactory bulb lesioned mice. Although the lesions were confirmed to cause substantial damage, it is difficult to be certain that the olfactory bulb tissue was removed entirely. Alternatively, mechanisms besides olfaction might play a role. TMT might evoke a physiologic response through an unpleasant taste. Or, TMT might irritate mucosal tissues such as the eyes or airway, for instance through bitter taste receptors in lung mucosa (Shah et al., 2009). Additionally, it is conceivable that a compound as volatile as TMT might be absorbed systemically which might exert a variety of potential effects.

These studies highlighted two effects of TMT, slowed breathing rate and freezing. Previous studies have debated whether TMT induces its effects because of its repugnant odor or its association with a predator (McGregor et al., 2002; Blanchard et al., 2003; Fendt and Endres, 2008). Our data suggest that the breathing effect of TMT might be related to its repugnant odor. Another repugnant odor, butyric acid,

also reduced breathing rate and by a magnitude similar to that induced by TMT. However, butyric acid did not induce freezing, indicating that the reduction in breathing rate alone was not sufficient to cause freezing and that the freezing effect is probably independent from the breathing effect. Furthermore, *Asic1a*$^{-/-}$ mice which froze significantly less in response to TMT exhibited a robust suppression of breathing that was equivalent to that observed in wild-type mice, suggesting that breathing, and freezing responses can be separable. Although the breathing response to TMT may be distinct from the fear-related freezing effects of TMT, understanding the underlying mechanisms may provide important insight into voluntary or involuntary control of breathing.

Our original objective was to investigate the potential sites of ASIC1A action in TMT-evoked behavior. Supporting our hypothesis that the BNST might be a critical site of ASIC1A action, we found that site-specific disruption of *Asic1a* in the BNST reduced freezing to TMT. Furthermore, viral-mediated restoration of ASIC1A expression in the BNST was sufficient to increase TMT-evoked freezing in *Asic1a*$^{-/-}$ mice. Although these observations strongly implicate the BNST, they do not rule out the possibility that ASIC1A in other brain regions might also play a role. Global *Asic1a* disruption altered TMT-evoked c-Fos activity in medial amygdala and periaqueductal gray, two structures that abundantly express ASIC1A (Coryell et al., 2007). ASIC1A might act at several sites in the circuit underlying TMT-evoked freezing, or loss of ASIC1A in the BNST may lead to changes in c-Fos activity elsewhere in the circuit. In addition, these studies raise questions about which BNST subnuclei are important for TMT-evoked freezing. Our viral manipulations altered ASIC1A in several BNST subnuclei, including the anterodorsal, and oval nuclei, which are thought to play opposing roles in anxiety-related behaviors (Kim et al., 2013). Further studies will be necessary to identify the key sites of ASIC1A action within the BNST and to evaluate other candidate sites of ASIC1A action in the TMT response.

A key question remains, how is this proton-gated channel ASIC1A involved in TMT-evoked freezing? One possible answer is that TMT might induce a systemic acidosis by reducing breathing rate and thus raising CO_2 levels. This seems to be an unlikely explanation, however, as butyric acid had a similar effect on breathing, but did not elicit freezing. Moreover, we did not observe an increase in respiratory rate, even after prolonged TMT exposure, which suggests that CO_2 did not reach a level sufficient to trigger the hypercapnic ventilatory response (Corcoran et al., 2009). Alternatively, TMT might lower pH in the circuit by inducing synaptic transmission. Neurotransmitter containing vesicles are acidic and during vesicle release can lower pH in the synaptic cleft (Du et al., 2014). Perhaps the two potential mechanisms highlighted above act in combination whereby systemic acidosis potentiates localized changes in pH due to synaptic transmission or another source such as local metabolic activity. Conversely, although ASIC1A is a proton-gated channel, it might contribute to the TMT response in some way other than by directly detecting acidosis. Recent studies suggest that ASIC1A may influence synaptic structure in brain regions other than the BNST (Zha et al., 2006; Du et al., 2014; Kreple et al., 2014). Therefore, it is possible that as in these other structures ASIC1A in the BNST might alter dendritic spine number and morphology, AMPA/NMDA ratio, and/or synaptic currents.

Finally, the observation that ASIC1A in the BNST contributes to both TMT- and CO_2-evoked freezing behaviors raises the possibility that ASIC1A and the BNST might contribute to other unconditioned fear-related responses in mice, and also possibly in humans. Unconditioned fear is thought to play a role in psychiatric diseases, particularly specific phobias, in which patients exhibit a strong unconditioned response to certain objects or situations (Kessler et al., 2005; Choy et al., 2007). ASIC1A in the BNST might also contribute to other anxiety disorders, as variations in the ASIC1A-encoding genomic locus have been recently associated with panic disorder (Smoller et al., 2014).

Acknowledgments

JW was supported by the Department of Veterans Affairs (Merit Award), the NIMH (5R01MH085724), NHLBI (R01HL113863) and a NARSAD Independent Investigator Award. RT was supported by an NIMH training grant (5T32MH019113-22). We thank George Richerson, Collin Kreple, Yimo Wang, Cory Massey, and YuJaung Kim for their recommendations and assistance. We also thank the University of Iowa Central Microscopy Facility and Gene Transfer Vector Core.

References

Asok, A., Ayers, L. W., Awoyemi, B., Schulkin, J., and Rosen, J. B. (2013). Immediate early gene and neuropeptide expression following exposure to the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT). *Behav. Brain Res.* 248, 85–93. doi: 10.1016/j.bbr.2013.03.047

Ayers, L. W., Asok, A., Heyward, F. D., and Rosen, J. B. (2013). Freezing to the predator odor 2,4,5 dihydro 2,5 trimethylthiazoline (TMT) is disrupted by olfactory bulb removal but not trigeminal deafferentation. *Behav. Brain Res.* 253, 54–59. doi: 10.1016/j.bbr.2013.06.034

Blanchard, D. C., Griebel, G., and Blanchard, R. J. (2003). Conditioning and residual emotionality effects of predator stimuli: some reflections on stress and emotion. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 1177–1185. doi: 10.1016/j.pnpbp.2003.09.012

Boiten, F. A. (1998). The effects of emotional behaviour on components of the respiratory cycle. *Biol. Psychol.* 49, 29–51. doi: 10.1016/S0301-0511(98)00025-8

Choy, Y., Fyer, A. J., and Lipsitz, J. D. (2007). Treatment of specific phobia in adults. *Clin. Psychol. Rev.* 27, 266–286. doi: 10.1016/j.cpr.2006.10.002

Corcoran, A. E., Hodges, M. R., Wu, Y., Wang, W., Wylie, C. J., Deneris, E. S., et al. (2009). Medullary serotonin neurons and central CO_2 chemoreception. *Respir. Physiol. Neurobiol.* 168, 49–58. doi: 10.1016/j.resp.2009.04.014

Coryell, M., Ziemann, A. E., Westmoreland, P. J., Haenfler, J. M., Kurjakovic, Z., Zha, X. M., et al. (2007). Targeting ASIC1a reduces innate fear and alters neuronal activity in the fear circuit. *Biol. Psychiatry* 62, 1140–1148. doi: 10.1016/j.biopsych.2007.05.008

Coryell, M. W., Wunsch, A. M., Haenfler, J. M., Allen, J. E., McBride, J. L., Davidson, B. L., et al. (2008). Restoring Acid-sensing ion channel-1a in the amygdala of knock-out mice rescues fear memory but not unconditioned

fear responses. *J. Neurosci.* 28, 13738–13741. doi: 10.1523/JNEUROSCI.3907-08.2008

Day, H. E., Masini, C. V., and Campeau, S. (2004). The pattern of brain c-fos mRNA induced by a component of fox odor, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), in rats, suggests both systemic and processive stress characteristics. *Brain Res.* 1025, 139–151. doi: 10.1016/j.brainres.2004.07.079

Du, J., Reznikov, L. R., Price, M. P., Zha, X. M., Lu, Y., Moninger, T. O., et al. (2014). Protons are a neurotransmitter that regulates synaptic plasticity in the lateral amygdala. *Proc. Natl. Acad. Sci. U.S.A.* 111, 8961–8966. doi: 10.1073/pnas.1407018111

Endres, T., and Fendt, M. (2009). Aversion- vs fear-inducing properties of 2,4,5-trimethyl-3-thiazoline, a component of fox odor, in comparison with those of butyric acid. *J. Exp. Biol.* 212, 2324–2327. doi: 10.1242/jeb.028498

Fendt, M., and Endres, T. (2008). 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox odor - just repugnant or really fear-inducing? *Neurosci. Biobehav. Rev.* 32, 1259–1266. doi: 10.1016/j.neubiorev.2008.05.010

Fendt, M., Endres, T., and Apfelbach, R. (2003). Temporary inactivation of the bed nucleus of the stria terminalis but not of the amygdala blocks freezing induced by trimethylthiazoline, a component of fox feces. *J. Neurosci.* 23, 23–28.

Fendt, M., Endres, T., Lowry, C. A., Apfelbach, R., and McGregor, I. S. (2005). TMT-induced autonomic and behavioral changes and the neural basis of its processing. *Neurosci. Biobehav. Rev.* 29, 1145–1156. doi: 10.1016/j.neubiorev.2005.04.018

Hodges, M. R., Tattersall, G. J., Harris, M. B., McEvoy, S. D., Richerson, D. N., Deneris, E. S., et al. (2008). Defects in breathing and thermoregulation in mice with near-complete absence of central serotonin neurons. *J. Neurosci.* 28, 2495–2505. doi: 10.1523/JNEUROSCI.4729-07.2008

Hotsenpiller, G., and Williams, J. (1997). A synthetic predator odor (TMT) enhances conditioned analgesia and fear when paired with a benzodiazepine receptor inverse agonist (FG-7142). *Psychobiology* 25, 83–88.

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., and Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch. Gen. Psychiatry* 62, 593–602. doi: 10.1001/archpsyc.62.6.593

Kim, S. Y., Adhikari, A., Lee, S. Y., Marshel, J. H., Kim, C. K., Mallory, C. S., et al. (2013). Diverging neural pathways assemble a behavioural state from separable features in anxiety. *Nature* 496, 219–223. doi: 10.1038/nature12018

Kreple, C. J., Lu, Y., Taugher, R. J., Schwager-Gutman, A. L., Du, J., Stump, M., et al. (2014). Acid-sensing ion channels contribute to synaptic transmission and inhibit cocaine-evoked plasticity. *Nat. Neurosci.* 17, 1083–1091. doi: 10.1038/nn.3750

Ledoux, J. E., Cicchetti, P., Xagoraris, A., and Romanski, L. M. (1990). The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. *J. Neurosci.* 10, 1062–1069.

McGregor, I. S., Schrama, L., Ambermoon, P., and Dielenberg, R. A. (2002). Not all 'predator odours' are equal: cat odour but not 2,4,5 trimethylthiazoline (TMT; fox odour) elicits specific defensive behaviours in rats. *Behav. Brain Res.* 129, 1–16. doi: 10.1016/S0166-4328(01)00324-2

Muller, M., and Fendt, M. (2006). Temporary inactivation of the medial and basolateral amygdala differentially affects TMT-induced fear behavior in rats. *Behav. Brain Res.* 167, 57–62. doi: 10.1016/j.bbr.2005.08.016

Nader, K., Majidishad, P., Amorapanth, P., and Ledoux, J. E. (2001). Damage to the lateral and central, but not other, amygdaloid nuclei prevents the acquisition of auditory fear conditioning. *Learn. Mem.* 8, 156–163. doi: 10.1101/lm.38101

Paxinos, G., and Franklin, K. B. J. (2001). *The Mouse Brain in Steriotaxic Coordinates.* San Diego, CA: Academic Press.

Price, M. P., Gong, H., Parsons, M. G., Kundert, J. R., Reznikov, L. R., Bernardinelli, L., et al. (2014). Localization and behaviors in null mice suggest that ASIC1 and ASIC2 modulate responses to aversive stimuli. *Genes Brain Behav.* 13, 179–194. doi: 10.1111/gbb.12108

Ren, J., Ding, X., Funk, G. D., and Greer, J. J. (2012). Anxiety-related mechanisms of respiratory dysfunction in a mouse model of Rett syndrome. *J. Neurosci.* 32, 17230–17240. doi: 10.1523/JNEUROSCI.2951-12.2012

Rosen, J. B., Pagani, J. H., Rolla, K. L., and Davis, C. (2008). Analysis of behavioral constraints and the neuroanatomy of fear to the predator odor trimethylthiazoline: a model for animal phobias. *Neurosci. Biobehav. Rev.* 32, 1267–1276. doi: 10.1016/j.neubiorev.2008.05.006

Santos, D. V., Reiter, E. R., Dinardo, L. J., and Costanzo, R. M. (2004). Hazardous events associated with impaired olfactory function. *Arch. Otolaryngol. Head Neck Surg.* 130, 317–319. doi: 10.1001/archotol.130.3.317

Shah, A. S., Ben-Shahar, Y., Moninger, T. O., Kline, J. N., and Welsh, M. J. (2009). Motile cilia of human airway epithelia are chemosensory. *Science* 325, 1131–1134. doi: 10.1126/science.1173869

Smoller, J. W., Gallagher, P. J., Duncan, L. E., McGrath, L. M., Haddad, S. A., Holmes, A. J., et al. (2014). The human ortholog of acid-sensing ion channel gene ASIC1a is associated with panic disorder and amygdala structure and function. *Biol. Psychiatry* 76, 902–910. doi: 10.1016/j.biopsych.2013.12.018

Takahashi, L. K., Nakashima, B. R., Hong, H., and Watanabe, K. (2005). The smell of danger: a behavioral and neural analysis of predator odor-induced fear. *Neurosci. Biobehav. Rev.* 29, 1157–1167. doi: 10.1016/j.neubiorev.2005.04.008

Taugher, R. J., Lu, Y., Wang, Y., Kreple, C. J., Ghobbeh, A., Fan, R., et al. (2014). The bed nucleus of the stria terminalis is critical for anxiety-related behavior evoked by CO_2 and acidosis. *J. Neurosci.* 34, 10247–10255. doi: 10.1523/JNEUROSCI.1680-14.2014

Vernet-Maury, E., Polak, E. H. P., and Demael, A. (1984). Structure–activity relationship of stress-inducing odorants in the rat. *J. Chem. Ecol.* 10, 1007–1018. doi: 10.1007/bf00987509

Wallace, K. J., and Rosen, J. B. (2001). Neurotoxic lesions of the lateral nucleus of the amygdala decrease conditioned fear but not unconditioned fear of a predator odor: comparison with electrolytic lesions. *J. Neurosci.* 21, 3619–3627.

Wemmie, J. A., Askwith, C. C., Lamani, E., Cassell, M. D., Freeman, J. H. Jr., and Welsh, M. J. (2003). Acid-sensing ion channel 1 is localized in brain regions with high synaptic density and contributes to fear conditioning. *J. Neurosci.* 23, 5496–5502.

Wemmie, J. A., Chen, J., Askwith, C. C., Hruska-Hageman, A. M., Price, M. P., Nolan, B. C., et al. (2002). The acid-activated ion channel ASIC contributes to synaptic plasticity, learning, and memory. *Neuron* 34, 463–477. doi: 10.1016/S0896-6273(02)00661-X

Zha, X. M., Wemmie, J. A., Green, S. H., and Welsh, M. J. (2006). Acid-sensing ion channel 1a is a postsynaptic proton receptor that affects the density of dendritic spines. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16556–16561. doi: 10.1073/pnas.0608018103

Ziemann, A. E., Allen, J. E., Dahdaleh, N. S., Drebot, II, Coryell, M. W., Wunsch, A. M., et al. (2009). The amygdala is a chemosensor that detects carbon dioxide and acidosis to elicit fear behavior. *Cell* 139, 1012–1021. doi: 10.1016/j.cell.2009.10.029

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 22 September 2015
doi: 10.3389/fnins.2015.00319

HDAC I inhibition in the dorsal and ventral hippocampus differentially modulates predator-odor fear learning and generalization

*Robin K. Yuan[1], Jenna C. Hebert[2], Arthur S. Thomas[3], Ellen G. Wann[1] and Isabel A. Muzzio[1,4]**

[1] Department of Psychology, University of Pennsylvania, Philadelphia, PA, USA, [2] Biological Basis of Behavior, University of Pennsylvania, Philadelphia, PA, USA, [3] Department of Biology, University of Pennsylvania, Philadelphia, PA, USA, [4] Department of Biology, University of Texas at San Antonio, San Antonio, TX, USA

OPEN ACCESS

Edited by:
Yasushi Kiyokawa, The University of Tokyo, Japan

Reviewed by:
D. Caroline Blanchard, University of Hawaii at Manoa, USA
Lorey K. Takahashi, University of Hawaii at Manoa, USA

***Correspondence:**
Isabel A. Muzzio, Biology Department, University of Texas at San Antonio, One UTSA Circle, San Antonio, TX 78249, USA
isabel.muzzio@utsa.edu

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *30 June 2015*
Accepted: *27 August 2015*
Published: *22 September 2015*

Citation:
Yuan RK, Hebert JC, Thomas AS, Wann EG and Muzzio IA (2015) HDAC I inhibition in the dorsal and ventral hippocampus differentially modulates predator-odor fear learning and generalization. Front. Neurosci. 9:319. doi: 10.3389/fnins.2015.00319

Although predator odors are ethologically relevant stimuli for rodents, the molecular pathways and contribution of some brain regions involved in predator odor conditioning remain elusive. Inhibition of histone deacetylases (HDACs) in the dorsal hippocampus has been shown to enhance shock-induced contextual fear learning, but it is unknown if HDACs have differential effects along the dorso-ventral hippocampal axis during predator odor fear learning. We injected MS-275, a class I HDAC inhibitor, bilaterally in the dorsal or ventral hippocampus of mice and found that it had no effects on innate anxiety in either region. We then assessed the effects of MS-275 at different stages of fear learning along the longitudinal hippocampal axis. Animals were injected with MS-275 or vehicle after context pre-exposure (pre-conditioning injections), when a representation of the context is first formed, or after exposure to coyote urine (post-conditioning injections), when the context becomes associated with predator odor. When MS-275 was administered after context pre-exposure, dorsally injected animals showed enhanced fear in the training context but were able to discriminate it from a neutral environment. Conversely, ventrally injected animals did not display enhanced learning in the training context but generalized the fear response to a neutral context. However, when MS-275 was administered after conditioning, there were no differences between the MS-275 and vehicle control groups in either the dorsal or ventral hippocampus. Surprisingly, all groups displayed generalization to a neutral context, suggesting that predator odor exposure followed by a mild stressor such as restraint leads to fear generalization. These results may elucidate distinct functions of the dorsal and ventral hippocampus in predator odor-induced fear conditioning as well as some of the molecular mechanisms underlying fear generalization.

Keywords: histone deacetylase inhibitors, MS-275, contextual fear conditioning, fear generalization, hippocampus, predator odor

Introduction

Predator odors are ethologically relevant stimuli that have been shown to elicit a variety of defensive responses in rodents (Blanchard and Blanchard, 1990; Zangrossi and File, 1992; Wallace and Rosen, 2000; Dielenberg and McGregor, 2001; Wang et al., 2013a), and, under some conditions, can also produce conditioning (Blanchard et al., 2001; Dielenberg et al., 2001; Takahashi et al., 2008). Contextual fear conditioning, including predator odor fear learning, involves the association of a context (the conditioned stimulus, CS) with a predator odor (unconditioned stimulus, US), which leads to the emergence of a conditioned fear response (CR) in response to the context CS (Fanselow, 2000; Maren and Holt, 2000; Anagnostaras et al., 2001; Rosen, 2004). We have recently developed and characterized a predator odor fear conditioning paradigm using coyote urine that is effective with mice. We showed that this paradigm produces moderate but consistent freezing, a stereotypic response to fear observed in rodents, during long-term retrieval tests. This response is not observed when animals are exposed to water (no odor) or an aversive non-fearful odor (2-methyl butyric acid), indicating that the freezing is a result of associative learning (Wang et al., 2013a, 2015). Furthermore, the conditioned fear response is context specific since freezing is observed only in the training context, and it requires both the dorsal and ventral hippocampus (Wang et al., 2013a).

Using this paradigm, we recently found that spatial representations formed in the dorsal hippocampus after predator odor fear conditioning are stable in the long term (Wang et al., 2012) but become unstable again during extinction (Wang et al., 2015), suggesting that predator odor learning alters the stability of the dorsal hippocampal representation of context. These findings correlate with numerous studies indicating that the dorsal hippocampus receives preprocessed spatial information (for review, see Witter et al., 2014) and thus plays a critical role forming representations of context during conditioning (for review, see Maren and Holt, 2000); however, the role of the ventral region remains unclear. Clarifying the role of the ventral hippocampus for predator odor fear learning is particularly important because this region receives most of the olfactory inputs from the medial and posterior amygdala (Pitkanen et al., 2000; Kemppainen et al., 2002), areas that receive projections from the main and accessory olfactory system involved in predator odor processing (Masini et al., 2010). Moreover, in addition to these neuroanatomical differences, ventral and dorsal cells display distinct firing characteristics, further suggesting that these regions may have different functions. Cells in the dorsal hippocampus fire in specific circumscribed locations, whereas ventral cells have large and overlapping receptive fields (O'Keefe and Dostrovsky, 1971; Kjelstrup et al., 2008; Keinath et al., 2014). Based on these differences, it has been suggested that the dorsal region may be important for minimizing memory interference by coding specific aspects of contexts, while the ventral hippocampus may play a role in contextual generalization (Komorowski et al., 2013; Keinath et al., 2014). However, no studies have directly tested if these differential functions play a role in contextual fear learning.

On the molecular level, the formation of new memories requires alterations in gene transcription, which lead to the translation of proteins necessary for the cellular changes implicated in long-term memory (for review, see Kandel, 2012). This occurs through modifications of chromatin, a DNA-protein complex. The basic unit of chromatin is the nucleosome, which consists of DNA wrapped around four histone proteins. Modifying these proteins through processes such as acetylation, phosphorylation, and methylation changes the state of the chromatin, influencing the rate of transcription by making the DNA more or less accessible to transcription factors (Levenson and Sweatt, 2005; Wood et al., 2006). The most studied and well understood of these modifications in relation to memory is histone acetylation, a process that facilitates gene transcription by relaxing chromatin structure. This, in turn, leads to synthesis of proteins necessary for long-term memory (Peixoto and Abel, 2013). Histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), enzymes that increase and decrease acetylation, respectively (for review, see Levenson and Sweatt, 2005; Day and Sweatt, 2011). Evidence from experiments investigating the correlation between histone modifications and long-term memory in mice suggests that changes in acetylation are essential for hippocampus-dependent fear learning using electrical shock (Vecsey et al., 2007; Bahari-Javan et al., 2012). However, it is unknown what role histone acetylation plays in predator odor conditioning and whether the dorsal and ventral hippocampus differentially respond to chromatin alterations.

Here, we investigated the effects of the HDAC inhibitor MS-275, a class I-specific inhibitor (Simonini et al., 2006; Beckers et al., 2007; Khan et al., 2008; Formisano et al., 2015), in the dorsal and ventral hippocampus on innate anxiety and during predator odor fear conditioning. We found that MS-275 had no effect on traditional anxiety tests. However, injections of MS-275 after context pre-exposure (pre-conditioning injections) had different roles in the dorsal and ventral hippocampus, leading to enhanced fear and generalization, respectively. Interestingly, although injections after conditioning did not have effects in any of the groups, all conditions displayed fear generalization, suggesting that animals generalize fear to neutral contexts when a stressor, such as restraint, follows immediately after predator odor exposure. These results extend our understanding of hippocampal function during fear learning and provide insights about the learning contingencies that could lead to fear generalization.

Materials and Methods

Animals

Male C57BL/6 mice 2–5 months of age (Jackson Laboratories, Bar Harbor, ME) were housed individually, kept on a 12-h light/dark cycle, and allowed access to food and water *ad libitum* for at least 1 week prior to beginning behavioral experiments. All experiments were approved by the Institution of Animal Care and Use Committee of the University of Pennsylvania, and were carried out in accordance with NIH guidelines.

Anxiety Measures

Animals were run in the open-field test and black-white box to determine the effects of MS-275 on innate anxiety. MS-275 was infused bilaterally into either the dorsal or ventral hippocampus 1–2 h before conducting anxiety tests. For the open field test, mice were placed in the center of a large cylindrical arena (70 cm in diameter). The arena was illuminated by a ceiling-mounted array of eight 60-watt lights arranged symmetrically around the perimeter, approximately 1.8 m above the base. Explorative behavior was recorded for 20 min. Additionally, we evaluated freezing by calculating the percent time the animals remain immobile, except for respiratory movements. For the black/white two compartment box, we used a plastic box divided into two equal compartments (22 × 24 cm) connected by a small opening. The black compartment was darkened with black contact paper and covered with a piece of cardboard, while the open-topped white compartment was lined with white contact paper and illuminated by three ceiling-mounted 60-watt lights approximately 1 m above the apparatus, aimed at the center of the compartment. Mice were placed in the center of the white compartment facing the black side, and explorative behavior was recorded for 3 min (see behavioral analysis for quantification details below).

Contextual Fear Conditioning and Context Discrimination

Prior to the start of behavioral experiments, animals were handled and restrained twice a day for 2 consecutive days. Animals were then conditioned using a predator odor contextual fear conditioning paradigm previously characterized in our lab (Wang et al., 2013a). We have demonstrated in several studies that this paradigm produces moderate but consistent increases in freezing, which are not seen when animals undergo the same schedule of context exposures with no odor exposure or with exposure to a non-fearful odor (Wang et al., 2012, 2013a, 2015). On day 1 (one day before conditioning), mice were habituated to a cylindrical training context (baseline context A, blA) and an equivalently sized neutral context (baseline context B, blB) for 10 min each. Both contexts were 35 cm in diameter and had distinct configurations of black visual cues on the cylinder's white walls; additionally, the contexts were placed in separate rooms. The next day (day 2, 24 h after baseline context exposures), a paper towel square (2 × 2 cm) saturated with 40 drops of coyote urine (Maine Outdoor Solutions, Harmon, MN) was placed in the center of context A, and mice were re-exposed to the context for 4 min in the presence of the odor (conditioning session, cond). A short-term retrieval test was conducted 1 h later in context A without odor for 10 min. We have previously shown that this retrieval session is important for the consolidation of the fear representation in the long term (Wang et al., 2012). The following day (day 3, 24 h after conditioning), a long-term retrieval test was conducted in both context A and context B for 10 min without odor (24h A and 24h B). The order of context A and context B during baseline and the 24 h retrieval test was counterbalanced across animals. At all time points other than the conditioning session, a paper towel square saturated with water was placed in the center of the context. MS-275 or DMSO (4%) was administered bilaterally into either the dorsal or ventral hippocampus immediately following the baseline context exposures (pre-conditioning injections) or immediately after the conditioning session (cond; post-conditioning injections). We evaluated freezing as a measure of learning as described below.

Behavioral Analysis

All behavioral measures were recorded and analyzed using the Limelight automated tracking system (Coulburn Instruments). For the open-field test, the context was divided into three equally spaced concentric circles and the percent time spent in these areas was measured. For the black-white box, the percent time spent in the white compartment of the white/black box was measured, along with the number of reentries to the white side. For conditioning and the open field, freezing was quantified as the percentage of time during which the velocity of the animal was lower than 0.6 cm/s. Freezing was evaluated using both Lime Light and custom-written MATLAB code. For the MATLAB analysis, the position data were smoothed with a 1 s boxcar to eliminate jitter in the tracking. Finally, we calculated percent freezing by calculating the percent ratio between freezing at 24 h and freezing during baseline for each context.

Surgery

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally and placed on a stereotaxic apparatus in a flat skull position (David Kopf Instruments, Tujunga, CA). 26 gauge guide cannulas (Plastics One, Roanoke, VA) were implanted bilaterally in either the dorsal or ventral hippocampus at the following coordinates, measured from Bregma in mm. Dorsal: AP, -1.7; ML, ± 1.5; DV: -1.0 (internal cannulas project an additional 0.7 mm beyond guides for an injection depth of -1.7). Ventral: AP, -3.0; ML, ± 2.8; DV: -2.0 (internal cannulas project an additional 1.5 mm beyond guides for an injection depth of -3.5). An anchor screw was placed just anterior to lambda, and cannulas were affixed to the skull with cyanoacrylate and dental cement. After surgery, animals were allowed to recover for 1 week prior to behavioral experiments.

Bilateral Hippocampal Injections and Drug Concentration in the Hippocampus

MS-275 (SelleckChem, Houston, TX) was diluted to 1 mM using 4% dimethyl sulfoxide (DMSO) in ACSF, then infused bilaterally into either the dorsal or ventral hippocampus through the implanted guide cannulas using a standard infusion syringe pump (Harvard Apparatus, Holliston, MA). Total volume injected was 0.5 µl on each side at a rate of 0.5 µl/min. Controls were injected with an equivalent volume of vehicle (4% DMSO in artificial cerebrospinal fluid). We estimated the concentration of MS-275 in the hippocampus to be roughly 71 µM, since the average volume of the hippocampus in C57bl6 mice is 28 mm^3, with each hemisphere being approximately 14 mm^3 and each dorsal and ventral sub-regions around 7 mm^3 (Peirce et al., 2003). This concentration is well above the dosages that are effective in inhibiting class I HDACs (Hu et al., 2003; Khan et al., 2008). The increase in acetylation

produced by MS-275 (Simonini et al., 2006; Formisano et al., 2015) is evident 2 h after drug treatment and persists for up to 8 h (Simonini et al., 2006). Injections were performed in a room separate from all behavioral experiments and were given after contextual pre-exposure (pre-conditioning injections) or after predator odor exposure (post-conditioning injections).

Histology

To verify cannula placements, animals were sacrificed after behavioral experiments. Brains were removed and fixed at 4°C with 10% formalin for at least 24 h. They were then transferred to a 30% sucrose solution and kept for at least 48 h at 4°C for cryoprotection. Brains were then cryosectioned (35 μm, coronal) and Nissl stained with cresyl violet using standard histological procedures (Powers and Clark, 1955).

Statistics

Independent *t*-tests were used to evaluate anxiety measures. Two-Way ANOVAS with repeated measures were used to compare baseline and post-conditioning freezing in the training and neutral context. Student Newman Keuls *post hoc* tests were used to determine which groups were significantly different. Independent and paired *t*-tests were used to evaluate percent of freezing relative to baseline.

Results

MS-275 has No Effect on Innate Anxiety

Since we wanted to establish the effects of HDAC inhibition on fear learning, we first investigated whether MS-275 had any effects on innate anxiety. We performed bilateral injections of MS-275 or vehicle into the dorsal or ventral hippocampus of animals 2–3 h prior to behavioral testing in the open field and the black/white box (**Figures 1A,B**). The open field test, which consists of free exploration in a large arena, evaluates the anxiety that rodents exhibit in open spaces. It is well established that rodents find the inner areas of the open field more anxiogenic than the outer areas (Hall, 1934; Prut and Belzung, 2003). Thus, differences in the amount of time spent in these areas normally reflect distinct levels of anxiety. Thirty-four animals were injected with MS-275 (dorsal: $N = 20$; ventral: $N = 14$), and 41 animals were injected with vehicle (dorsal: $N = 21$; ventral: $N = 20$). We divided the open field in three concentric areas and calculated the time spent in each of these zones. We did not find any differences between the groups in the percentage of time spent in the three concentric regions comprising the open field (**Figures 1C,D**; dorsal: center: $p = 0.80$, inner: $p = 0.36$, outer: $p = 0.62$; ventral: center: $p = 0.57$, inner: $p = 0.30$, outer: $p = 0.46$). Additionally, we did not find differences in the levels of freezing in the open field (**Figures 1E,F**; dorsal: $p = 0.45$; ventral: $p = 0.66$).

Next, we evaluated the effects of MS-275 on behavior in a black/white two-compartment box (Crawley and Goodwin, 1980; Sánchez, 1997). The animals are initially placed in the open white compartment, and the time they spend in this compartment vs. the dark covered side of the chamber is measured while animal freely move across these areas. Rodents find bright, open environments more anxiogenic than closed, dark ones (Sánchez, 1995); therefore, increased anxiety is typically seen through a reduction in the amount of time spent exploring the white compartment. Twenty-three animals were injected with MS-275 (dorsal: $N = 12$; ventral: $N = 11$) and 23 animals were injected with vehicle (dorsal: $N = 12$; ventral: $N = 11$). We did not find significant differences between the groups in the percentage of time spent in the white compartment (**Figures 1G,H**, dorsal: $p = 0.99$; ventral: $p = 0.17$) or the number of reentries to the white compartment (dorsal: $p = 0.29$; ventral: $p = 0.24$; data not shown). These results suggest that MS-275 does not affect innate anxiety in the dorsal or ventral hippocampus.

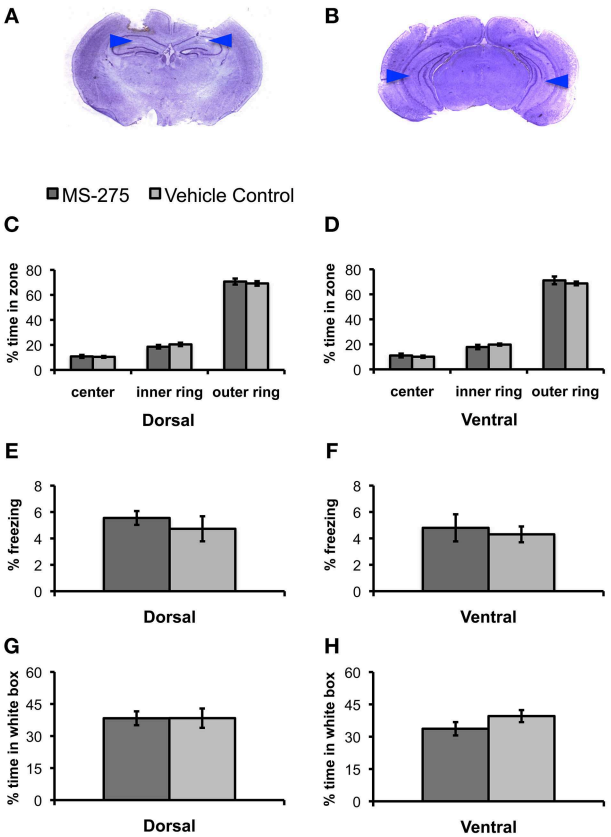


FIGURE 1 | (A,B) Photomicrograph of Nissl-stained coronal section showing position of dorsal **(A)** and ventral **(B)** guide cannulas. Note that internal cannulas for dorsal injections project an additional 0.7 mm beyond guide cannulas, and internal cannulas for ventral injections project an additional 1.5 mm beyond guide cannulas. Blue arrows indicate estimated injection depth. **(C–H)** Animals injected with MS-275 or vehicle in either the dorsal or ventral hippocampus showed no significant differences in tests of anxiety. **(C–F)** Animals injected with MS-275 (dorsal: $N = 20$; ventral: $N = 14$) or vehicle (dorsal: $N = 21$; ventral: $N = 20$) showed no significant differences in behavior in the open field test. This was evident in the percentage of time spent in the center, inner ring, and outer ring of an open field in the dorsal **(C)** and ventral **(D)** groups, as well as the level of freezing observed in the open field in the dorsal **(E)** and ventral **(F)** groups. **(G,H)** Animals injected with MS-275 (dorsal: $N = 12$; ventral: $N = 11$) or vehicle (dorsal: $N = 12$; ventral: $N = 11$) in the dorsal **(G)** or ventral **(H)** hippocampus showed no significant difference in the percentage of time they spent in the white compartment of the black/white box. Means ± SEM are shown.

Effects of MS-275 on Fear Learning

Inhibition of Class I HDACs Following Contextual Pre-exposure Produces Enhanced Fear in the Dorsal Hippocampus and Generalization in the Ventral Region

Several researchers have suggested that successful contextual conditioning consists of two stages. In the first stage, a representation of the context is formed, while in the second stage, the context is associated with the US (Young et al., 1994; Fanselow and Rudy, 1998; Rudy and O'Reilly, 1999, 2001; Fanselow, 2000; O'Reilly and Rudy, 2001; Rudy et al., 2004). This view is based on the observation that animals display learning deficits in the absence of contextual pre-exposure (Fanselow, 1990). Our predator odor paradigm is ideal for testing the contributions of the hippocampus to these two learning stages because animals are exposed to the context one day prior to conditioning, which provides an optimal time window to explore the effects of HDAC inhibition either during the context pre-exposure (pre-conditioning) or the associative phase of learning (post-conditioning).

To determine the role of histone acetylation during the formation of a representation of context, MS-275 or vehicle (DMSO, 4%) was injected in either the dorsal or ventral hippocampus after exposure to the training context (baseline context A) and a neutral context (baseline context B) on Day 1 (pre-conditioning injections). Twenty-four hours after the injections (Day 2), these groups were conditioned and tested 1 h after conditioning in the training context A. On day 3, the four groups were retested in the training (A) and neutral (B) context 24 h after conditioning (long-term retrieval test; see **Figure 2A**). The order of exposure to context A and B during baseline and the 24 h retrieval tests was counterbalanced across animals.

Since the dorsal hippocampus has been implicated in coding specific information about contexts (Nadel et al., 2013), and cells in this region are sensitive to subtle contextual changes (Colgin et al., 2008), we hypothesized that dorsal injections of MS-275 after context pre-exposure (pre-conditioning injections) should lead to enhanced learning because animals would remember the training context in more detail. These injections, however, would not substantially decrease the ability of the animals to discriminate the training and neutral contexts because the specific information about each context would be remembered. Conversely, since spatial representations in the ventral hippocampus are large and overlapping, a characteristic that may facilitate generalization (Komorowski et al., 2013; Keinath et al., 2014), we hypothesized that ventral injections after context pre-exposure (pre-conditioning injections) would lead to fear generalization to a neutral context.

There were no differences in baseline freezing prior to conditioning in the dorsal groups (MS-275: $N = 13$; vehicle control: $N = 16$). In support of our hypothesis, we found that dorsal injections significantly increased freezing in the training context (context A) in the MS-275 group in comparison to the vehicle control group. (**Figure 2B**; effect of group: $F_{(1, 27)} = 4.50$, $p < 0.05$, effect of session: $F_{(3, 81)} = 17.56$, $p < 0.001$; interaction: $F_{(3, 81)} = 2.95$, $p < 0.04$). *Post hoc* Student-Newman-Keuls tests (SNKTs) showed that freezing levels were comparable in the control and MS-275 groups during baseline (blA: $p = 0.40$; blB: $p = 0.99$), but were significantly different during the post-conditioning 24 h test in context A (24 h: $p < 0.003$). Furthermore, although MS-275 also produced a significant increase in freezing in the neutral context (B) (SNKTs: 24 h B: $p < 0.03$), both the vehicle and MS-275 groups, were able to discriminate the training context A from the neutral context B, which was evident in significantly higher levels of freezing in context A (vehicle control: $p < 0.05$; MS-275: $p < 0.005$). The differences between the MS-275 and vehicle groups are also evident in a significant percent increase in freezing observed 24 h after conditioning relative to baseline in context A (**Figure 2C**; $t_{27} = -2.07$, $p < 0.05$) and a trend in context B ($t_{28} = -1.82$, $p = 0.08$). Again, when we compared the percent increase in freezing from baseline within each group, both the MS-275 and vehicle control groups displayed higher freezing in context A than B at 24 h relative to the freezing baseline in each context (MS-275 (trend toward significance): $t_{11} = 2.13$, $p = 0.056$; vehicle control: $t_{19} = 2.63$, $p < 0.02$). These results suggest that MS-275 enhances the memory of the training context without disrupting the ability of the animals to discriminate this context from a neutral one.

In the ventral hippocampus, we did not observe differences in baseline freezing between the groups receiving injections after contextual pre-exposure (pre-conditioning; MS-275: $N = 17$; vehicle control: $N = 15$). Even though MS-275 did not significantly affect learning, the HDAC inhibitor produced fear generalization in response to the neutral context B. This was evident in higher freezing levels in the neutral context in animals injected with MS-275 than vehicle-injected controls (**Figure 2D**; effect of group: $F_{(1, 31)} = 0.09$, $p = 0.77$, effect of session: $F_{(3, 89)} = 25.19$, $p < 0.001$; interaction: $F_{(3, 89)} = 2.75$, $p < 0.05$). SNKTs showed no differences prior to conditioning or after conditioning in the training context ($P > 0.05$). However, the groups were significantly different in the neutral context B (24 h B: $p < 0.04$). Furthermore, while the control animals discriminated between the training and neutral context ($p < 0.003$), the MS-275 group did not ($p = 0.865$). These effects are also evident when we examined the percent increase in freezing at 24 h after conditioning relative to baseline in each context, showing that there were no differences in the training context A ($t_{35} = -0.97$, $p = 0.34$) but significantly higher freezing in the MS-275 group in context B ($t_{35} = 2.36$, $p < 0.03$; **Figure 2E**). Importantly, while vehicle control animals clearly discriminated between the contexts ($t_{16} = 2.36$, $p = 0.03$), MS-275 animals showed no difference in freezing in context A and B ($t_{19} = 0.08$, $p = 0.94$). These results suggest that HDAC I inhibition in the dorsal and ventral hippocampus plays different roles in predator odor fear memory. MS-275 leads to enhanced fear memory in the dorsal hippocampus and fear generalization in the ventral region.

Inhibition of Class I HDACs Following Conditioning has No Effect on Fear Learning

To determine the role of histone acetylation along the longitudinal hippocampal axis after coyote exposure, we then examined groups of mice injected with MS-275 or vehicle (DMSO, 4%) after conditioning (see **Figure 3A**). The amygdala

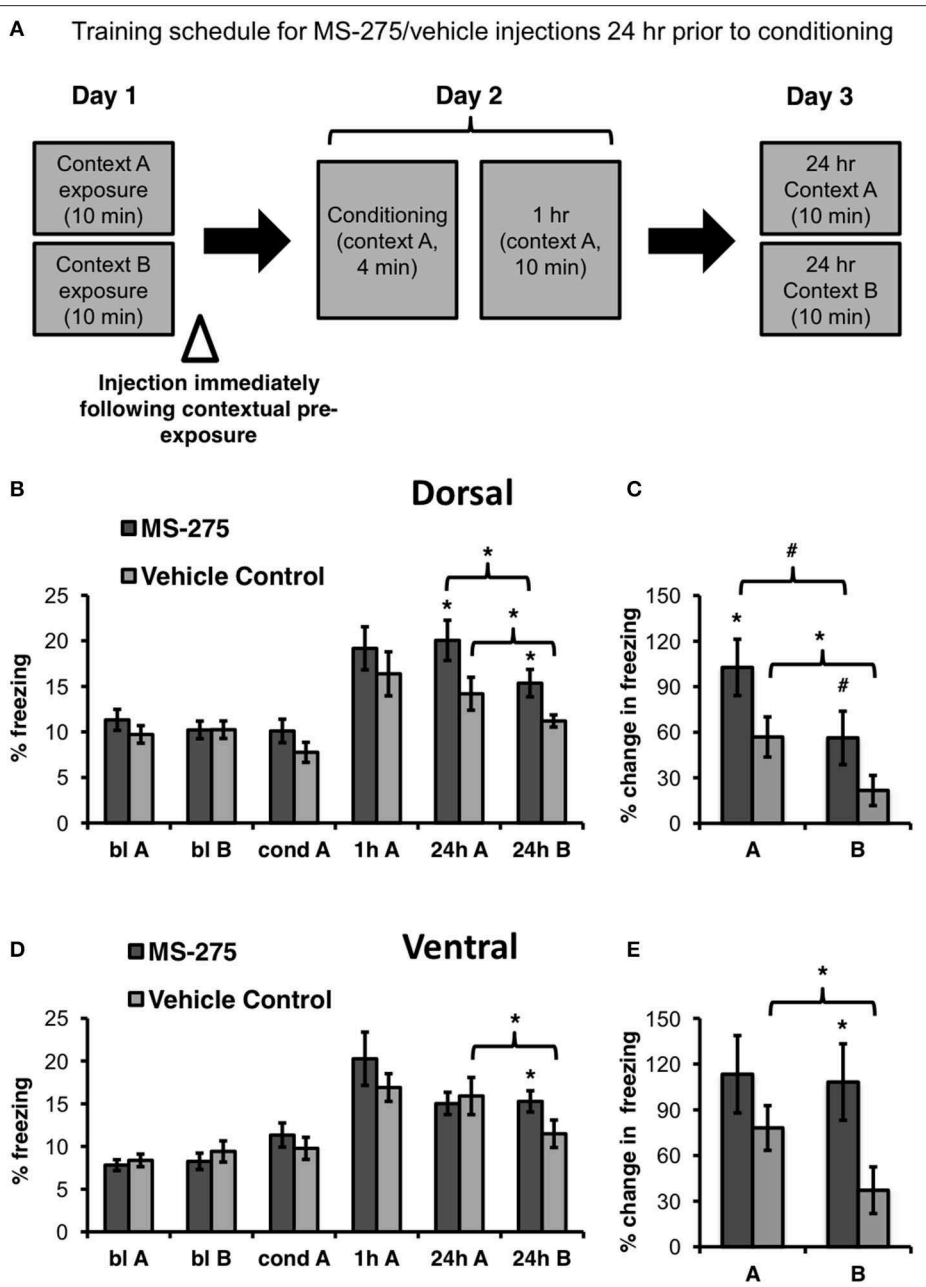


FIGURE 2 | (A) Schematic representation of behavioral paradigm and timing of injections. Animals were injected with MS-275 or vehicle immediately following exposure to contexts A and B, 24 h prior to fear conditioning. **(B)** Animals injected with MS-275 ($N = 13$) in the dorsal hippocampus after contextual pre-exposure (bl A, bl B) exhibited enhanced fear learning in context A and elevated freezing in context B 24 h after conditioning in comparison to the vehicle-injected controls ($N = 16$). However, freezing was significantly higher in context A, the conditioning context, than in the neutral context B, indicating that these animals still discriminated between the environments. **(C)** Animals injected with MS-275 in the dorsal hippocampus exhibited a significantly greater percent change in freezing 24 h post-conditioning in context A and a trend toward enhanced freezing in context B relative to baseline freezing in each of these contexts in comparison to controls. **(D)** Animals injected with MS-275 ($N = 17$) in the ventral hippocampus after contextual pre-exposure exhibited fear generalization without showing enhanced fear learning 24 h after conditioning; This generalization effect was not observed in control animals injected with vehicle ($N = 15$). **(E)** Animals injected with MS-275 in the ventral hippocampus exhibit significantly greater percent change in freezing 24 h post-conditioning in context B relative to baseline in that context in comparison to vehicle-injected controls. No difference in percent change in freezing was observed in the training context A between the MS-275 and vehicle control animals. Means ± SEM are shown, $*p < 0.05$, $\#p < 0.09$.

has been identified as a critical brain region for the associative phase of predator odor fear learning (for review, see Takahashi et al., 2008). In particular, it has been demonstrated that the medial amygdala plays a role during acquisition (Blanchard et al., 2005; Takahashi et al., 2007) and the basolateral amygdala during consolidation (Takahashi et al., 2007). Since the dorsal

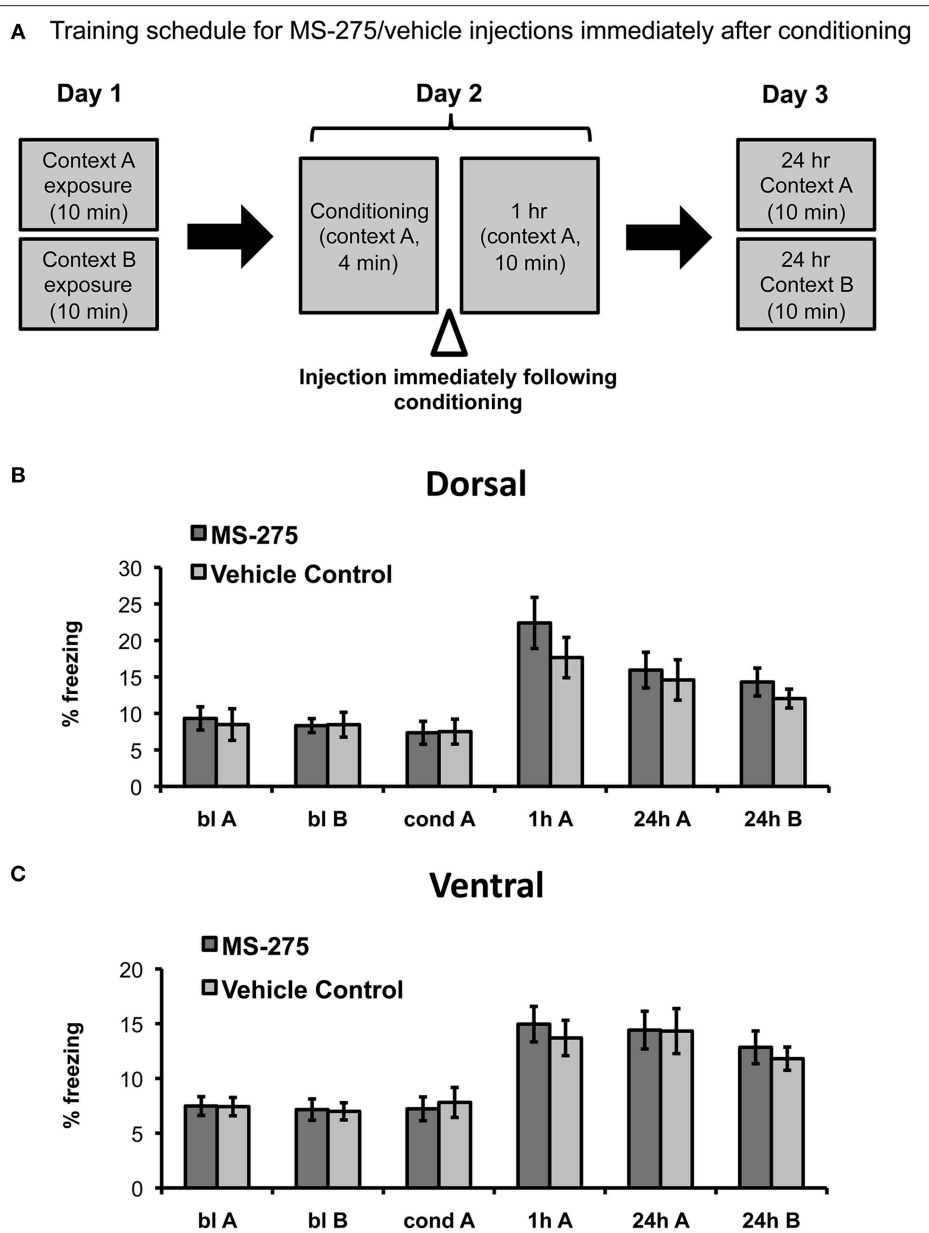


FIGURE 3 | (A) Schematic representation of behavioral paradigm and timing of injections. Animals were injected with MS-275 or vehicle immediately following fear conditioning in context A. Animals injected in the dorsal (**B**; MS-275: $N = 11$, vehicle: $N = 11$) or ventral (**C**; MS-275: $N = 13$, vehicle: $N = 14$) hippocampus after the conditioning session exhibited fear generalization 24 h after conditioning. Means ± SEM are shown, $^{*}p < 0.05$.

hippocampus receives very few projections from the basolateral amygdala and no projections from the medial amygdala, the area where olfactory information converges (Pikkarainen et al., 1999), we hypothesized that dorsal injections of MS-275 would not affect predator odor fear learning. Conversely, the ventral hippocampus receives strong projections from the posterior, medial, and basolateral amygdala (Pitkanen et al., 2000; Kemppainen et al., 2002) as well as structures associated with the hypothalamic-pituitary adrenal axis (Witter, 1986), which suggests that the ventral hippocampus may also be involved in processing anxiety and fear. However, since we did not observe any effects of MS-275 on anxiety measures in the ventral region, we hypothesized that HDAC I inhibition after conditioning would not have an effect on the conditioning phase in the ventral hippocampus.

In agreement with our predictions, we found no significant effect of MS-275 in the dorsal hippocampus (effect of group: $F_{(1, 33)} = 0.25$, $p = 0.62$, interaction: $F_{(3, 91)} = 0.06$, $p = 0.98$). The MS-275 ($N = 11$) and vehicle control ($N = 11$) groups only showed an effect of session reflecting that

freezing was significantly higher after learning (**Figure 3B**; effect of session: $F_{(3, 91)} = 17.04$, $p < 0.001$; *post hoc* comparisons showed that freezing was significantly higher after conditioning in comparison to baseline in both context A and B, $p < 0.05$). In the ventral groups, we observed a similar pattern. There were also no significant differences between the MS-275 ($N = 13$) and control ($N = 14$) groups (effect of group: $F_{(1, 28)} = 0.51$, $p = 0.48$, interaction: $F_{(3, 75)} = 1.13$, $p = 0.34$), though the groups displayed a significant effect of session, which reflected that freezing changed after learning (**Figure 3C**; effect of session: $F_{(3, 75)} = 27.19$, $p < 0.001$, *post hoc* comparisons showed that freezing was significantly higher after conditioning in comparison to baseline in both context A and B, $p < 0.05$). The fact that all groups exhibited significantly higher freezing in the neutral context B at 24 h compared to baseline indicates that generalization is not produced by HDAC inhibition. Since all animals are restrained during the injections, we suggest that the observed generalization occurs as a result of having an additional stressor following predator odor exposure. These findings indicate that predator odor fear learning provides a good model to study fear generalization.

Discussion

Using predator odor conditioning, we tested the effects of the class I HDAC inhibitor MS-275 in the dorsal and ventral hippocampus on innate anxiety and at different stages of fear learning. We found that HDAC I inhibition after contextual pre-exposure (pre-conditioning) has different effects in the dorsal and ventral hippocampus. In the dorsal hippocampus, HDAC inhibition enhances fear learning, whereas in the ventral hippocampus, it leads to fear generalization. However, this epigenetic mechanism does not affect learning when HDAC inhibition takes place after conditioning. Instead, the presentation of a predator odor followed by a stressor results in fear generalization to a neutral context.

There are several advantages associated with the use of predator odors in fear learning. First, odors are extremely relevant cues to rodents because identifying dangerous odors is critical for survival (Brennan and Keverne, 1997; Luo et al., 2003; Restrepo et al., 2004). Second, predator odors produce innate fear in many species, and thus, are ethologically relevant models (Apfelbach et al., 2005; Rosen et al., 2008; Ferrero et al., 2011). Moreover, predator stress produces long-term changes in behavior, and these changes correlate with persistent alterations in molecular fear and stress pathways (Blanchard and Blanchard, 1989; Adamec and Shallow, 1993; Adamec et al., 1998, 2001; Wiedenmayer, 2004). Here, we demonstrate that predator urine is a good model to study the molecular mechanisms underlying fear generalization.

Field studies have shown that long-lasting smells, such as predator urine, produce long-term avoidance of spatial locations in many mammalian species (Swihart et al., 1991; Rosell, 2001), suggesting that animals can recall spatial locations where these odors have been encountered for long periods of time. In this study, as well as previous ones (Wang et al., 2012, 2013a, 2015), we found that coyote urine produces consistent but moderate levels of freezing, which, at first glance, appears surprising in the context of the effects on behavior observed in field studies. However, we have also previously demonstrated that exposure to predator urine has profound and long-lasting effects on hippocampal spatial representations. Specifically, we showed that the spatial map formed after predator odor exposure stabilizes in the long term (Wang et al., 2012), and these changes can only be reversed when animals learn to perceive the context as safe after extinction (Wang et al., 2015). These findings suggest that while conditioned freezing in response to predator urine is moderate, the neurological changes associated with fear learning induced with long lasting predator smells are persistent.

Prey animals are under significant evolutionary pressure to rapidly identify and avoid novel predators, since unguarded encounters may result in death. Consequently, it has been demonstrated that after exposure to a specific predator, many species are capable of generalizing fear responses to completely novel predators that resemble the one initially encountered (Griffin et al., 2001; Ferrari et al., 2007, 2008). It follows that it would also be important for prey animals to generalize defensive fear responses from a particular dangerous context to novel but similar contexts that may also be unsafe. Fear generalization to environments that resemble one in which a threat is originally encountered may be evolutionarily advantageous, since particular types of predators are frequently found in similar habitats. Our data suggest that epigenetic mechanisms within the ventral hippocampus play a role in this process.

It is important to note that although animals injected with MS-275 in the dorsal hippocampus after contextual pre-exposure (pre-conditioning) display increased freezing in a neutral context, the level of generalization observed in these animals is minimal in comparison to ventrally injected mice. This suggests that these animals still differentiate between the training and neutral contexts. Conversely, animals injected with MS-275 in the ventral region cannot discriminate between neutral and fearful contexts. Several studies support the idea that the ventral hippocampus may play a role in fear generalization. In rats, hippocampal place cells have receptive fields of increasing size moving from the dorsal to the ventral pole (Kjelstrup et al., 2008). We recently showed that the broadly tuned nature of the cells' receptive fields favors the involvement of this region in generalization processes (Keinath et al., 2014). Furthermore, the ability of ventral cells to generalize across situations is modulated by learning (Komorowski et al., 2013). These data suggest that in rodents, the ventral region may be critical for extracting commonalities across situations. Interestingly, studies investigating anatomical differences in humans found that in healthy adults, the anterior hippocampus (ventral in rodents) contains a smaller proportion of dentate gyrus than the posterior hippocampus (dorsal in rodents) (Malykhin et al., 2010). This distinction is remarkable because the dentate gyrus is implicated in pattern separation, the process of distinguishing between similar memories (Marr, 1971; Rolls and Kesner, 2006; Bakker et al., 2008), suggesting that the ability of the hippocampus to discriminate between similar memories may decrease along the dorso-ventral longitudinal axis. In addition, fMRI studies in humans have shown that

the anterior (ventral) and posterior (dorsal) hippocampi are activated in different kinds of recall tasks. Thinking of specific spatial details of an event activates the posterior (dorsal) region, while thinking about the general location of the same event activates the anterior (ventral) area (Poppenk et al., 2013). Therefore, data from both rodents and humans suggest that the dorsal and ventral hippocampus may serve different roles in encoding a representation of context. The dorsal hippocampus encodes particular features and allows animals to discriminate between similar situations, whereas the ventral hippocampus appears to facilitate generalization processes. Here we show that the consolidation of these memories involves epigenetic mechanisms.

Our data indicate that inhibition of class I HDACs does not have an effect on innate anxiety and/or the conditioning phase of predator fear learning. Since previous studies suggest that the ventral hippocampus plays a role in anxiety (Bannerman et al., 2004; Kheirbek et al., 2013), it is possible that other epigenetic mechanims modulate anxiety in this region. MS-275 preferentially inhibits HDAC1 over HDAC2/3, and has no effect on other HDACs (Khan et al., 2008; Formisano et al., 2015). Previous studies have found that HDAC1 regulates DNA repair in neurons (Wang et al., 2013b) and modulates fear extinction (Bahari-Javan et al., 2012), HDAC2 plays an important role in several forms of spatial memory (Guan et al., 2009), and HDAC3 enhances long-term contextual fear memory (McQuown et al., 2011), all of which suggest that distinct epigenetic mechanisms modulate different aspects of memory consolidation. Interestingly, a recent study demonstrated that Class II HDAC inhibitors also regulate hippocampus-dependent learning and plasticity (Kim et al., 2012). It will be important to assess if class II HDACs play a role in the ventral hippocampus and whether this epigenetic pathway affects the conditioning phase or anxiety responses. Understanding the roles played by different hippocampal regions and epigenetic markers in fear learning may shed light on the mechanisms that lead to post-traumatic stress disorder (PTSD) and other anxiety disorders stemming from deficits in contextual learning. Here, we demonstrate that predator odor fear conditioning provides a useful paradigm for understanding these processes.

Author Contributions

JCH, AST, and EGW performed experiments; RKY performed experiments, analyzed data, and wrote the manuscript; IAM designed experiments and supervised analysis and writing of the manuscript.

Funding

This work was supported by the National Science Foundation (NSF) CAREER award to IM (grant number 1256941) and a Ruth L. Kirschstein predoctoral National Research Service Award (NRSA) to RY (grant number 1F31MH105161-01A1).

Acknowledgments

The authors wish to thank Run Jin, Sarah Ly, and Akiff Premjee for their assistance in collecting preliminary data for the manuscript, and Alexander Keinath for his assistance writing code for analysis.

References

Adamec, R. E., Blundell, J., and Collins, A. (2001). Neural plasticity and stress induced changes in defense in the rat. *Neurosci. Biobehav. Rev.* 25, 721–744. doi: 10.1016/S0149-7634(01)00053-7

Adamec, R. E., Burton, P., Shallow, T., and Budgell, J. (1998). NMDA receptors mediate lasting increases in anxiety-like behavior produced by the stress of predator exposure—implications for anxiety associated with posttraumatic stress disorder. *Physiol. Behav.* 65, 723–737. doi: 10.1016/S0031-9384(98)00226-1

Adamec, R. E., and Shallow, T. (1993). Lasting effects on rodent anxiety of a single exposure to a cat. *Physiol. Behav.* 54, 101–109. doi: 10.1016/0031-9384(93)90050-P

Anagnostaras, S. G., Gale, G. D., and Fanselow, M. S. (2001). Hippocampus and contextual fear conditioning: recent controversies and advances. *Hippocampus* 11, 8–17. doi: 10.1002/1098-1063(2001)11:1<8::AID-HIPO1015>3.0.CO;2-7

Apfelbach, R., Blanchard, C. D., Blanchard, R. J., Hayes, R. A., and McGregor, I. S. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neurosci. Biobehav. Rev.* 29, 1123–1144. doi: 10.1016/j.neubiorev.2005.05.005

Bahari-Javan, S., Maddalena, A., Kerimoglu, C., Wittnam, J., Held, T., Bähr, M., et al. (2012). HDAC1 regulates fear extinction in mice. *J. Neurosci.* 32, 5062–5073. doi: 10.1523/jneurosci.0079-12.2012

Bakker, A., Kirwan, C. B., Miller, M., and Stark, C. E. (2008). Pattern separation in the human hippocampal CA3 and dentate gyrus. *Science* 319, 1640–1642. doi: 10.1126/science.1152882

Bannerman, D. M., Rawlins, J. N., McHugh, S. B., Deacon, R. M., Yee, B. K., Bast, T., et al. (2004). Regional dissociations within the hippocampus–memory and anxiety. *Neurosci. Biobehav. Rev.* 28, 273–283. doi: 10.1016/j.neubiorev.2004.03.004

Beckers, T., Burkhardt, C., Wieland, H., Gimmnich, P., Ciossek, T., Maier, T., et al. (2007). Distinct pharmacological properties of second generation HDAC inhibitors with the benzamide or hydroxamate head group. *Int. J. Cancer* 121, 1138–1148. doi: 10.1002/ijc.22751

Blanchard, D. C., Canteras, N. S., Markham, C. M., Pentkowski, N. S., and Blanchard, R. J. (2005). Lesions of structures showing FOS expression to cat presentation: effects on responsivity to a Cat, Cat odor, and nonpredator threat. *Neurosci. Biobehav. Rev.* 29, 1243–1253. doi: 10.1016/j.neubiorev.2005.04.019

Blanchard, R. J., and Blanchard, D. C. (1989). Antipredator defensive behaviors in a visible burrow system. *J. Comp. Psychol.* 103, 70. doi: 10.1037/0735-7036.103.1.70

Blanchard, R. J., and Blanchard, D. C. (1990). "Anti-predator defense as models of animal fear and anxiety," in *Fear and Defence,* eds P. F. Brain, S. Parmigiani, R. J. Blanchard, and D. Mainardi (Amsterdam: Harwood Academic Publishers), 89–108.

Blanchard, R. J., Yang, M., Li, C.-I., Gervacio, A., and Blanchard, D. C. (2001). Cue and context conditioning of defensive behaviors to cat odor stimuli. *Neurosci. Biobehav. Rev.* 25, 587–595. doi: 10.1016/S0149-7634(01)00043-4

Brennan, P. A., and Keverne, E. B. (1997). Neural mechanisms of mammalian olfactory learning. *Prog. Neurobiol.* 51, 457–481. doi: 10.1016/S0301-0082(96)00069-X

Colgin, L. L., Moser, E. I., and Moser, M. B. (2008). Understanding memory through hippocampal remapping. *Trends Neurosci.* 31, 469–477. doi: 10.1016/j.tins.2008.06.008

Crawley, J., and Goodwin, F. K. (1980). Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines.

Pharmacol. Biochem. Behav. 13, 167–170. doi: 10.1016/0091-3057(80)90067-2

Day, J. J., and Sweatt, J. D. (2011). Epigenetic mechanisms in cognition. *Neuron* 70, 813–829. doi: 10.1016/j.neuron.2011.05.019

Dielenberg, R. A., Carrive, P., and McGregor, I. S. (2001). The cardiovascular and behavioral response to cat odor in rats: unconditioned and conditioned effects. *Brain Res.* 897, 228–237. doi: 10.1016/S0006-8993(01)02227-2

Dielenberg, R. A., and McGregor, I. S. (2001). Defensive behavior in rats towards predatory odors: a review. *Neurosci. Biobehav. Rev.* 25, 597–609. doi: 10.1016/S0149-7634(01)00044-6

Fanselow, M., and Rudy, J. (1998). "Convergence of experimental and developmental approaches to animal learning and memory processes," in *Mechanistic Relationships Between Development and Learning*, eds T. J. Carew, R. Menzel, and C. J. Shatz (New York, NY: Wiley), 15–28.

Fanselow, M. S. (1990). Factors governing one-trial contextual conditioning. *Anim. Learn. Behav.* 18, 264–270. doi: 10.3758/BF03205285

Fanselow, M. S. (2000). Contextual fear, gestalt memories, and the hippocampus. *Behav. Brain Res.* 110, 73–81. doi: 10.1016/S0166-4328(99)00186-2

Ferrari, M. C., Gonzalo, A., Messier, F., and Chivers, D. P. (2007). Generalization of learned predator recognition: an experimental test and framework for future studies. *Proc. R. Soc. Lond. B Biol. Sci.* 274, 1853–1859. doi: 10.1098/rspb.2007.0297

Ferrari, M. C., Messier, F., and Chivers, D. P. (2008). Can prey exhibit threat-sensitive generalization of predator recognition? Extending the predator recognition continuum hypothesis. *Proc. R. Soc. Lond. B Biol. Sci.* 275, 1811–1816. doi: 10.1098/rspb.2008.0305

Ferrero, D. M., Lemon, J. K., Fluegge, D., Pashkovski, S. L., Korzan, W. J., Datta, S. R., et al. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11235–11240. doi: 10.1073/pnas.1103317108

Formisano, L., Guida, N., Valsecchi, V., Cantile, M., Cuomo, O., Vinciguerra, A., et al. (2015). Sp3/REST/HDAC1/HDAC2 complex represses and Sp1/HIF-1/p300 complex activates ncx1 gene transcription, in brain ischemia and in ischemic brain preconditioning, by epigenetic mechanism. *J. Neurosci.* 35, 7332–7348. doi: 10.1523/jneurosci.2174-14.2015

Griffin, A. S., Evans, C. S., and Blumstein, D. T. (2001). Learning specificity in acquired predator recognition. *Anim. Behav.* 62, 577–589. doi: 10.1006/anbe.2001.1781

Guan, J.-S., Haggarty, S. J., Giacometti, E., Dannenberg, J.-H., Joseph, N., Gao, J., et al. (2009). HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature* 459, 55–60. doi: 10.1038/nature07925

Hall, C. S. (1934). Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. *J. Comp. Psychol.* 18, 385. doi: 10.1037/h0071444

Hu, E., Dul, E., Sung, C. M., Chen, Z., Kirkpatrick, R., Zhang, G. F., et al. (2003). Identification of novel isoform-selective inhibitors within class I histone deacetylases. *J. Pharmacol. Exp. Ther.* 307, 720–728. doi: 10.1124/jpet.103.055541

Kandel, E. R. (2012). The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. *Mol. Brain* 5:14. doi: 10.1186/1756-6606-5-14

Keinath, A. T., Wang, M. E., Wann, E. G., Yuan, R. K., Dudman, J. T., and Muzzio, I. A. (2014). Precise spatial coding is preserved along the longitudinal hippocampal axis. *Hippocampus* 24, 1533–1548. doi: 10.1002/hipo.22333

Kemppainen, S., Jolkkonen, E., and Pitkänen, A. (2002). Projections from the posterior cortical nucleus of the amygdala to the hippocampal formation and parahippocampal region in rat. *Hippocampus* 12, 735–755. doi: 10.1002/hipo.10020

Khan, N., Jeffers, M., Kumar, S., Hackett, C., Boldog, F., Khramtsov, N., et al. (2008). Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. *Biochem. J.* 409, 581–589. doi: 10.1042/BJ20070779

Kheirbek, M. A., Drew, L. J., Burghardt, N. S., Costantini, D. O., Tannenholz, L., Ahmari, S. E., et al. (2013). Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus. *Neuron* 77, 955–968. doi: 10.1016/j.neuron.2012.12.038

Kim, M. S., Akhtar, M. W., Adachi, M., Mahgoub, M., Bassel-Duby, R., Kavalali, E. T., et al. (2012). An essential role for histone deacetylase 4 in synaptic plasticity and memory formation. *J. Neurosci.* 32, 10879–10886. doi: 10.1523/jneurosci.2089-12.2012

Kjelstrup, K. B., Solstad, T., Brun, V. H., Hafting, T., Leutgeb, S., Witter, M. P., et al. (2008). Finite scale of spatial representation in the hippocampus. *Science* 321, 140–143. doi: 10.1126/science.1157086

Komorowski, R. W., Garcia, C. G., Wilson, A., Hattori, S., Howard, M. W., and Eichenbaum, H. (2013). Ventral hippocampal neurons are shaped by experience to represent behaviorally relevant contexts. *J. Neurosci.* 33, 8079–8087. doi: 10.1523/jneurosci.5458-12.2013

Levenson, J. M., and Sweatt, J. D. (2005). Epigenetic mechanisms in memory formation. *Nat. Rev. Neurosci.* 6, 108–118. doi: 10.1038/nrn1604

Luo, M., Fee, M. S., and Katz, L. C. (2003). Encoding pheromonal signals in the accessory olfactory bulb of behaving mice. *Sci. Signal.* 299, 1196. doi: 10.1126/science.1082133

Malykhin, N. V., Lebel, R. M., Coupland, N., Wilman, A. H., and Carter, R. (2010). *In vivo* quantification of hippocampal subfields using 4.7 T fast spin echo imaging. *Neuroimage* 49, 1224–1230. doi: 10.1016/j.neuroimage.2009.09.042

Maren, S., and Holt, W. (2000). The hippocampus and contextual memory retrieval in Pavlovian conditioning. *Behav. Brain Res.* 110, 97–108. doi: 10.1016/S0166-4328(99)00188-6

Marr, D. (1971). Simple memory: a theory for archicortex. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 262, 23–81. doi: 10.1098/rstb.1971.0078

Masini, C. V., Garcia, R. J., Sasse, S. K., Nyhuis, T. J., Day, H. E., and Campeau, S. (2010). Accessory and main olfactory systems influences on predator odor-induced behavioral and endocrine stress responses in rats. *Behav. Brain Res.* 207, 70–77. doi: 10.1016/j.bbr.2009.09.038

McQuown, S. C., Barrett, R. M., Matheos, D. P., Post, R. J., Rogge, G. A., Alenghat, T., et al. (2011). HDAC3 is a critical negative regulator of long-term memory formation. *J. Neurosci.* 31, 764–774. doi: 10.1523/jneurosci.5052-10.2011

Nadel, L., Hoscheidt, S., and Ryan, L. R. (2013). Spatial cognition and the hippocampus: the anterior–posterior axis. *J. Cogn. Neurosci.* 25, 22–28. doi: 10.1162/jocn_a_00313

O'Keefe, J., and Dostrovsky, J. (1971). The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res.* 34, 171–175. doi: 10.1016/0006-8993(71)90358-1

O'Reilly, R. C., and Rudy, J. W. (2001). Conjunctive representations in learning and memory: principles of cortical and hippocampal function. *Psychol. Rev.* 108, 311. doi: 10.1037/0033-295X.108.2.311

Peirce, J. L., Chesler, E. J., Williams, R. W., and Lu, L. (2003). Genetic architecture of the mouse hippocampus: identification of gene loci with selective regional effects. *Genes Brain Behav.* 2, 238–252. doi: 10.1034/j.1601-183X.2003.00030.x

Peixoto, L., and Abel, T. (2013). The role of histone acetylation in memory formation and cognitive impairments. *Neuropsychopharmacology* 38, 62–76. doi: 10.1038/npp.2012.86

Pikkarainen, M., Rönkkö, S., Savander, V., Insausti, R., and Pitkänen, A. (1999). Projections from the lateral, basal, and accessory basal nuclei of the amygdala to the hippocampal formation in rat. *J. Comp. Neurol.* 403, 229–260.

Pitkanen, A., Pikkarainen, M., Nurminen, N., and Ylinen, A. (2000). Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. *Ann. N.Y. Acad. Sci.* 911, 369–391. doi: 10.1111/j.1749-6632.2000.tb06738.x

Poppenk, J., Evensmoen, H. R., Moscovitch, M., and Nadel, L. (2013). Long-axis specialization of the human hippocampus. *Trends Cogn. Sci.* 17, 230–240. doi: 10.1016/j.tics.2013.03.005

Powers, M. M., and Clark, G. (1955). An evaluation of cresyl echt violet acetate as a Nissl stain. *Stain Technol.* 30, 83–88. doi: 10.3109/10520295509113749

Prut, L., and Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur. J. Pharmacol.* 463, 3–33. doi: 10.1016/S0014-2999(03)01272-X

Restrepo, D., Arellano, J., Oliva, A. M., Schaefer, M. L., and Lin, W. (2004). Emerging views on the distinct but related roles of the main and accessory olfactory systems in responsiveness to chemosensory signals in mice. *Horm. Behav.* 46, 247–256. doi: 10.1016/j.yhbeh.2004.02.009

Rolls, E. T., and Kesner, R. P. (2006). A computational theory of hippocampal function, and empirical tests of the theory. *Prog. Neurobiol.* 79, 1–48. doi: 10.1016/j.pneurobio.2006.04.005

Rosell, F. (2001). Effectiveness of predator odors as gray squirrel repellents. *Can. J. Zool.* 79, 1719–1723. doi: 10.1139/z01-116

Rosen, J. B. (2004). The neurobiology of conditioned and unconditioned fear: a neurobehavioral system analysis of the amygdala. *Behav. Cogn. Neurosci. Rev.* 3, 23–41. doi: 10.1177/1534582304265945

Rosen, J. B., Pagani, J. H., Rolla, K. L., and Davis, C. (2008). Analysis of behavioral constraints and the neuroanatomy of fear to the predator odor trimethylthiazoline: a model for animal phobias. *Neurosci. Biobehav. Rev.* 32, 1267–1276. doi: 10.1016/j.neubiorev.2008.05.006

Rudy, J. W., Huff, N. C., and Matus-Amat, P. (2004). Understanding contextual fear conditioning: insights from a two-process model. *Neurosci. Biobehav. Rev.* 28, 675–685. doi: 10.1016/j.neubiorev.2004.09.004

Rudy, J. W., and O'Reilly, R. C. (2001). Conjunctive representations, the hippocampus, and contextual fear conditioning. *Cogn. Affect. Behav. Neurosci.* 1, 66–82. doi: 10.3758/CABN.1.1.66

Rudy, J. W., and O'Reilly, R. C. (1999). Contextual fear conditioning, conjunctive representations, pattern completion, and the hippocampus. *Behav. Neurosci.* 113, 867. doi: 10.1037/0735-7044.113.5.867

Sánchez, C. (1995). Serotonergic mechanisms involved in the exploratory behaviour of mice in a fully automated two−compartment black and white test box. *Pharmacol. Toxicol.* 77, 71–78. doi: 10.1111/j.1600-0773.1995.tb01916.x

Sánchez, C. (1997). Acute stress enhances anxiolytic-like drug responses of mice tested in a black and white test box. *Eur. Neuropsychopharmacol.* 7, 283–288. doi: 10.1016/S0924-977X(97)00035-7

Simonini, M. V., Camargo, L. M., Dong, E., Maloku, E., Veldic, M., Costa, E., et al. (2006). The benzamide MS-275 is a potent, long-lasting brain region-selective inhibitor of histone deacetylases. *Proc. Natl. Acad. Sci. U.S.A.* 103, 1587–1592. doi: 10.1073/pnas.0510341103

Swihart, R. K., Pignatello, J. J., and Mattina, M. J. (1991). Aversive responses of white-tailed deer, *Odocoileus virginianus*, to predator urines. *J. Chem. Ecol.* 17, 767–777. doi: 10.1007/BF00994199

Takahashi, L. K., Chan, M. M., and Pilar, M. L. (2008). Predator odor fear conditioning: current perspectives and new directions. *Neurosci. Biobehav. Rev.* 32, 1218–1227. doi: 10.1016/j.neubiorev.2008.06.001

Takahashi, L. K., Hubbard, D. T., Lee, I., Dar, Y., and Sipes, S. M. (2007). Predator odor-induced conditioned fear involves the basolateral and medial amygdala. *Behav. Neurosci.* 121, 100. doi: 10.1037/0735-7044.121.1.100

Vecsey, C. G., Hawk, J. D., Lattal, K. M., Stein, J. M., Fabian, S. A., Attner, M. A., et al. (2007). Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. *J. Neurosci.* 27, 6128–6140. doi: 10.1523/jneurosci.0296-07.2007

Wallace, K. J., and Rosen, J. B. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behav. Neurosci.* 114, 912. doi: 10.1037/0735-7044.114.5.912

Wang, M. E., Fraize, N. P., Yin, L., Yuan, R. K., Petsagourakis, D., Wann, E. G., et al. (2013a). Differential roles of the dorsal and ventral hippocampus in predator odor contextual fear conditioning. *Hippocampus* 23, 451–466. doi: 10.1002/hipo.22105

Wang, M. E., Wann, E. G., Yuan, R. K., Álvarez, M. M., Stead, S. M., and Muzzio, I. A. (2012). Long-term stabilization of place cell remapping produced by a fearful experience. *J. Neurosci.* 32, 13. doi: 10.1523/jneurosci.0480-12.2012

Wang, M. E., Yuan, R. K., Keinath, A. T., Álvarez, M. M., and Muzzio, I. A. (2015). Extinction of learned fear induces Hippocampal place cell remapping. *J. Neurosci.* 35, 9122–9136. doi: 10.1523/jneurosci.4477-14.2015

Wang, W. Y., Pan, L., Su, S. C., Quinn, E. J., Sasaki, M., Jimenez, J. C., et al. (2013b). Interaction of FUS and HDAC1 regulates DNA damage response and repair in neurons. *Nat. Neurosci.* 16, 1383–1391. doi: 10.1038/nn.3514

Wiedenmayer, C. P. (2004). Adaptations or pathologies? Long-term changes in brain and behavior after a single exposure to severe threat. *Neurosci. Biobehav. Rev.* 28, 1–12. doi: 10.1016/j.neubiorev.2003.09.005

Witter, M. P. (1986). A survey of the anatomy of the hippocampal formation, with emphasis on the septotemporal organization of its intrinsic and extrinsic connections. *Adv. Exp. Med. Biol.* 203, 67–82. doi: 10.1007/978-1-4684-7971-3_5

Witter, M. P., Canto, C. B., Couey, J. J., Koganezawa, N., and O'Reilly, K. C. (2014). Architecture of spatial circuits in the hippocampal region. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369:20120515. doi: 10.1098/rstb.2012.0515

Wood, M. A., Hawk, J. D., and Abel, T. (2006). Combinatorial chromatin modifications and memory storage: a code for memory? *Learn. Mem.* 13, 241–244. doi: 10.1101/lm.278206

Young, S. L., Bohenek, D. L., and Fanselow, M. S. (1994). NMDA processes mediate anterograde amnesia of contextual fear conditioning induced by hippocampal damage: immunization against amnesia by context preexposure. *Behav. Neurosci.* 108, 19. doi: 10.1037/0735-7044.108.1.19

Zangrossi, H. Jr., and File, S. E. (1992). Behavioral consequences in animal tests of anxiety and exploration of exposure to cat odor. *Brain Res. Bull.* 29, 381–388. doi: 10.1016/0361-9230(92)90072-6

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 06 October 2015
doi: 10.3389/fnins.2015.00336

Avoidance and contextual learning induced by a kairomone, a pheromone and a common odorant in female CD1 mice

Lluís Fortes-Marco[1,2†], *Enrique Lanuza*[2], *Fernando Martínez-García*[1] and *Carmen Agustín-Pavón*[1*]

[1] Unitat Pre-departamental de Medicina, Facultad de Ciencias de la Salud, Universitat Jaume I, Castelló de la Plana, Spain,
[2] Departament de Biologia Cel·lular, Facultat de Ciències Biològiques, Universitat de València, València, Spain

OPEN ACCESS

Edited by:
Thomas Endres,
Otto-von-Guericke University,
Germany

Reviewed by:
Jeffrey B Rosen,
University of Delaware, USA
Pablo Chamero,
University of Saarland, Germany

***Correspondence:**
Carmen Agustín-Pavón,
Unitat Pre-departamental de Medicina, Universitat Jaume I, Av. de Vicent Sos Baynat, s/n, 12071 Castelló de la Plana, Spain
deagusti@uji.es

†Present Address:
Lluís Fortes-Marco,
Departamento de Neurobiología Molecular, Celular y del Desarrollo. Instituto Cajal, Madrid, Spain

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: 25 June 2015
Accepted: 07 September 2015
Published: 06 October 2015

Citation:
Fortes-Marco L, Lanuza E, Martínez-García F and Agustín-Pavón C (2015) Avoidance and contextual learning induced by a kairomone, a pheromone and a common odorant in female CD1 mice. Front. Neurosci. 9:336. doi: 10.3389/fnins.2015.00336

Chemosignals mediate both intra- and inter-specific communication in most mammals. Pheromones elicit stereotyped reactions in conspecifics, whereas kairomones provoke a reaction in an allospecific animal. For instance, predator kairomones elicit anticipated defensive responses in preys. The aim of this work was to test the behavioral responses of female mice to two chemosignals: 2-heptanone (2-HP), a putative alarm pheromone, and 2,4,5-trimethylthiazoline (TMT), a fox-derived putative kairomone, widely used to investigate fear and anxiety in rodents. The banana-like odorant isoamyl acetate (IA), unlikely to act as a chemosignal, served as a control odorant. We first presented increasing amounts of these odorants in consecutive days, in a test box in which mice could explore or avoid them. Female mice avoided the highest amounts of all three compounds, with TMT and IA eliciting avoidance at lower amounts (3.8 pmol and 0.35 μmol, respectively) than 2-HP (35 μmol). All three compounds induced minimal effects in global locomotion and immobility in this set up. Further, mice detected 3.5 pmol of TMT and IA in a habituation–dishabituation test, so avoidance of IA started well beyond the detection threshold. Finally, both TMT and IA, but not 2-HP, induced conditioned place avoidance and increased immobility in the neutral compartment during a contextual memory test. These data suggest that intense odors can induce contextual learning irrespective of their putative biological significance. Our results support that synthetic predator-related compounds (like TMT) or other intense odorants are useful to investigate the neurobiological basis of emotional behaviors in rodents. Since intense odorants unlikely to act as chemosignals can elicit similar behavioral reactions than chemosignals, we stress the importance of using behavioral measures in combination with other physiological (e.g., hormonal levels) or neural measures (e.g., immediate early gene expression) to establish the ethological significance of odorants.

Keywords: aversion, isoamyl acetate, 2,4,5-trimethylthiazoline, 2-heptanone, kairomones, pheromones, place conditioning, vomeronasal

Introduction

Rodents are widely used in studies on the neurobiological basis of emotional behaviors. Chemical signals are the most relevant sensory cues for rodents, capable of eliciting strong emotional responses in them. For example, chemical signals such as alarm pheromones and predator kairomones are anxiogenic for mice and rats. Alarm pheromones are substances released by an injured or threatened animal and detected by conspecifics (Gutiérrez-García et al., 2007; Brechbühl et al., 2013), whereas predator kairomones elicit defensive responses in preys (for a review, see Fortes-Marco et al., 2013). Thus, experiments exposing mice and rats to alarm pheromones and kairomones are valuable to investigate the neural circuits controlling fear and anxiety, key features of pathologies such as generalized anxiety disorder, depression, or post-traumatic stress disorder.

The volatile chemical 2-heptanone (2-HP) has been proposed to act as an alarm substance in bees (Collins et al., 1989) and rats (Gutiérrez-García et al., 2007). This ketone is a component of the urine of rats, and its concentration is higher in stressed individuals. Urine from stressed rats or 2-HP alone induce stress-like reactions in recipient subjects (Gutiérrez-García et al., 2006), and despair in rats subjected to the forced swim paradigm (Gutiérrez-García et al., 2007). In mice, the concentration of 2-HP in urine is dependent on the adrenal gland (Novotny et al., 1986), and it acts as a puberty modulator in females in a blend with other chemicals (Novotny et al., 1986; Jemiolo et al., 1989). Thus, 2-HP might be classified as a trigger pheromone for rats and a primer pheromone for mice, following the definition by McClintock (2002). To our knowledge, the behavioral responses of mice to 2-HP have not been characterized yet, and most of the studies using 2-HP have focused on the mechanism of detection (Boschat et al., 2002; Thompson et al., 2004). Interestingly, a recent paper suggests that, in mice, some substances proposed as alarm pheromones, like 2-sec-butyl-4,5-dihydrothiazole, share structural similarity with kairomones (but see Jemiolo et al., 1985 on the possible role of this chemical as a sexual pheromone). Thus, alarm pheromones and kairomones might act using the same neural circuits conveying signals of danger (Brechbühl et al., 2013).

Cat fur and feces are potential sources of kairomones for rodents. Indeed, cat odors consistently induce biochemical and behavioral measures of stress in mice and rats, such as elevation of plasma glucocorticoids, fear responses such as freezing, avoidance, and contextual memory (Berton et al., 1998; Muñoz-Abellán et al., 2008, 2009). However, the components of cat fur odor and feces are diverse, not yet fully characterized, and the reaction to cat odor depends on the donor cat (Muñoz-Abellán et al., 2008) and its diet (Berton et al., 1998; Ferrero et al., 2011). Conversely, specific odorants derived from predator sources would offer the advantage of a controllable presentation to produce reproducible results.

The synthetic molecule 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), a component isolated from fox feces (Vernet-Maury, 1980), offers the mentioned advantages. TMT induces freezing, contextual learning, and anxiety-related behavioral changes in rodents (Endres and Fendt, 2007, 2009). Moreover, TMT activates brain regions related to stress and anxiety, although the activation pattern differs from that induced by cat odor (Dielenberg et al., 2001; Staples et al., 2008; Janitzky et al., 2015; Pérez-Gómez et al., 2015). This fact, together with the fact that both unconditioned responses to TMT and its ability to support conditioning are dependent on the environment and protocol used (Wallace and Rosen, 2000; Morrow et al., 2002; Blanchard et al., 2003; Endres and Fendt, 2007), have led to some debate on the kairomonal or simply aversive nature of this molecule (Fendt and Endres, 2008; Fortes-Marco et al., 2013).

In this vein, we have previously hypothesized that the exposure to intense odorants might be indeed very aversive, so it could elicit similar anxiety-like responses to the ones elicited by kairomones and alarm pheromones in mice—not necessarily accompanied by behavioral components of fear i.e., freezing (Fortes-Marco et al., 2013).

Thus, we sought to compare the behavioral reactions of female mice to increasing amounts of TMT, 2-HP and isoamyl acetate (IA), a banana-like odorant frequently used as a control odor (Wallace and Rosen, 2000; Root et al., 2014) in two-choice tests. Previous studies from our lab have validated these tests to investigate the attractive properties of male pheromones for female mice (Agustín-Pavón et al., 2007, 2014; Martínez-Ricós et al., 2007). We hypothesized that the putative kairomone TMT would be avoided at detectable concentrations. The putative pheromone 2-HP might be either attractive or aversive depending on its role—alarm pheromone or puberty modulator, see above. Finally, the control odorant should not elicit avoidance, except perhaps at high concentrations. We also hypothesized that sufficiently aversive olfactory stimulation might be able to support learning in the animals. Thus, we tested whether repeated exposure to the concentrations eliciting the maximum behavioral reaction could induce a conditioned contextual avoidance in mice.

Materials and Methods

Animals

For the present experiments, we used 125 adult female CD1 mice (8–18 weeks old, Janvier SAS, St Berthevin Cedex, France). Animals were housed under controlled temperature (22–24°C) and a light-dark cycle 12:12 (light from 08:00 to 20:00), with food and water *ad libitum*. Animals were treated throughout according to the European Communities Council Directive of (86/609/EEC), and the protocols were approved by the Committee of Ethics on Animal Experimentation of the University of Valencia.

Odorants

We used three odorants: 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; Contech, Victoria, Canada), a putative kairomone found in fox feces and detected by the main olfactory system and the Grueneberg ganglion (Brechbühl et al., 2013), 2-heptanone (2-HP, Sigma-Aldrich, Schnelldorf, Germany), a putative mouse pheromone detected by the main and accessory olfactory epithelia (Thompson et al., 2004; Xu et al., 2005), and isoamyl acetate (IA, Panreac Quimica SA, Barcelona, Spain), a control

odorant detected by the main olfactory system (Xu et al., 2005) and unlikely to participate in intra or interspecific communication (Root et al., 2014). We diluted the odorants to the desired concentration, with PBS with Triton X-100 1%, (pH = 7.4) for 2-HP and TMT and mineral oil for IA. We selected these solvents because the odorants were more readily diluted in them than in distilled water. In addition, mice were not able to detect differences between the odorants diluted in the solvents or in distilled water (Supplementary Material, Experiment S1). Also, the solvents did not elicit enhanced chemoinvestigation (see Section Results and **Figure 2**).

Behavioral Tests

Experiment 1. Behavioral Effects of Increasing Amount of Odorants

To check the behavioral reaction of mice toward each amount of odorant in two-choice tests, subjects were randomly assigned to four groups. Each group was presented with one of the odorants or PBS (control) (TMT $n = 12$, 2-HP $n = 11$, IA $n = 12$, PBS $n = 12$). The behavioral test was performed in a methacrylate opaque box (45 × 47.5 × 22.5 cm), divided in two identical chambers by a panel with a door to minimize diffusion of the odors. Each chamber had one perforated stainless steel capsule (JP Selecta, Abrera, Barcelona, Spain) attached to the floor with double-sided adhesive tape. We prepared serial dilutions of each of the odorants, from the pure substance to 10^{-8}, and pipetted 5 μl of the corresponding solution in a piece of filter paper (2 × 2 cm) inside each capsule. The capsule on the stimulus chamber contained 5 μl of the corresponding odorant, whereas the capsule on the neutral chamber contained the corresponding solvent, except in the PBS group, in which both capsules contained 5 μl of PBS.

Animals were habituated to the experimenter and apparatus for 10 min for 3 days. The fourth day, we performed a 5-min test (control), in which both capsules contained the correspondent solvent (PBS with Triton X-100 or mineral oil, see above) or PBS. On the following days, mice were exposed to the 5 μL of the relevant chemical stimulus at increasing concentrations at the stimulus chamber (exposure days 1–9), and 5 μL of the corresponding solvent in the capsule at the neutral chamber. All odorants have similar molecular weight and density (TMT 129.2, 1 g/ml; 2-HP 114.2, 0.8 g/ml; IA 130.2, 0.9 g/ml), so the molar concentration of the pure substance is similar (TMT 7.7 M, 2-HP and IA, 7 M). Thus, the first exposure day, mice had access to 0.38 pmol of TMT or 0.35 pmol of 2-HP and IA, and the amount of odorant was increased 10-fold each day to the pure substance, i.e., 38 μmol of TMT or 35 μmol of 2-HP and IA. To avoid diffusion of the odorant and facilitate exhausting the volatiles of the room, we performed the tests in a room with mild negative air pressure. Experimental cages were thoroughly cleaned after each test, and each stimulus was used in a different room.

Experiment 2. Habituation–Dishabituation Tests

To establish the detection threshold of each of the odorants, we submitted three separate groups of mice ($n = 6$ for each odorant) to habituation–dishabituation tests, following Agustín-Pavón et al. (2007).

The tests were performed in a squared opaque methacrylate box (25 cm^2) with a hole at 8 cm from the floor in one of the walls. Females were placed in the test box 3 min before the test for habituation. The stimuli were presented to the mice in a stick with a cotton swab at the tip, which was introduced through the hole and fixed to the box wall. We presented the mice with three consecutive 1-min presentations of the swab impregnated with 5 μl of water, followed by three consecutive presentations of 5 μl each odorant amount. Between each odorant amount, mice were presented with three consecutive 1-min presentations of the corresponding solvent. Mice investigate more each stick when presented for the first time or when its odor changes, and investigation decreases in successive presentations. Thus, this is a reliable method to investigate the detection threshold of odorants.

Experiment 3. Place Conditioning Test

To test whether repeated exposure to the odorants in the same location would induce learning, we performed a place conditioning test following the protocol described in Martínez-Ricós et al. (2007). Animals were randomly distributed in three groups, (TMT, $n = 11$; 2-HP, $n = 12$, IA, $n = 12$). We used the same test box as in Experiment 1, so that animals could freely explore both chambers. Animals were habituated to the experimental conditions for 10 min for 2 days. The third day, we put 5 μL PBS in each capsule and recorded the behavior of the animals for 5 min (control). From the next day, mice had access for four consecutive days (training days, 1–4) to 5 μL of their corresponding odorant at pure concentration in the stimulus chamber and to PBS in the neutral chamber, for 5 min. The day after the last training session, we evaluated the induction of contextual memory (test) with PBS in each capsule.

Behavioral Measures

All tests were video-recorded and the videos were analyzed with the video-tracking software SMART v2.5.11 (Panlab, Cornella, Spain). For experiments 1 and 3, we defined an area of interest covering 25% of each chamber surface, as a circular region of 12 cm of radius surrounding the center of the capsule (stimulus or neutral zones). This zone ensured the detection of the animal in close proximity of the stimuli.

For each stimulus and test, we obtained data from time spent in the area of interest and distance traveled in cm. Informal observations by a trained observer who was blind to the experimental conditions revealed lack of risk assessment postures or freezing, so we used the percentage of time that the animal moved at a speed <1 mm/s as an approximate measure of immobility (Fortes-Marco et al., 2013). As a measure of attraction/avoidance, we calculated an avoidance ratio as the ratio between the time spent on the stimulus zone and the total time spent in the neutral plus the stimulus zone (see Agustín-Pavón et al., 2014). A value of 0.5 of this avoidance ratio indicates that the stimulus is neither attractive nor avoided, whereas an avoidance ratio <0.5 reveals avoidance of the stimuli. For experiment 2, the tests were videotaped and an observer blind to the experimental conditions measured the time that females

spent rearing on their hind limbs and actively sniffing at the cotton tip.

Statistical Analysis

Data were analyzed with R statistical software (v. 3.1.2, http://www.R-project.org/) and IBM SPSS 22.0. We checked the normality and homocedasticity of the data by means of a Kolmogorov–Smirnov and Levene test. Data from Experiment 1 were analyzed by means of ANOVA for repeated measures, with the factors DAY/CONCENTRATION (for the avoidance ratio) and ZONE (for time spent in the zones) as within-subject factors, followed by Dunnet *post-hoc* comparisons (to compare exposure days with the control condition) or *post-hoc* pairwise comparisons with the Bonferroni correction. Data from Experiment 2 were analyzed by means of paired Student's *t*-tests between the last presentation of each solvent and the first presentation of the following odorant. Data from Experiment 3 were analyzed by means of paired Student's *t*-test (avoidance ratio in control vs. place avoidance test), repeated measures ANOVA (distance traveled and immobility) with DAY and ZONE as within-subjects factors, and repeated measures ANOVA (avoidance ratio during exposure days) with DAY as within-subject factor and ODOR as between-subjects factor.

Results

Experiment 1. Behavioral Effects of Increasing Amount of Odorants

The aim of the first experiment was to determine the range of amounts at which each odor would elicit a measurable behavioral reaction in mice, including attraction/avoidance, distance traveled, and immobility, in two-choice tests. To ensure that there was no *a priori* preference of the animals for any compartment—this was unlikely, since both were identical-, or some habituation process that would affect the activity of the animals across tests, we run group of animals that were exposed to PBS in each zone for 10 consecutive days. None of the behavioral measures varied across days in these mice exposed to PBS alone, so any behavioral changes in the mice exposed to the different odorants could be attributed to the effects of the stimuli. (Supplementary Material, Experiment S2).

The avoidance ratio for the group exposed to TMT was significantly different from control at the highest amount of TMT used [repeated measures ANOVA, factor CONCENTRATION, $F_{(9,\ 99)} = 4.9$, $p < 0.001$; *post-hoc* comparison between exposure day 9 (38 μmol of TMT) vs. control (no TMT), $p = 0.009$; **Figure 1A**]. We further checked whether there was a difference in the raw time that animals spent in each zone. There was a significant decrease of the time spent in the stimulus zone when it contained 38 μmol of TMT with respect to the stimulus zone in the control day [repeated measures ANOVA, CONCENTRATION × ZONE, $F_{(9,\ 99)} = 3.6$, $p < 0.001$; *post-hoc* comparison for exposure day 9, stimulus zone vs. control, $p = 0.001$; **Figure 1B**]. Still, *post-hoc* pairwise comparisons between zones showed that time spent in the TMT zone was lower with respect to the neutral zone during exposure days 2, 4, 8, and 9 (3.8 pmol, $p = 0.023$; 0.38 nmol, $p = 0.015$; 3.8 μmol, $p = 0.002$; and 38 μmol, $p < 0.001$; **Figure 1B**). These results suggest that mice were able to detect TMT from 3.8 pmol, since this amount induced a slight avoidance reaction (**Figure 1B**), but TMT was strongly avoided at pure concentration only as measured with both time spent in zones and avoidance ratio.

The putative pheromone 2-HP induced avoidance at the highest amount presented [repeated measures ANOVA of the avoidance ratio, CONCENTRATION, $F_{(9,\ 90)} = 9.4$, $p < 0.001$, *post-hoc* comparison exposure day 9 vs. control, $p = 0.001$; **Figure 1C**]. Further, the ANOVA of the raw time spent in zones and subsequent *post-hoc* tests revealed an increase in time spent in the neutral zone and a decrease in the stimulus zone with 35 μmol of 2-HP [CONCENTRATION × ZONE, $F_{(9,\ 90)} = 7.4$, $p < 0.001$, *post-hoc* exposure day 9 vs. control, neutral zone $p = 0.009$, stimulus zone $p = 0.03$; **Figure 1D**]. Pairwise *post-hoc* comparisons between zones confirmed a significant difference of time spent in stimulus vs. neutral zone in exposure day 9 only ($p < 0.001$).

Finally, mice also avoided IA at the highest amount presented [repeated measures ANOVA for the avoidance ratio, CONCENTRATION, $F_{(9,\ 90)} = 9.1$, $p < 0.001$; *post-hoc* comparison of exposure day 9 vs. control, $p = 0.048$; **Figure 1E**]. For the time spent in each zone, there was a decrease in time spent in the stimulus zone in the presence of 35 μmol of IA with respect to the control [ANOVA, CONCENTRATION × ZONE, $F_{(9,\ 90)} = 5.83$, $p < 0.001$, *post-hoc* comparison of exposure day 9 vs. control, $p = 0.002$]. In addition, *post-hoc* pairwise comparisons revealed significantly lower time spent in the stimulus zone as compared to the neutral zone when IA was presented from 0.35 to 35 μmol ($p = 0.014$; $p = 0.004$; $p < 0.001$, respectively; **Figure 1F**). Thus, the pattern of avoidance of IA resembles that of TMT.

Surprisingly, an ANOVA between tests revealed that TMT did not significantly affect distance traveled [$F_{(9,90)} = 1.7$, $p = 0.11$] or percentage of immobility [$F_{(9,90)}$=1.5,$p = 0.22$]. By contrast, distance traveled was, overall, significantly decreased by the increasing concentrations of 2-HP [DAY effect, $F_{(9,\ 90)} = 4.0$, $p < 0.001$]. *Post-hoc* comparisons revealed significant decreases in the presence of 0.35 nmol to 3.5 μmol of 2-HP with respect to the control (all $p < 0.05$). The percentage of immobility was also significantly different across tests [DAY, $F_{(9,\ 90)} = 2.2$, $p = 0.027$]. Thus, 2-HP was avoided by mice at the highest presented amount, but concentration-dependently affected the activity of the animals. Finally, the exposure to IA significantly decreased the distance traveled and increased immobility globally across tests [$F_{(9,\ 90)} = 3.4$, $p = 0.018$, $F_{(9,\ 90)} = 3.3$, $p = 0.026$; **Table 1**].

Experiment 2. Habituation–dishabituation test

Results from Experiment 1 suggested that mice could detect 3.8 pmol of TMT, since this was the minimal amount that elicited a slight avoidance response. However, both 2-HP and IA were not avoided until we presented 35 and 0.35 μmol, respectively. To investigate whether odorants affected the behavior of mice at their olfactory detection threshold or beyond, we carried

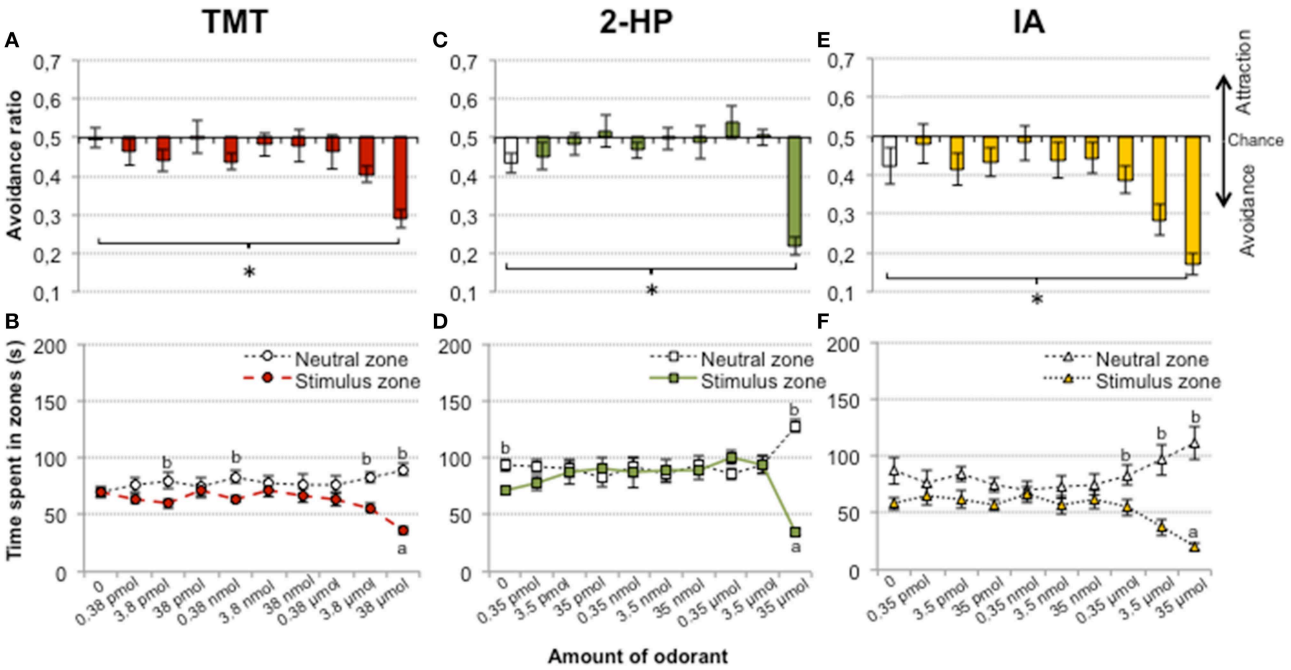


FIGURE 1 | TMT, 2-HP, and IA induced avoidance in a concentration-dependent way. Bar and line charts representing the avoidance ratio and time spent in each zone for increasing concentrations of each odorant. For all three odorants, the avoidance ratio was significantly different from the control situation (no odor) only when pure substance was presented (**A,C,E**, *$p < 0.05$). Time spent by the animals at the stimulus zone was dose-dependently decreased by TMT with respect to the neutral zone **(B)**, whereas 2-HP did not induce avoidance except when presented pure **(D)**. Finally, IA produced a pattern of avoidance similar to that of TMT **(F)**. Comparison between the stimulus zone with the same zone in the control (no odor): a, $p < 0.05$. Comparison between the stimulus and the neutral zones: b, $p < 0.05$. Data are represented as mean ± SEM.

out a habituation–dishabituation test. The analysis comparing the last presentation of the cotton swab impregnated with solvent with the first presentation of each amount of odorant tested revealed that female mice detected 3.8 pmol of TMT, since this amount increased investigation of the cotton swab ($p = 0.002$; **Figure 2A**). By contrast, 2-HP did not significantly increased investigation until 35 µmol were presented ($p = 0.039$; **Figure 2B**). Finally, 3.5 pmol of IA significantly increased chemoinvestigation ($p = 0.005$). Thus, we confirmed that mice detected 3.8 pmol of TMT, although avoidance behavior was not strong with this amount of odorant. Further, in agreement with the avoidance expressed by mice in Experiment 1, 2-HP did not significantly enhanced chemoinvestigation until presented at the maximum amount (see also Supplementary Material). By contrast, IA was detected in the habituation–dishabituation test much more diluted than it was avoided in Experiment 1. These results also raise the possibility that learning could contribute to the strong avoidance displayed toward TMT and IA at the highest amount used. Finally, this experiment confirmed that the solvents did not elicit chemoinvestigation (**Figure 2**).

Experiment 3. Place Conditioning Test

We next checked whether the repeated exposure to the different stimuli was able to induce the formation of a contextual memory. To do so, we used the undiluted odorants, which were equally avoided by mice, in a place conditioning experiment.

Mice of the TMT group expressed a conditioned avoidance for the stimulus chamber after four consecutive days of exposure to this kairomone (Student's *t*-test of the avoidance ratio control vs. place conditioning test, $p = 0.029$; **Figure 3A**). Moreover, a Student's *t*-test against the chance value 0.5 revealed that the avoidance ratio was significantly different from chance in the test ($p = 0.027$) but not the pre-training control ($p = 0.64$). However, neither distance traveled nor global percentage of immobility were significantly different between the control and the place conditioning test ($p = 0.7$, **Figure 3B**). We further explored whether the percentage of immobility would be dependent on the zone, i.e., whether mice would stay inactive in the neutral or the stimulus zone (Fortes-Marco et al., 2013). Indeed, there was a significant difference between zones in the place conditioning test, driven by an increase in the percentage of immobility in the neutral zone in the test with respect to control [repeated measures ANOVA, DAY × ZONE, $F_{(1, 10)} = 8.7$, $p = 0.014$, *post-hoc* stimulus vs. neutral zone in the test, $p = 0.002$; *post-hoc* time spent in neutral zone in control vs. test, $p = 0.017$; **Figure 3C**].

In spite of being strongly avoided at the highest amount presented, 2-HP did not induce contextual memory (avoidance ratio, control vs. test $p = 0.99$; **Figure 3D**). Distance traveled and immobility were not significantly affected either ($p = 0.068$ and 0.094, respectively; **Figures 3E,F**).

Finally, in the group exposed to IA the decrease of the avoidance ratio did not reach statistical significance with respect to the control ($p = 0.067$; **Figure 3G**). However, a Student's

TABLE 1 | Exposure to the TMT did not significantly affect distance traveled or immobility, whereas both 2-HP and IA decreased distance traveled and increased percentage of immobility across tests.

Odorant		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
		0	0.35–0.38 pmol	3.5–3.8 pmol	35–38 pmol	0.35–0.38 nmol	3.5–3.8 nmol	35–38 nmol	0.35–0.38 µmol	3.5–3.8 µmol	35–38 µmol
TMT	Distance (cm)	2080 ± 156	1995 ± 143	1914 ± 124	1881 ± 118	1781 ± 83	1817 ± 97	1944 ± 111	1883 ± 85	1895 ± 73	1919 ± 80
	Percentage of immobility	13.7 ± 1.3	14.8 ± 1.4	15.2 ± 1.3	16.1 ± 1.4	16.7 ± 1.0	16.3 ± 1.0	14.9 ± 1.1	15.1 ± 1.0	15.3 ± 0.9	14.2 ± 0.9
2-HP	Distance (cm)	2380 ± 114	2278 ± 103	2156 ± 124	2147 ± 84	2000 ± 60	1939 ± 50	1946 ± 51	2133 ± 76	1914 ± 78	2143 ± 73
	Percentage of immobility	11.6 ± 0.9	12.6 ± 1.1	14.1 ± 1.1	13.3 ± 0.7	14.2 ± 0.9	14.5 ± 0.8	14.8 ± 0.9	13.5 ± 0.8	15.0 ± 0.8	12.7 ± 0.8
IA	Distance (cm)	2024 ± 109	1836 ± 109	1853 ± 97	1919 ± 140	1786 ± 111	1752 ± 101	1725 ± 106	1812 ± 111	1762 ± 131	1639 ± 118
	Percentage of immobility	17.8 ± 1.3	20.7 ± 1.8	19.9 ± 1.8	19.6 ± 1.8	20.9 ± 1.6	20.9 ± 1.5	22.3 ± 1.6	21.0 ± 1.8	21.2 ± 2.0	23.9 ± 2.7

t-test against the chance value revealed that the avoidance ratio was significantly different from chance at the test ($p = 0.001$) but not the control ($p = 0.115$), so this measure indicates the formation of a conditioned avoidance. Further, distance traveled was significantly lower in the test with respect to the control ($p = 0.034$; **Figure 3H**). The percentage of immobility in each zone was different in the memory test, due to an increase in immobility in the neutral zone [DAY × ZONE, $F_{(1,11)} = 4.9$ $p = 0.049$, stimulus vs. neutral chamber, $p = 0.004$; time in immobility in the neutral zone control vs. test, $p = 0.012$; **Figure 3I**).

In summary, TMT and IA induced a contextual memory after repeated exposure, so that mice avoided the stimulus zone even in the absence of the odorants. Exposure to IA significantly decreased distance traveled in the contextual memory test, and both TMT and IA increased the immobility of the animals in the neutral zone, paralleling the significant avoidance of the stimulus zone. Conversely, exposure to 2-HP did not produce statistically significant behavioral differences between the control and the memory test.

To check whether the different effects of each odorant in memory and activity were due to differential effects of the repeated exposure to them, we compared the avoidance ratio, distance traveled and immobility during the training days between groups (TMT, 2-HP and IA). The avoidance ratio of the groups exposed to TMT and 2-HP was significantly different to those of IA [repeated measures ANOVA, DAY × ODOR, $F_{(6, 96)} = 3.1$, $p = 0.01$; *post-hoc* comparison TMT vs. IA and 2-HP and 2-HP vs. IA, both $p = 0.023$; **Figure 4A**]. Further *post-hoc* pairwise comparisons between the individual exposure days revealed that all odors were equally avoided during the first two training days, but from day 3, the avoidance ratio of TMT decreased and became significantly different from both 2-HP and IA ($p < 0.05$ in all cases; **Figure 4A**). In fact, the avoidance ratio in the TMT group was significantly different between day 2 and 3 ($p = 0.042$). Conversely, the avoidance ratio of the group exposed to 2-HP was significantly lower in day 2 than in day 1 ($p = 0.02$). Finally, the avoidance ratio of the group exposed to IA did not vary across days. These findings suggest that mice were slightly habituated to the aversive properties of TMT, whereas they expressed a higher avoidance of 2-HP in consecutive tests.

Distance traveled was significantly different across exposure days and between groups [repeated measures ANOVA, ODOR, $F_{(2, 32)} = 5.4$, $p = 0.009$; TEST × ODOR, $F_{(6, 96)} = 3.1$, $p = 0.01$; **Figure 4B**]. *Post-hoc* comparisons of the factor ODOR showed that, overall, the distance traveled by the mice exposed to TMT was significantly different to distance traveled by the mice exposed to 2-HP ($p = 0.007$). Further *post-hoc* pairwise comparisons revealed that distance traveled by animals exposed to 2-HP was lower at day 4 as compared to day 1 ($p = 0.014$), whereas mice exposed to IA traveled less distance all three last exposure days as compared to the first one (all $p < 0.05$). We thus checked whether these differences were due to basal differences in distance traveled between the groups. Importantly, distance traveled was similar for all groups at the control day ($p > 0.1$). Further, the ANOVA followed by Dunnet *post-hoc* comparison revealed that TMT increased distance traveled during the first exposure with respect to the control ($p = 0.019$), whereas 2-HP

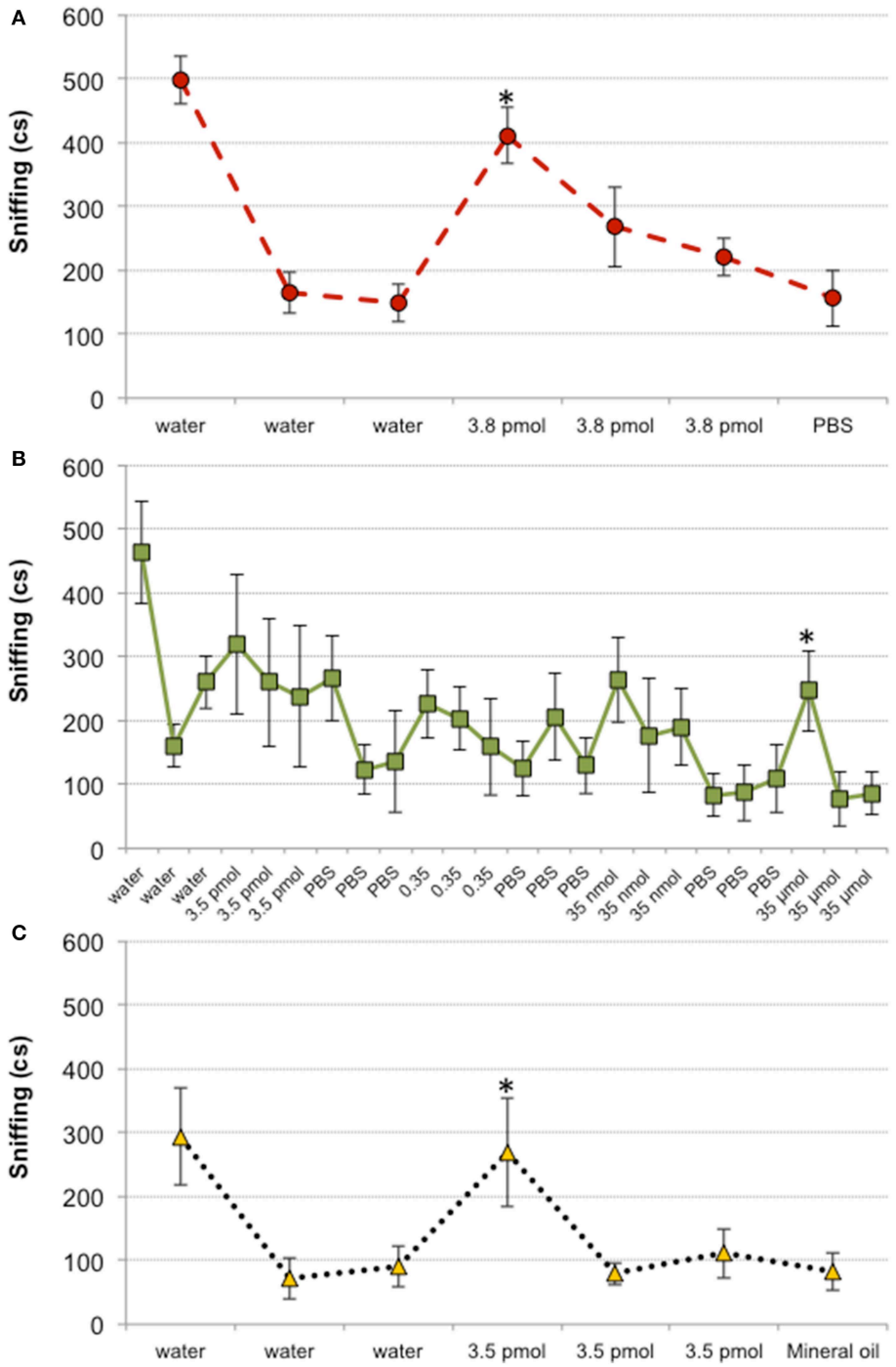


FIGURE 2 | Mice are able to detect TMT, 2-HP and IA in habituation–dishabituation tests. Line charts representing the chemoinvestigation of scented cotton swabs in cs. 3.8 pmol of TMT elicited increased chemoinvestigation **(A)**, whereas 2-HP did not significantly increase chemoinvestigation until we presented 35 µmol **(B)**. The detection threshold of IA was similar to that of TMT **(C)**. Comparison between last presentation of a solvent (water, PBS with Triton X-100 1% or mineral oil) and first presentation of each odorant amount, $^*p < 0.05$. Data are expressed as mean ± SEM.

and IA decreased distance traveled with respect to the control at days 3 and 4, respectively (both $p = 0.04$).

As expected, immobility followed a pattern that was complementary to that of distance traveled [repeated measures ANOVA, TEST × ODOR, $F_{(6, 96)} = 2.3$, $p = 0.042$]. Overall, immobility was significantly different in the TMT and 2-HP groups ($p = 0.037$). Again, these differences were not due to different initial levels of immobility, since this measure was not

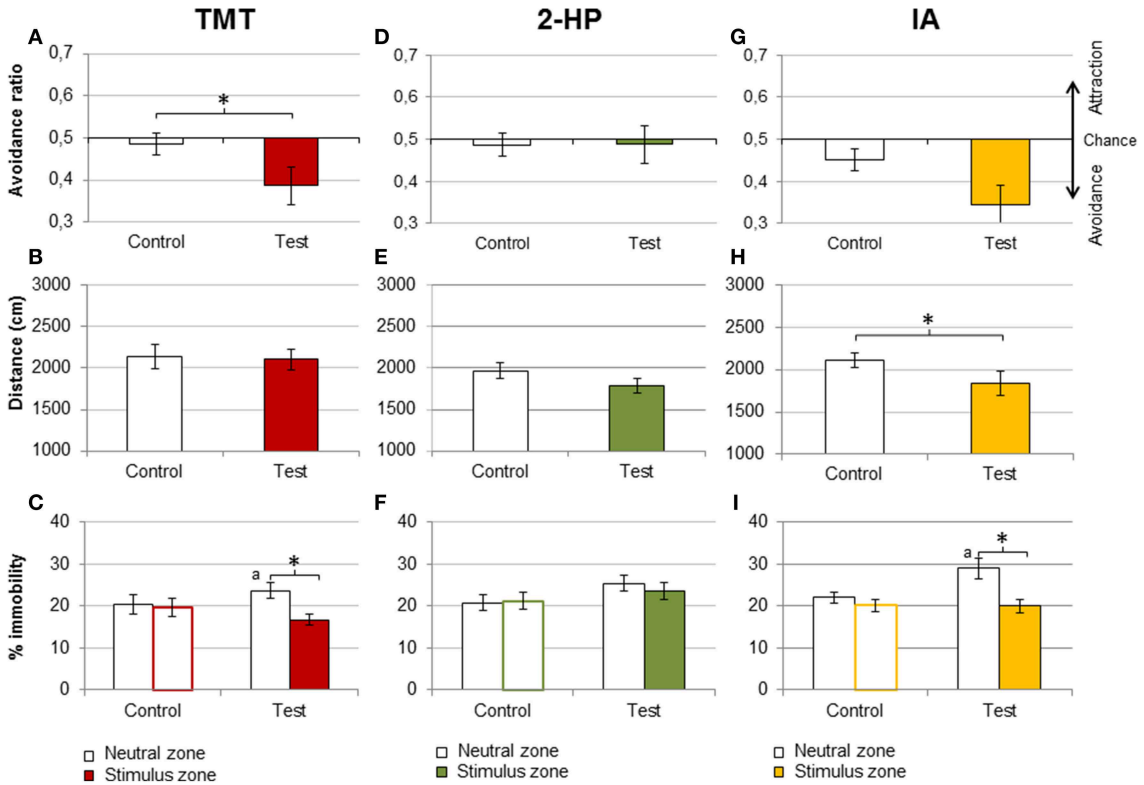


FIGURE 3 | TMT and IA, but not 2-HP, induced contextual learning. Bar charts representing behavioral responses in the contextual learning experiment, in the pre-exposure control (Control) and the contextual memory test (Test). Mice exposed to pure TMT during four consecutive sessions expressed conditioned place avoidance to the zone paired with the stimulus, as shown by a significant decrease of the avoidance ratio in the test **(A)**. In addition, TMT failed to affect distance traveled **(B)**, but increased immobility in the neutral zone during the test **(C)**, suggesting that animals avoided TMT and stayed immobile in the neutral zone. By contrast, 2-HP did not induce a contextual memory, since it did not affect the avoidance ratio **(D)**, but, overall, it slightly decreased distance traveled **(E),** and did not affect immobility **(F)**. Finally, IA produced a marginally significant decrease in the avoidance ratio **(G)**, and significantly decreased distance traveled **(H)**. Like TMT, IA increased immobility in the neutral zone **(I)**. *$p < 0.05$. Comparison between the neutral zones in control and test: a, $p < 0.05$. Data are expressed as mean ± SEM.

significantly different between groups in the control day (all $p > 0.9$). In contrast to most reports showing an increase in freezing in animals exposed to TMT, this putative kairomone decreased immobility during the first day as compared to control ($p = 0.017$). Conversely, 2-HP increased immobility at day 4 as compared to control ($p = 0.004$), whereas animals exposed to IA did not show any significant variation in the percentage of time they spent inactive (**Figure 4C**).

Discussion

Our results indicate that female mice displayed specific behavioral changes when exposed to substances thought to be implicated in inter (TMT) and intraspecific communication (2-HP), but also to a common odorant unlikely to act as a chemosignal (IA). Mice avoided these chemicals in a concentration-dependent way, but showed opposite changes in activity in response to them. Further, avoidance of both TMT and IA was expressed beyond the detection threshold of the odorants. In addition, only TMT and IA induced conditioned, context-dependent behavioral changes. We discuss these results by comparing them with the behavioral responses of mice to the common odorant IA.

Exposure to TMT Elicits Avoidance but not Freezing

Mice mainly rely on their sense of smell to avoid danger. Thus, when given the opportunity, they readily avoid spots containing predator kairomones (Root et al., 2014; Wernecke et al., 2015). Our results in female CD1 mice, showing that they avoid a zone containing TMT, a putative kairomone, are in agreement with this view. In fact, in Experiment 1, mice spent less time in the zone of the cage containing TMT than in the opposite neutral zone when TMT was present at amounts as low as 3.8 pmol and 0.38 nmol (**Figure 1A**). However, in the ensuing sessions at higher concentrations (3.8 nmol to 0.38 µmol) mice seemed to habituate and spent the same amount of time in both zones. Avoidance reappeared at the highest concentrations (3.8 and 0.38 µmol). In Experiment 2, we confirmed the olfactory detection of 3.8 pmol TMT, so that mice can detect and avoid TMT at low concentrations, supporting its role as a kairomone.

However, the time spent in the stimulus zone was significantly reduced with respect to the control situation (no odor) only when 38 µmol TMT were presented (**Figure 1B**). In addition, mice displayed a similar avoidance pattern to TMT and to IA, an odorant unlikely to have a kairomonal role. In fact, IA is frequently used as a control odor for TMT studies (Wallace

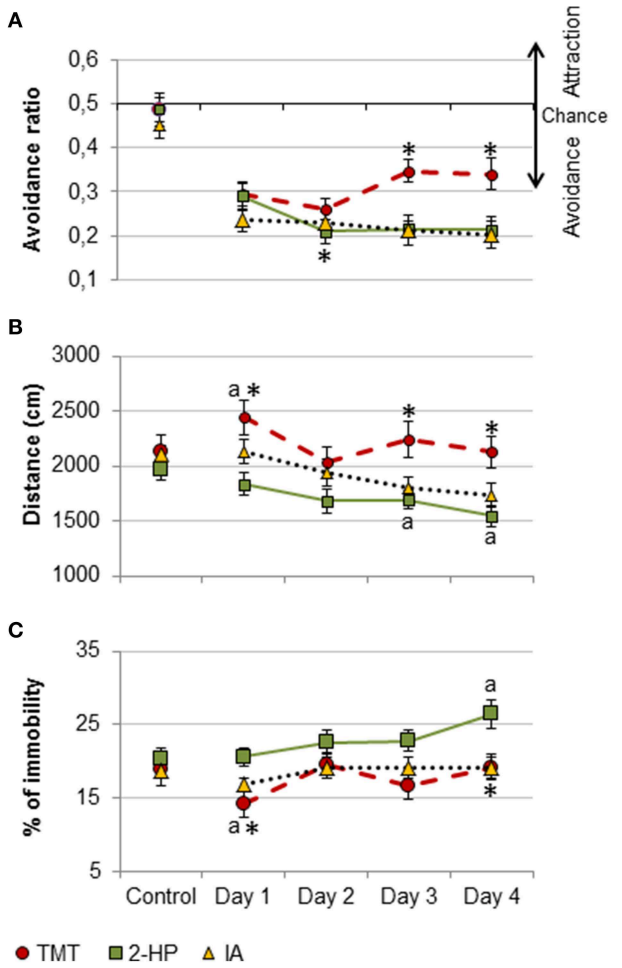


FIGURE 4 | Pure TMT, 2-HP and IA were avoided and differently affected locomotion. Line charts representing behavioral responses during the training days of the contextual conditioning experiment for each odorant. All three odors were significantly avoided, but the avoidance of TMT was blunted across sessions, whereas avoidance of 2-HP increased in the second day **(A)**. Distance traveled was differentially affected by the three odors: whereas TMT increased locomotion during the first exposure day and then this parameter remained stable, 2-HP and IA decreased locomotion across tests **(B)**. TMT reduced immobility the first training day, and 2-HP increased it during the last training day, whereas IA did not affect to this measure **(C)**. Comparison between odorants: $^*p < 0.05$. Comparison between exposure days and the control situation: a, $p < 0.05$. Data are expressed as mean ± SEM.

and Rosen, 2000; Root et al., 2014), and our results showing that low amounts of IA are detected but not avoided by mice support its use as such, albeit only at those low concentrations. Our findings suggest that the behavioral reactions that mice display to supra-threshold, very intense odorants might not be odorant-specific, and therefore they may not be informative about the role of that substance as a chemical signal. In this line, a recent study by Dewan et al. (2013), showed that genetic deletion of specific olfactory receptors blocked the aversion that mice expressed for low, but not high, concentration of amines found in the urine of predators and for the urine itself. The high concentration of amines used in that study were pungent to humans, so the aversion could be due to either overactivation of non-specific olfactory receptors or trigeminal stimulation (see below), supporting that that aversion toward very intense odors might not only depend on the biological significance of the odorant. Second, this study highlights that biologically significant chemosignals are detected at very low concentrations.

Further, behavioral avoidance of a given stimulus does not necessarily reflect fear, but rather that the stimulus has aversive properties for the subject due to its repugnant, pungent or disgusting properties. In this sense, Endres and Fendt (2009) demonstrated that whereas both TMT and butyric acid elicited avoidance in rats, only TMT elicited freezing, a more accurate behavioral measure of fear.

However, TMT did not produce any freezing in our mice as measured by a trained observer. Immobility, a behavioral measure that could approximate freezing, was not affected either by the increasing concentrations of TMT. Quite the opposite, animals repeatedly exposed to pure TMT displayed heightened locomotion and reduced immobility, as compared to both the control situation (no odor) and to animals exposed to 2-HP. These findings contrast with the above mentioned study by Endres and Fendt (2009) and several other reports showing that rats (Wallace and Rosen, 2000) and mice (Hebb et al., 2004; Hacquemand et al., 2013) display enhanced immobility and freezing in the presence of TMT, but are in agreement with our previous findings in two strains of mice (Fortes-Marco et al., 2013). In fact, sensitivity to TMT is dependent on the strain in rats, so that, under the same conditions, Sprague–Dawley, but not Wistar rats display freezing in the presence of TMT (Rosen et al., 2006). In agreement, we have previously shown that mice of the C57BL/J6 strain displayed enhanced immobility in the presence of the TMT as compared to CD1 (Fortes-Marco et al., 2013).

The contrasting findings about freezing to TMT found in the literature also reflect that responses of animals toward a given aversive stimulus are critically dependent on the protocol used. To investigate this issue, Morrow et al. (2002) exposed rats to TMT either in a low anxiety (a comfortable, dimly lit open field) or a high anxiety environment (a bigger apparatus intensely lit). Their results showed that rats displayed enhanced immobility in the high anxiety environment only. Thus, the behavioral strategy that animals select to cope with an aversive stimulus is dependent on the possibilities offered by the environment. In a single-chamber open field or in the home cage, where the animals cannot escape or hide, it is more likely that they freeze than in our set up, a box divided in two chambers with a neutral compartment where the animals can escape. This is also true in response to a footshock, the most common fear-inducing stimulus in the laboratory. When rats are given the opportunity to escape to a save chamber, they do not freeze but quickly escape following the footshock (Blanchard et al., 2006).

Additionally, the conditions in which volatile compounds are tested determine their effective concentration in the air. Thus, if a given amount of a volatile substance is presented in a small cage covered by a lid, the concentration in the air will be higher than in an open or larger cage. This might also explain the differences found between the different studies in the behavioral response of animals to volatiles.

Exposure to pure 2-HP Elicits Avoidance and Enhanced Immobility

To our knowledge, this is the first report analyzing the behavioral effects of 2-HP in adult female mice. Although sometimes quoted as a "known mouse pheromone" (Xu et al., 2005), studies on the behavioral and endocrine effects of 2-HP in mice are scarce, and restricted to early reports about its puberty-affecting properties (Novotny et al., 1986; Jemiolo et al., 1989). Conversely, more recent studies in rats have shown that 2-HP is elevated in the urine of stressed rats (Gutiérrez-García et al., 2006) and increases immobility in the forced swimming test (Gutiérrez-García et al., 2007), suggesting that 2-HP acts as an alarm pheromone in this species (but see also Zhang et al., 2008; Zhang and Zhang, 2011 for studies about the role of 2-HP as a sexual pheromone in rats).

The alarming properties of 2-HP have been also demonstrated in bees (Collins et al., 1989). Even if it might seem surprising that the same compound acts as a pheromone in vertebrates and insects, this is not an isolated case. For example, (Z)-7-dodecen-l-yl acetate works as a pheromone in several species of moths and in elephants (Rasmussen et al., 1996). Anyway, although the avoidance observed to pure 2-HP in mice would fit its role as an alarm pheromone, it is possible that these aversive properties are due to pungent or disgusting properties of the pure substance—which the animals are unlikely to encounter in nature—rather than to its pheromonal actions. This might represent another case of substance-unspecific aversion to an intense odor (see above).

Although 2-HP elicited avoidance only when presented pure, it decreased the distance traveled and increased immobility in a concentration-dependent way. This finding suggests that 2-HP was detected at relatively low concentrations (0.35 nmol). However, data from habituation–dishabituation tests (Experiment 2 and Experiment S1) shows that amounts lower than 35 μmol of 2-HP did not significantly elicit chemoinvestigation. These results raise the possibility that 2-HP might have subthreshold behavioral effects. In fact, the vomeronasal organ of mice responds to 2-HP at a concentration of 10^{-11} *in vitro* (Leinders-Zufall et al., 2000). Moreover, this response is sex-specific, so that female-derived vomeronasal preparations, but not male-derived ones, responded to 2-HP by increasing the intracellular concentration of inositol-3-phosphate (Thompson et al., 2004), thus indicating that the aversion to the pure substance described in the present work might not be the only biological effect of this chemical signal. In summary, it is possible that the effects of 2-HP depend on the age and sex of the animals, so that it might act as a puberty regulator in pre-puber females, and as an alarm pheromone in adult females and males. The effects of 2-HP might also be strongly dependent on its concentration and on the chemical blend in which it is encountered (Novotny et al., 1986). Future studies are needed to tests these hypothesis.

Finally, the modulation of locomotor activity by IA and 2-HP was similar. Our data about the responses of mice to IA are in agreement to a previous study showing that IA induced avoidance and increased immobility in rats (Wallace and Rosen, 2000). Thus, although IA is commonly used as a control odor, and our data shows that it is neutral to mice at its detection threshold, our data also suggest that it should not be used at high concentrations for that purpose, since a strong olfactory stimulation results in strong avoidance.

In summary, the similarity in the behavioral responses of mice to high concentrations of two biologically significant chemosignals and a common odorant suggest that strong odorants might induce odorant-unspecific behavioral responses of avoidance. In mice, pure TMT elicits corticosterone secretion (see Fendt et al., 2005), and a pattern of brain c-fos expression suggestive of intense stress (Janitzky et al., 2015). These findings together with our present results suggest that intense odors might be used to study stress and anxiety in addition to other unspecific stimuli such as loud noises (Burow et al., 2005; Mikheenko et al., 2010). Studies of the endocrine response to and central effects of high concentrations of control odorants such as isoamyl acetate are needed to check this hypothesis.

Conditioned Contextual Responses of Mice after Repeated Exposure to TMT and IA

The lack of consistent freezing to TMT, together with the failure to induce the contextual learning that is observed after exposure to other predator cues, i.e., cat odor (Wallace and Rosen, 2000; Blanchard et al., 2003; Muñoz-Abellán et al., 2009) have been considered by several researchers as a challenge to the kairomonal nature of this substance. Nonetheless, Endres and Fendt (2007) explored whether TMT could support learning by using different conditioning protocols. Their data showed that whereas TMT failed to induce conditioning in rats trained in a box with a single compartment [in agreement with the results obtained by Blanchard et al. (2003)], it induced conditioned avoidance in a box divided in two compartments. Our results replicate and extend these findings, since mice in our experiment expressed a conditioned avoidance of a zone paired with TMT, as well as a specific increase in immobility in the neutral zone. This latter result is striking, since TMT decreased immobility during training days. Nevertheless, as noted by (Endres and Fendt, 2007), the conditioned behavioral responses are not necessarily the same as the unconditioned ones. In addition, it is possible that our mice were able to express conditioned avoidance because they could use TMT as a discriminative stimulus due to the lack of strong innate responses toward TMT, which could interfere with conditioning in other strains. Finally, as we have stressed before, differences in the set up and protocols used might contribute to differences in the experiments outcome.

On the contrary, 2-HP failed to induce a conditioned avoidance, albeit it was, overall, significantly more avoided than TMT during the exposure days. Moreover, whereas the avoidance toward 2-HP increased the second training day, the avoidance toward TMT was slightly reduced after repeated exposures, suggesting a slight habituation. These differences are open to several interpretations.

First, it is possible that, at the high concentration used, TMT activated the trigeminal nerve (Galliot et al., 2012) producing an aversion strong enough to form an avoidance memory. In fact, although trigeminal deafferentiation does not block freezing to TMT (Ayers et al., 2013), TMT induces trigeminal activation at concentrations higher than 10% (Hacquemand et al., 2010), at least in some experimental conditions. The exposure to IA,

also known to stimulate the trigeminal nerve (Doty et al., 1978), mimicked the results obtained with TMT in our experiments. This suggests that a strong olfactory stimulation, maybe along with trigeminal activation, is enough to support learning, even if the odorant has, in principle, no special innate meaning for the animals. Future studies investigating memory induction with lower amounts of TMT and IA in control and anosmic animals would shed light on whether the olfactory and/or trigeminal properties of these odorants are responsible for their ability to support learning.

However, although 2-HP could also stimulate the trigeminal nerve at high concentrations (Cometto-Muñiz and Cain, 1995), and elicited aversion in mice when presented pure, it failed to induce contextual learning. Maybe 2-HP is recognized as a mouse-derived odor and, hence, it is not tagged as dangerous. Finally, whereas TMT and IA activate the main olfactory bulb only (Bepari et al., 2012), 2-HP is detected by both, the olfactory epithelium (Spehr et al., 2006) and V1R vomeronasal receptors (Boschat et al., 2002), thus activating both the main and the accessory olfactory systems (Xu et al., 2005). Given the overlapping but complementary roles of the main and accessory olfactory systems (Martínez-García et al., 2009), it is possible that higher order brain structures processing the olfactory and vomeronasal stimuli result in different unconditioned and learnt responses.

Neural Basis of Biologically Significant Odor Processing

The brain circuits processing TMT and other predator-derived cues (e.g., cat odor) might be, in fact, underlying the diversity of responses toward each type of chemosignals. Thus, cat fur odor activates the vomeronasal system (Dielenberg et al., 2001; Staples et al., 2008), including the posteroventral medial amygdala, a key center for defensive anti-predatory responses (Day et al., 2004; Pérez-Gómez et al., 2015). By contrast, TMT is not able to induce c-fos in this nucleus (Day et al., 2004; Janitzky et al., 2015; Pérez-Gómez et al., 2015). In agreement with the activation of defensive nuclei of the brain, mice show robust risk assessment responses toward cat odor (Pérez-Gómez et al., 2015), but fail to do so when exposed to TMT (Pérez-Gómez et al., 2015, present results). In this sense, cat fur odor seems a more valuable stimulus than TMT to study antipredatory responses in rodents.

On the other hand, the bed nucleus of the stria terminalis (BNST) seems key in controlling the behavioral responses to TMT. Thus, TMT elicits robust and specific increases of Fos induction in the BNST (Janitzky et al., 2015), and temporary inactivation of this nucleus with muscimol injections abolished freezing to TMT in rats (Fendt et al., 2003). The BNST has been related to anxiety rather than fear (Davis et al., 2010). Fear is a response to an explicit threat, whereas anxiety involves uncertainty as to the expectancy of threat, and predator-derived odors are, indeed, poor predictors of the presence of a predator itself (Blanchard et al., 2003). Thus, we suggest that TMT could certainly be regarded asan anxiogenic rather than as a fear-provoking stimulus, but this hypothesis needs further investigation.

Regarding 2-HP, to our knowledge there are not studies looking at the activation of central brain structures beyond the olfactory bulbs (Xu et al., 2005). Mapping of alarm pheromones obtained from the anal glands of rats revealed Fos increases in the BNST and other nuclei involved in stress processing (Kiyokawa et al., 2005). It would be interesting that future studies directly compare the brain activation patterns elicited by both biologically significant and common of odors under the same conditions.

Conclusions

In conclusion, our results suggest that some intense odors are avoided by mice irrespective of their possible role in inter- or intra-species communication. Second, some of these intense odors, including fox-derived TMT and the odorant IA, which in principle is devoid of value for intra- or interspecies communication, induce conditioned avoidance when repeatedly presented at high concentration. By contrast, 2-HP is avoided at high concentrations but it does not induce contextual learning, maybe reflecting its role as an intraspecific chemosignal. These results support the widely use of synthetic predator-related compounds (like TMT) to investigate the neurobiological basis of emotional behaviors in rodents. However, since IA, a common odorant frequently used as control, elicits similar behavioral reactions to TMT, we stress the importance of using behavioral measures in combination with other physiological or neural manipulations (e.g., measurements of hormonal levels, expression of immediate early genes) to establish the ethological significance of chemosignals.

Author Contributions

EL, FM, and CA, designed research; LF and CA performed research; LF and CA analyzed data; LF, FM, and CA wrote the paper, EL, FM, and CA revised the final version and approved the manuscript.

Acknowledgments

This study was funded by the Spanish MINECO-FEDER (BFU2010-16656; BFU2013-47688-P), Junta de Comunidades de Castilla-La Mancha/FEDER (PEIC11-0045-4490), and Vicerrectorado de Investigación, Universitat Jaume I de Castelló. We would like to thank the reviewers for their fruitful suggestions, which help us improve the manuscript. The authors are indebted to Adoración Hernández-Martínez for technical assistance.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2015.00336

References

Agustín-Pavón, C., Martínez-García, F., and Lanuza, E. (2014). Focal lesions within the ventral striato-pallidum abolish attraction for male chemosignals in female mice. *Behav. Brain Res.* 259, 292–296. doi: 10.1016/j.bbr.2013.11.020

Agustín-Pavón, C., Martínez-Ricós, J., Martínez-García, F., and Lanuza, E. (2007). Effects of dopaminergic drugs on innate pheromone-mediated reward in female mice: a new case of dopamine-independent "liking". *Behav. Neurosci.* 121, 920–932. doi: 10.1037/0735-7044.121.5.920

Ayers, L. W., Asok, A., Heyward, F. D., and Rosen, J. B. (2013). Freezing to the predator odor 2,4,5 dihydro 2,5 trimethylthiazoline (TMT) is disrupted by olfactory bulb removal but not trigeminal deafferentation. *Behav. Brain Res.* 253, 54–59. doi: 10.1016/j.bbr.2013.06.034

Bepari, A. K., Watanabe, K., Yamaguchi, M., Tamamaki, N., and Takebayashi, H. (2012). Visualization of odor-induced neuronal activity by immediate early gene expression. *BMC Neurosci.* 13:140. doi: 10.1186/1471-2202-13-140

Berton, F., Vogel, E., and Belzung, C. (1998). Modulation of mice anxiety in response to cat odor as a consequence of predators diet. *Physiol. Behav.* 65, 247–254. doi: 10.1016/S0031-9384(98)00126-7

Blanchard, D. C., Markham, C., Yang, M., Hubbard, D., Madarang, E., and Blanchard, R. J. (2003). Failure to produce conditioning with low-dose trimethylthiazoline or cat feces as unconditioned stimuli. *Behav. Neurosci.* 117, 360–368. doi: 10.1037/0735-7044.117.2.360

Blanchard, D. C., Yang, M., Herbert, M. A., and Blanchard, R. J. (2006). "Defensive behaviours," in *Encyclopaedia of Stress*, ed G. Fink (New York, NY: Academic Press), 652–656.

Boschat, C., Pélofi, C., Randin, O., Roppolo, D., Lüscher, C., Broillet, M.-C., et al. (2002). Pheromone detection mediated by a V1r vomeronasal receptor. *Nat. Neurosci.* 5, 1261–1262. doi: 10.1038/nn978

Brechbühl, J., Moine, F., Klaey, M., Nenniger-Tosato, M., Hurni, N., Sporkert, F., et al. (2013). Mouse alarm pheromone shares structural similarity with predator scents. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4762–4767. doi: 10.1073/pnas.1214249110

Burow, A., Day, H. E., and Campeau, S. (2005). A detailed characterization of loud noise stress: intensity analysis of hypothalamo-pituitary-adrenocortical axis and brain activation. *Brain Res.* 1062, 63–73. doi: 10.1016/j.brainres.2005.09.031

Collins, A. M., Rinderer, T. E., Daly, H. V., Harbo, J. R., and Pesante, D. (1989). Alarm pheromone production by two honeybee (*Apis mellifera*) types. *J. Chem. Ecol.* 15, 1747–1756. doi: 10.1007/BF01012262

Cometto-Muñiz, J. E., and Cain, W. S. (1995). Relative sensitivity of the ocular trigeminal, nasal trigeminal and olfactory systems to airborne chemicals. *Chem. Senses* 20, 191–198.

Davis, M., Walker, D. L., Miles, L., and Grillon, C. (2010). Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. *Neuropsychopharmacology* 35, 105–135. doi: 10.1038/npp.2009.109

Day, H. E., Masini, C. V., and Campeau, S. (2004). The pattern of brain c-fos mRNA induced by a component of fox odor, 2,5-dihydro-2,4,5-Trimethylthiazoline (TMT), in rats, suggests both systemic and processive stress characteristics. *Brain Res.* 1025, 139–151. doi: 10.1016/j.brainres.2004.07.079

Dewan, A., Pacifico, R., Zhan, R., Rinberg, D., and Bozza, T. (2013). Non-redundant coding of aversive odours in the main olfactory pathway. *Nature* 497, 486–489. doi: 10.1038/nature12114

Dielenberg, R. A., Hunt, G. E., and McGregor, I. S. (2001). "When a rat smells a cat": the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor. *Neuroscience* 104, 1085–1097. doi: 10.1016/S0306-4522(01)00150-6

Doty, R. L., Brugger, W. E., Jurs, P. C., Orndorff, M. A., Snyder, P. J., and Lowry, L. D. (1978). Intranasal trigeminal stimulation from odorous volatiles: psychometric responses from anosmic and normal humans. *Physiol. Behav.* 20, 175–185. doi: 10.1016/0031-9384(78)90070-7

Endres, T., and Fendt, M. (2007). Conditioned behavioral responses to a context paired with the predator odor trimethylthiazoline. *Behav. Neurosci.* 121, 594–601. doi: 10.1037/0735-7044.121.3.594

Endres, T., and Fendt, M. (2009). Aversion- vs fear-inducing properties of 2,4,5-trimethyl-3-thiazoline, a component of fox odor, in comparison with those of butyric acid. *J. Exp. Biol.* 212, 2324–2327. doi: 10.1242/jeb.028498

Fendt, M., and Endres, T. (2008). 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox odor - just repugnant or really fear-inducing? *Neurosci. Biobehav. Rev.* 32, 1259–1266. doi: 10.1016/j.neubiorev.2008.05.010

Fendt, M., Endres, T., and Apfelbach, R. (2003). Temporary inactivation of the bed nucleus of the stria terminalis but not of the amygdala blocks freezing induced by trimethylthiazoline, a component of fox feces. *J. Neurosci.* 23, 23–28. Available online at: http://www.jneurosci.org/content/23/1/23.long

Fendt, M., Endres, T., Lowry, C. A., Apfelbach, R., and McGregor, I. S. (2005). TMT-induced autonomic and behavioral changes and the neural basis of its processing. *Neurosci. Biobehav. Rev.* 29, 1145–1156. doi: 10.1016/j.neubiorev.2005.04.018

Ferrero, D. M., Lemon, J. K., Fluegge, D., Pashkovski, S. L., Korzan, W. J., Datta, S. R., et al. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11235–11240. doi: 10.1073/pnas.1103317108

Fortes-Marco, L., Lanuza, E., and Martinez-Garcia, F. (2013). Of pheromones and kairomones: what receptors mediate innate emotional responses? *Anat. Rec.* 296, 1346–1363. doi: 10.1002/ar.22745

Galliot, E., Laurent, L., Hacquemand, R., Pourié, G., and Millot, J. L. (2012). Fear-like behavioral responses in mice in different odorant environments: trigeminal versus olfactory mediation under low doses. *Behav. Processes* 90, 161–166. doi: 10.1016/j.beproc.2012.01.002

Gutiérrez-García, A. G., Contreras, C. M., Mendoza-López, M. R., Cruz-Sánchez, J. S., García-Barradas, O., and Rodríguez-Landa, J. (2006). A single session of emotional stress produces anxiety in Wistar rats. *Behav. Brain Res.* 167, 30–35. doi: 10.1016/j.bbr.2005.08.011

Gutiérrez-García, A. G., Contreras, C. M., Mendoza-López, M. R., García-Barradas, O., and Cruz-Sánchez, J. S. (2007). Urine from stressed rats increases immobility in receptor rats forced to swim: role of 2-heptanone. *Physiol. Behav.* 91, 166–172. doi: 10.1016/j.physbeh.2007.02.006

Hacquemand, R., Choffat, N., Jacquot, L., and Brand, G. (2013). Comparison between low doses of TMT and cat odor exposure in anxiety- and fear-related behaviors in mice. *Behav. Brain Res.* 238, 227–231. doi: 10.1016/j.bbr.2012.10.014

Hacquemand, R., Jacquot, L., and Brand, G. (2010). Comparative fear-related behaviors to predator odors (TMT and Natural Fox Feces) before and after intranasal ZnSO(4) treatment in mice. *Front. Behav. Neurosci.* 4:188. doi: 10.3389/fnbeh.2010.00188

Hebb, A. L., Zacharko, R. M., Gauthier, M., Trudel, F., Laforest, S., and Drolet, G. (2004). Brief exposure to predator odor and resultant anxiety enhances mesocorticolimbic activity and enkephalin expression in CD-1 mice. *Eur. J. Neurosci.* 20, 2415–2429. doi: 10.1111/j.1460-9568.2004.03704.x

Janitzky, K., D'Hanis, W., Kröber, A., and Schwegler, H. (2015). TMT predator odor activated neural circuit in C57BL/6J mice indicates TMT-stress as a suitable model for uncontrollable intense stress. *Brain Res.* 1599, 1–8. doi: 10.1016/j.brainres.2014.12.030

Jemiolo, B., Alberts, J., Sochinski-Wiggins, S., Harvey, S., and Novotny, M. (1985). Behavioural and endocrine responses of female mice to synthetic analogues of volatile compounds in male urine. *Anim. Behav.* 33, 1114–1118. doi: 10.1016/S0003-3472(85)80170-6

Jemiolo, B., Andreolini, F., Xie, T. M., Wiesler, D., and Novotny, M. (1989). Puberty-affecting synthetic analogs of urinary chemosignals in the house mouse, *Mus domesticus*. *Physiol. Behav.* 46, 293–298. doi: 10.1016/0031-9384(89)90270-9

Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2005). Mapping the neural circuit activated by alarm pheromone perception by c-Fos immunohistochemistry. *Brain Res.* 1043, 145–154. doi: 10.1016/j.brainres.2005.02.061

Leinders-Zufall, T., Lane, A. P., Puche, A. C., Ma, W., Novotny, M. V., Shipley, M. T., et al. (2000). Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature* 405, 792–796. doi: 10.1038/35015572

Martínez-García, F., Martínez-Ricós, J., Agustín-Pavón, C., Martínez-Hernández, J., Novejarque, A., and Lanuza, E. (2009). Refining the dual olfactory hypothesis: pheromone reward and odour experience. *Behav. Brain Res.* 200, 277–286. doi: 10.1016/j.bbr.2008.10.002

Martínez-Ricós, J., Agustín-Pavón, C., Lanuza, E., and Martínez-García, F. (2007). Intraspecific communication through chemical signals in female mice: reinforcing properties of involatile male sexual pheromones. *Chem. Senses* 32, 139–148. doi: 10.1093/chemse/bjl039

McClintock, M. K. (2002). "Pheromones, odors and vasanas: the neuroendocrinology of social chemosignals in humans and animals," in *Hormones, Brain and Behavior*, eds D. W. Pfaff, A. P. Arnold, A. M. Etgen, S. E. Fahrbach, and R. T. Rubin (San Diego, CA: Academic Press), 139–214.

Mikheenko, Y., Man, M.-S., Braesicke, K., Johns, M. E., Hill, G., Agustín-Pavón, C., et al. (2010). Autonomic, behavioral, and neural analyses of mild conditioned negative affect in marmosets. *Behav. Neurosci.* 124, 192–203. doi: 10.1037/a0018868

Morrow, B. A., Elsworth, J. D., and Roth, R. H. (2002). Fear-like biochemical and behavioral responses in rats to the predator odor, TMT, are dependent on the exposure environment. *Synapse* 46, 11–18. doi: 10.1002/syn.10109

Muñoz-Abellán, C., Andero, R., Nadal, R., and Armario, A. (2008). Marked dissociation between hypothalamic-pituitary-adrenal activation and long-term behavioral effects in rats exposed to immobilization or cat odor. *Psychoneuroendocrinology* 33, 1139–1150. doi: 10.1016/j.psyneuen.2008.06.002

Muñoz-Abellán, C., Daviu, N., Rabasa, C., Nadal, R., and Armario, A. (2009). Cat odor causes long-lasting contextual fear conditioning and increased pituitary-adrenal activation, without modifying anxiety. *Horm. Behav.* 56, 465–471. doi: 10.1016/j.yhbeh.2009.08.002

Novotny, M., Jemiolo, B., Harvey, S., Wiesler, D., and Marchlewska-Koj, A. (1986). Adrenal-mediated endogenous metabolites inhibit puberty in female mice. *Science* 231, 722–725. doi: 10.1126/science.3945805

Pérez-Gómez, A., Bleymehl, K., Stein, B., Pyrski, M., Birnbaumer, L., Munger, S. D., et al. (2015). Innate predator odor aversion driven by parallel olfactory subsystems that converge in the ventromedial hypothalamus. *Curr. Biol.* 25, 1340–1346. doi: 10.1016/j.cub.2015.03.026

Rasmussen, L. E. L., Lee, T. D., Roelofs, W. L., Zhang, A., and Daves, G. D. (1996). Insect pheromone in elephants. *Nature* 379, 684. doi: 10.1038/379684a0

Root, C. M., Denny, C. A., Hen, R., and Axel, R. (2014). The participation of cortical amygdala in innate, odor-driven behavior. *Nature* 515, 269–273. doi: 10.1038/nature13897

Rosen, J. B., West, E. A., and Donley, M. P. (2006). Not all rat strains are equal: differential unconditioned fear responses to the synthetic fox odor 2,4,5-trimethylthiazoline in three outbred rat strains. *Behav. Neurosci.* 120, 290–297. doi: 10.1037/0735-7044.120.2.290

Spehr, M., Kelliher, K. R., Li, X.-H., Boehm, T., Leinders-Zufall, T., and Zufall, F. (2006). Essential role of the main olfactory system in social recognition of major histocompatibility complex peptide ligands. *J. Neurosci.* 26, 1961–1970. doi: 10.1523/JNEUROSCI.4939-05.2006

Staples, L. G., McGregor, I. S., Apfelbach, R., and Hunt, G. E. (2008). Cat odor, but not trimethylthiazoline (fox odor), activates accessory olfactory and defense-related brain regions in rats. *Neuroscience* 151, 937–947. doi: 10.1016/j.neuroscience.2007.11.039

Thompson, R. N., Robertson, B. K., Napier, A., and Wekesa, K. S. (2004). Sex-specific responses to urinary chemicals by the mouse vomeronasal organ. *Chem. Senses* 29, 749–754. doi: 10.1093/chemse/bjh076

Vernet-Maury, E. (1980). Trimethyo-thiazoline in fox feces: a natural alarming substance for the rat. *Olfaction Tast.* 7, 407.

Wallace, K. J., and Rosen, J. B. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behav. Neurosci.* 114, 912–922. doi: 10.1037/0735-7044.114.5.912

Wernecke, K. E. A., Vincenz, S., Storsberg, S., D'Hanis, W., Goldschmidt, J., and Fendt, M. (2015). Fox urine exposure induces avoidance behavior in rats and activates the amygdalar olfactory cortex. *Behav. Brain Res.* 279, 76–81. doi: 10.1016/j.bbr.2014.11.020

Xu, F., Schaefer, M., Kida, I., Schafer, J., Liu, N., Rothman, D. L., et al. (2005). Simultaneous activation of mouse main and accessory olfactory bulbs by odors or pheromones. *J. Comp. Neurol.* 489, 491–500. doi: 10.1002/cne.20652

Zhang, J. X., Sun, L., Zhang, J. H., and Feng, Z. Y. (2008). Sex- and gonad-affecting scent compounds and 3 male pheromones in the rat. *Chem. Senses* 33, 611–621. doi: 10.1093/chemse/bjn028

Zhang, Y. H., and Zhang, J. X. (2011). Urine-derived key volatiles may signal genetic relatedness in male rats. *Chem. Senses* 36, 125–135. doi: 10.1093/chemse/bjq103

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 11 August 2015
doi: 10.3389/fnins.2015.00283

Lack of spatial segregation in the representation of pheromones and kairomones in the mouse medial amygdala

Vinicius M. A. Carvalho[1,2‡], *Thiago S. Nakahara*[1,2‡], *Leonardo M. Cardozo*[1,2†], *Mateus A. A. Souza*[1,2], *Antonio P. Camargo*[1,3], *Guilherme Z. Trintinalia*[1,2], *Eliana Ferraz*[4] *and Fabio Papes*[1*]

[1] *Department of Genetics and Evolution, Institute of Biology, University of Campinas, Campinas, Brazil,* [2] *Graduate Program in Genetics and Molecular Biology, Institute of Biology, University of Campinas, Campinas, Brazil,* [3] *Undergraduate Program in the Biological Sciences, Institute of Biology, University of Campinas, Campinas, Brazil,* [4] *Campinas Municipal Zoo, Campinas, Brazil*

The nervous system is organized to detect, internally represent and process sensory information to generate appropriate behaviors. Despite the crucial importance of odors that elicit instinctive behaviors, such as pheromones and kairomones, their neural representation remains little characterized in the mammalian brain. Here we used expression of the immediate early gene product c-Fos as a marker of neuronal activity to find that a wide range of pheromones and kairomones produces activation in the medial nucleus of the amygdala, a brain area anatomically connected with the olfactory sensory organs. We see that activity in this nucleus depends on vomeronasal organ input, and that distinct vomeronasal stimuli activate a dispersed ensemble of cells, without any apparent spatial segregation. This activity pattern does not reflect the chemical category of the stimuli, their valence or the induced behaviors. These findings will help build a complete understanding of how odor information is processed in the brain to generate instinctive behaviors.

Keywords: olfaction, vomeronasal organ, amygdala, higher-order representation, pheromone, kairomone

OPEN ACCESS

Edited by:
Markus Fendt,
Otto-von-Guericke University
Magdeburg, Germany

Reviewed by:
Qi Yuan,
Memorial University, Canada
Mario Engelmann,
Otto-von-Guericke-Universität
Magdeburg, Germany

****Correspondence:***
Fabio Papes,
Department of Genetics and Evolution, Institute of Biology, University of Campinas, Rua Monteiro Lobato, 255, Campinas, 13083-862 Sao Paulo, Brazil
papesf@unicamp.br

†Present Address:
Leonardo M. Cardozo, Neurosciences Graduate Program, University of California, San Diego, 9500 Gilman Drive 0634, La Jolla, CA 92093-0634, USA

‡These authors have contributed equally to this work.

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *26 June 2015*
Accepted: *28 July 2015*
Published: *11 August 2015*

Citation:
Carvalho VMA, Nakahara TS, Cardozo LM, Souza MAA, Camargo AP, Trintinalia GZ, Ferraz E and Papes F (2015) Lack of spatial segregation in the representation of pheromones and kairomones in the mouse medial amygdala. Front. Neurosci. 9:283. doi: 10.3389/fnins.2015.00283

Introduction

In mammals, sensory information is detected by specialized sensory cells at the periphery and is then sent to the brain, where it must be systematically represented by coherent patterns of neural activity (Luo and Flanagan, 2007). Though still poorly understood, it is assumed that these patterns of activity are interpreted, resulting in output behaviors or endocrine changes.

In the visual and somatosensory systems, the discrimination of stimuli located in different positions in the sensed environment is achieved by their representation in topographic (or continuous) maps in the brain. For example, neighboring activated retina cells, representing adjacent sources of light in the visual field, send projections to neighboring neurons in the thalamus and visual cortices, such that the ordering of sensory stimuli in the external world is represented by ordered maps of neural activity in the brain (Luo and Flanagan, 2007). In contrast, gustatory information is represented in a non-continuous, or discrete, fashion, where different taste qualities, such as sweet, bitter, umami, and salty, resulting from the detection of the corresponding tastants

in the upper digestive system, are each represented by cohorts of activated neurons in discrete sub-areas of the primary taste cortex in the brain (Chen et al., 2011).

Less is known about how olfactory information is internally represented in the brain. The olfactory system is specialized in the sensory detection of a variety of chemical stimuli, which indicate the presence and quality of food, potential mates, competitors, and dangers in the environment (reviewed in Munger et al., 2009). Though the central representation of regular volatile odorant stimuli in the brain has been recently investigated (Stettler and Axel, 2009; Sosulski et al., 2011), very little is known about how odors able to elicit instinctive responses, such as pheromones and kairomones, are internally represented by coherent activity in olfactory brain areas. Pheromones (released by an individual and detected by the same species) and kairomones (released by an individual and detected by another species) are chemosignals that mediate a range of instinctive behavioral responses, including aggression (Chamero et al., 2007), mating, gender discrimination (Stowers et al., 2002; Kimchi et al., 2007), and fear (Papes et al., 2010). Since these cues crucially regulate the interactions between individuals, the study of their neural representation is central to understanding how the brain controls animal behavior, indirectly impacting life cycle, natural history, and evolution.

The vomeronasal organ (VNO), an olfactory structure in the nasal cavity, has been implicated in the detection of some pheromones and kairomones (Munger et al., 2009). Substances detected by VNO sensory neurons include urine-derived small organic molecules (Sam et al., 2001; Trinh and Storm, 2003), sulfated steroids (Nodari et al., 2008; Isogai et al., 2011), MHC peptides (Leinders-Zufall et al., 2004), peptides in the ESP family (Haga et al., 2010; Ferrero et al., 2013) and conspecific and heterospecific small proteins in the Major Urinary Protein family (Mup) (Chamero et al., 2007; Papes et al., 2010; Kaur et al., 2014; Dey et al., 2015), which mediate the instinctive behaviors mentioned above.

Vomeronasal sensory neurons connect directly with the accessory olfactory bulb (AOB) in the brain via the vomeronasal nerve (Wagner et al., 2006). In turn, the AOB is anatomically connected to several brain areas, including nuclei in the amygdala, such as the posteromedial cortical nucleus of the amygdala (PMCO) and the medial nucleus of the amygdala (MeA) (Canteras et al., 1995; Petrovich et al., 2001).

Pheromones and kairomones lead to behaviors instinctively, and are thus believed to be processed by hard-wired brain circuits. Moreover, the behaviors mediated by the VNO are stereotypical (conserved between individuals), and therefore we assumed that pheromone/kairomone information is represented by coherent activity along those circuits. However, the organizing principles behind such brain activity remain poorly characterized. Prior studies have shown that activity in the AOB reflects sensory input from the VNO (Wagner et al., 2006). In contrast, it is thought that other regions are organized to reflect behavioral output. For example, cat-odorized pieces of collar, which induce defensive behaviors in mice, lead to prominent neural activity in the ventral part of the MeA (Dielenberg et al., 2001; Choi et al., 2005), while female mouse odors, which trigger reproductive responses, activate the dorsal MeA (Fernandez-Fewell and Meredith, 1994; Kollack-Walker and Newman, 1997; Choi et al., 2005). These findings led to the idea that the MeA is divided into dorsal and ventral sub-areas, activated by predatory and social stimuli, respectively, composing distinct pathways involved in defensive or reproductive behaviors (Swanson, 2000; Canteras, 2002; Choi et al., 2005; Lin et al., 2011; Silva et al., 2013). However, these notions arose from the use of a very limited set of olfactory stimuli and no systematic comparison has been made among a range of stimuli eliciting various behavioral outputs, to understand how and where sensory information represented along the circuit initiated at the VNO transitions to the generation of behavior by downstream brain areas.

Here we investigated and compared how different olfactory stimuli, each able to induce instinctive behaviors following detection mediated by the VNO, generate activity in the amygdala. Because the MeA is one of the first higher-order brain regions to receive information collected by the VNO, we decided to study the patterns of activity in this nucleus, in groups of animals exposed to distinct types of olfactory cues. In order to create a detailed view of how MeA activity is organized, we chose a comprehensive approach, where activity was evaluated in mice exposed to a wide range of pheromones and kairomones. First, we analyzed activity in the AOB, as a confirmation that the VNO has been activated by each employed stimulus. We then showed that a large set of intra- and interspecies signals leads to activation in the MeA, in a VNO-dependent manner. We also obtained evidence that neural activity in this nucleus is not organized to reflect the valence or behavioral consequences of each detected stimulus, as previously thought. Instead, we found a lack of any discernible spatial map in the MeA, where each stimulus activates dispersed ensembles of neurons and distinct stimuli activate intermingled sets of cells. Therefore, it is unlikely that the amygdala contains a spatial map to represent different pheromones and kairomones. This knowledge will be key to comprehending how the brain's limbic system represents external olfactory information important for the survival of the individual and the species.

Materials and Methods

Animals

Animals were 8 weeks old male mice (females, where indicated). In some experiments, MeA activity was evaluated in animals where VNO neurons were genetically ablated by a null mutation in the gene *TrpC2*, coding for the primary vomeronasal sensory transduction channel (Stowers et al., 2002). TrpC2$^{+/+}$ and TrpC2$^{-/-}$ littermates were obtained from heterozygous mating couples, which were produced by backcrossing the TrpC2$^{-/-}$ knockout line (Stowers et al., 2002) into the C57BL/6J background for at least 10 generations (Papes et al., 2010). Animals had no previous exposure to odors from other animal species, and subjects exposed to conspecific chemosignals were kept individually caged for at least 4 days. All subjects were exposed to odor, monitored for behavior, and subsequently processed for immunostaining or *in situ* hybridization, ensuring

that the cellular responses and behaviors were analyzed from the same individuals and no animals were re-used. This study was carried out in accordance with Animal Protocol no. 1883-1, approved on June 2009 by the Institute of Biology's Institutional Animal Care and Use Committee (Committee for Ethics in Animal Use in Research), at the University of Campinas. This protocol follows the guidelines established by the National Council for Animal Experimentation Control (CONCEA-Brazil).

Stimuli

Mouse subjects were separately exposed to a wide range of intra- and interspecies odor stimuli, known to induce biologically relevant instinctive behaviors, such as odors from adult female and male mice, juveniles, odors from other mouse strains (BALB/c and 128S/J), and numerous species that are natural or occasional mouse predators (rat, domestic cat, leopard cat, Cougar mountain lion, African lion, several snake species, great horned owl, caracara hawk, and tarantula spider). In principle, olfactory stimuli should ideally be presented in the same form and amount, such as equal volumes of scented bedding. However, because home cage bedding may contain noxious compounds from feces or urine, we decided to use gauze scented with bodily secretions (urine, skin secretions) or bodily shedding (feathers, fur, skin) whenever possible. Table S1 presents a complete list of stimuli and collection methods. Cat-scented gauze was obtained by rubbing a medical gauze against the fur of a domestic cat, particularly around the neck region (Papes et al., 2010). Fifty milliliters of scented bedding (fine wood chips) were used as odors from leopard cat, mountain lion, African lion, tarantula spider, rat, and male and female mice. Alternatively, rat urine was used in some experiments by placing 1 ml of urine on pieces of medical gauze. For all stimuli deposited on gauzes, the gauze was unscented in a desiccator under vacuum overnight before adding the stimulus. For each snake species, we used 1 g of stimulus (around four 5 × 5 cm pieces of shed skin). Avian predator stimuli (hawk and owl) were 1 g of feathers, cut into small pieces. All stimuli (solid or liquid deposited on gauze) were attached to "binder clips" to visually confirm their position and prevent the spreading of stimuli in the cage. Control mice were exposed to unscented control odors, as indicated in Table S2. Some of the aforementioned odors were presented in different forms and most are composed of complex mixtures of largely uncharacterized ligands, some of which may also activate other sensory systems. Thus, additional experiments were performed to determine with certainty that activity in the MeA was due to pheromones and kairomones, using Mup proteins as pure ligands. These Mups are contained within the corresponding complex mixtures and could be presented in comparable amounts. For Mup experiments (**Figure 3**), gauze was scented with 10 mg of recombinant protein as fusion with Maltose-Binding Protein (MBP), and gauze scented with MBP alone was used as control.

Behavioral Assays

For defensive avoidance behavior, individually caged mice were habituated for 2 days in the dark in the procedure room and assayed on day 3. The amounts used for each stimulus are indicated in Table S1. In all cases, the animal was placed in the procedure room on day 3 and the stimulus was deposited on the side opposite to the air inlet. Mice were exposed and filmed for 30 min in the dark. Movies were scored blindly for avoidance time, following previously published protocol (Papes et al., 2010). Avoidance behavior was defined as the amount of time animals spent more than 20 cm away from the stimulus. To compare avoidance times where needed, ANOVA was applied, followed by Tukey-Kramer Honest Significantly Different (HSD) *post-hoc* analysis. Other types of defensive behaviors were also assayed (not shown), including freezing and risk assessment (Papes et al., 2010); in the latter, the animal approaches the stimulus with an extended body posture and arched back, a behavior seen for all predatory stimuli; during these episodes, the animals closely approached the stimulus in the first 5 min of stimulation; close contact was eventually seen after 25 min from the onset of stimulation, at which time the defensive behaviors became of progressively lower magnitude; eventual licking and biting the gauze was observed at the end of the exposure sessions, but preliminary experiments determined that physical contact with the stimulus is not necessary to trigger the aversive behaviors or brain activity, suggesting that the stimuli employed (even non-volatile Mup proteins) accessed the VNO lumen as airborne aerosol particles. For aggressive behavior assays, C57BL/6J male mice (8–12 weeks old) were isolated for 1 week and then exposed to castrated adult mice swabbed with 40μl of test solution (male mouse urine or equivalent amounts of rat or rabbit urine) for 10 min in their home cages. Tests were videotaped and analyzed to measure total duration of aggressive contact (biting, wrestling, and kicking). One round of urine and no-urine controls were performed with each resident mouse before and after sample testing. For reproductive behavior assay, the resident was a male mouse, prepared the same way as in the aggressive behavior assays. Subjects were exposed to 8 weeks old sexually naive, receptive females. Each assay ran for 15 min and the filmed behaviors were scored for mounting time (not shown).

Recombinant Mup Protein Expression

The cDNA for rat major urinary protein, Mup13 (Logan et al., 2008), was amplified by PCR from a Sprague-Dawley liver sample using oligonucleotides 5′ ATCGGATCCCATGC AGAAGAAGCTAGTTCCACAAGAG 3′ and 5′ ATCAAGC TTTCATCCTCGGGCCTGGAGACAG 3′. The amplicon was cloned into pMAL-c2x bacterial expression vector (New England Biolabs) into *Bam*HI and *Eco*RI restriction sites, and expressed as a fusion protein with MBP, following the manufacturer's recommendations. Protein was eluted from an amylose affinity resin using maltose and then exchanged into 1x PBS using a YM10 column (Millipore) prior to exposures. Recombinant MBP was used as a control. The same procedure was applied for production of the mouse Mups (nomenclature following Logan et al., 2008), and for the production of recombinant cat Mup, except that the corresponding cDNA was synthesized *in vitro* based on the published sequence of Fel-d-4 (cat Mup; GenBank accession number NM_001009233) (Smith et al., 2004).

c-Fos Immunostaining

For brain activity analyses, the expression of the surrogate marker of neuronal activity c-Fos was assayed by immunostaining. Inspection of neural activity by electrophysiological methods is very difficult for the MeA, due to its small size and deep location, but direct recording of activity was shown to be highly correlated with expression of the immediate early gene *c-Fos* in previous reports (Lin et al., 2011).

Each animal was individually caged and habituated to the procedure room where the exposures were conducted for 2 h on 2 consecutive days, in the dark. On the third day, each cage was brought to the procedure room and the stimulus was introduced in the animal's home cage on the side opposite to the air inlet. Each animal was exposed for 30 min, and the stimulus was removed from the cage at the end of the session, after which the subject remained in the dark without further stimulation for an additional period of 60 min; the animal was then quickly euthanized and dissected to remove the brain. Brains were fixed overnight in 4% paraformaldehyde, equilibrated in 20% sucrose/1x PBS and sectioned on a Leica 1000S vibrating-blade microtome. Fifty micrometer coronal sections were collected for the entire brain, and suitable sections were chosen for subsequent c-Fos immunostaining based on comparisons to a reference brain atlas (Paxinos and Franklin, 2004). Sections were blocked as free-floating sections for 1 h with 1% blocking reagent (Invitrogen), pre-incubated in 1% BSA/1x PBS/0.3% Triton X-100, followed by incubation with the anti-c-Fos primary antibody (rabbit polyclonal; Ab5; Millipore) diluted 1:1500 in 1% BSA/1x PBS/0.3% Triton X-100 for 36 h at 4°C under gentle agitation. Sections were washed three times in 1xPBS/0.1% Triton X-100, 15 min each, and incubated for 3 h at room temperature with Alexa 488-conjugated goat anti-rabbit secondary antibody (Invitrogen) diluted 1:500 in 1% BSA/1x PBS/0.3% Triton X-100. After two washes in 1x PBS/0.1% Triton X-100, 15 min each, sections were counterstained with To-Pro-3 nuclear stain (Invitrogen) diluted 1:1000 in 1x PBS, washed twice in 1x PBS, 15 min each, and mounted onto glass microscope slides with ProLong Gold (Invitrogen). Dry mounted sections were imaged on a Leica TCS SP5 confocal fluorescence microscope. The number of c-Fos positive nuclei was counted blindly for each individual. After image acquisition, the nuclear stain channel was computationally false-colored as purple, to facilitate contrast with the green fluorescence channel (c-Fos).

RNA *In situ* Hybridization

For VNO activity analyses, expression of the surrogate marker of vomeronasal neuron activity *Egr1* (Isogai et al., 2011) was used. Animals were exposed to stimulus for 45 min, sacrificed and the VNOs immediately collected, immersed in 4% paraformaldehyde fixative overnight, equilibrated in sucrose and sectioned on a cryostat (Leica) to produce 16 μm transversal sections. Slides were air-dried for 10 min, followed by fixation with 4% paraformaldehyde for 20 min, and treatment with 0.1 M HCl for 10 min, with 0.1% H_2O_2 for 30 min and with 250 mL of 0.1 M triethanolamine (pH 8.0) containing 1 mL of acetic anhydride for 10 min, with gentle stirring. Slides were always washed twice in 1x PBS between incubations. Hybridization was then performed with DNP (1 μg/mL) or DIG (600 ng/mL) labeled cRNA probes (Isogai et al., 2011) at 58°C in hybridization solution (50% formamide, 10% dextran sulfate, 600 mM NaCl, 200 μg/ml yeast tRNA, 0.25% SDS, 10 mM Tris-HCl pH8.0, 1x Denhardt's solution, 1 mM EDTA pH 8.0) for 16 h. Slides were washed once in 2x SSC, once in 0.2x SSC and once in 0.1x SSC at 60°C (30, 20, and 20 min, respectively), followed by a quick incubation in 0.1x SSC at room temperature. Slides were then permeabilized in 1x PBS, 0.1% Tween-20 for 10 min, and washed twice in TN buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl) for 5 min at room temperature, followed by blocking in TNB buffer [100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% blocking reagent (Perkin Elmer)], and incubation with rabbit anti-DNP (Invitrogen) primary antibody diluted 1:600 in TNB buffer overnight at 4°C. Signal development proceeded with the tyramide signal amplification kit (Perkin Elmer), following the manufacturer's instructions. Briefly, slides were incubated in tyramide-biotin [1:50 in amplification diluent with 0.0015% H_2O_2 (Perkin Elmer)] for 15 min, followed by incubation in streptavidin-HRP (1:100 in TNB) for 1 h, followed by incubation in tyramide-Alexa Fluor 546 [1:100 in amplification diluent (Life Technologies) with 0.0015% H_2O_2] for 15 min. Prior to each incubation, slides were washed 6 times with TNT buffer for 5 min under mild agitation. Sections were then treated with 3% H_2O_2 in 1x PBS for 1 h to block peroxidases from the first signal development. Slides were then blocked in TNB for 90 min, followed by incubation overnight at 4°C with anti-DIG-POD (Roche) diluted in TNB (1:400). Signal development was performed using tyramide-Alexa Fluor 488 dye (Invitrogen). Samples were counter-stained with To-Pro 3 nuclear stain (Invitrogen) diluted 1:1000 in 1x PBS, washed twice in 1x PBS and mounted with ProLong Gold (Invitrogen). Dry mounted sections were imaged on a Leica TCS SP5 confocal fluorescence microscope.

Dual Immunostaining/*In situ* Hybridization

We developed a dual staining protocol to parse out, in the same animal, the sets of active neurons related to two sequential exposure events separated by a period with no stimulation. In brief, mice were first exposed to a stimulus, then transferred back to their own soiled home cages for some time, followed by a second exposure to stimulus. Brains were then subjected to a dual immunostaining/*in situ* hybridization protocol to detect immature nuclear *c-Fos* mRNA derived from the second exposure period, concomitant with the detection of nuclear c-Fos protein resulting from the first exposure period. Specifically, animals were individually caged in Cage #1 and habituated in the dark for 90 min per day on the previous 2 days before the exposure. The habituation protocol guarantees that the animals are exposed to olfactory stimuli in a context relevant to the generation of behaviors. On the exposure day, half of the soiled bedding from the animal's cage was transferred to another cage (Cage #2). The first exposure to olfactory stimulus was performed in Cage #1 for 20 min, and the animals were then transferred to Cage #2 for 60 min without olfactory stimulation. They were then transferred back to Cage #1 for a second period of olfactory stimulation for 20 min (same or different stimulus). All of these steps were conducted in the dark. At the end of

the exposures, animals were anesthetized with ketamine/xylazine, and quickly perfused with 4% paraformaldehyde fixative. Brains were further fixed overnight with RNAse-free fixative and equilibrated in RNase-free 20% sucrose. Forty micrometer sections were collected on a VT100S vibratome (Leica) in 1x PBS-DEPC. Chosen sections encompassing the MeA were subjected to a new method for the combined detection of *c-Fos* mRNA and protein, as follows. *Pre-treatment of sections and probe hybridization:* Free-floating sections were fixed with 4% paraformaldehyde for 20 min, permeabilized in 0.2 M HCl/H_2O-DEPC for 10 min, incubated in 0.1% H_2O_2/1x PBS-DEPC to inactivate endogenous peroxidases for 30 min and acetylated in 0.1 M Triethanolamine-HCl pH 8.0 with acetic anhydride for 10 min. Sections were then incubated in hybridization solution containing 400 ng/mL of each of two 1 kb digoxigenin-labeled cRNA probes in a 5x SSC humidified chamber for 16 h, at 58–60°C. Probes correspond to two fragments of the *c-Fos* mRNA (fragments were obtained by reverse transcription-PCR using the following oligonucleotides: Probe 1: 5′ CAGCGAGCAACTG AGAAGAC 3′ and 5′ GCTGCATAGAAGGAACCGGAC 3′; Probe 2: 5′ GGAGCCAGTCAAGAGCATCAG 3′ and 5′ AATGA ACATTGACGCTGAAGGAC 3′). Hybridization solution also contained 50% deionized formamide, 600 mM NaCl, 200 μg/mL yeast tRNA, 0.25% SDS, 10 mM Tris-HCl pH 8.0, 1x Denhardt's solution, 1 mM EDTA pH 8.0 and 10% dextran sulfate. *Washes and antibody incubation:* Sections were washed in 2x, 0.2x, and 0.1x SSC solutions (20 min each), permeabilized in 0.1% Tween 20/1x PBS and blocked in 100 mM Tris-HCl/150 mM NaCl/0.5% Blocking Reagent (Perkin Elmer). Next, sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche; 1:400) for 2 nights, at 4°C. *Signal amplification and immunostaining:* Sections were incubated in tyramide-biotin (Perkin Elmer; 1:50) in amplification diluent containing 0.0015% H_2O_2, then incubated with horseradish peroxidase-conjugated streptavidin (Perkin Elmer; 1:100) in 100 mM Tris-HCl/150 mM NaCl/0.5% Blocking Reagent, and finally incubated in Alexa Fluor 546-tyramide (Invitrogen; 1:100) in amplification diluent containing 0.0015% H_2O_2. Peroxidase from the first signal was inactivated by treatment with 3% hydrogen peroxide for 30 min and 0.1 HCl for 10 min, prior to blocking and anti-c-Fos primary antibody (Ab5; Millipore) incubation in regular c-Fos immunostaining as detailed before. Nuclear counterstaining was performed with To-Pro-3 (Invitrogen; 1:1000) and sections were mounted on glass slides with ProLong Gold anti-fade reagent (Invitrogen) and imaged on a Leica TCS SP5 confocal microscope.

Since time elapsing between the two episodes of stimulation is sufficiently long in our protocol, this strategy enabled us to parse out the activation profiles derived from both stimulations with great precision, such that c-Fos mRNA is indicative of activation during the last exposure and c-Fos protein is indicative of the first exposure (**Figures 9D–G**). This method was based on the catFISH procedure (Guzowski et al., 1999, 2001; Lin et al., 2011). However, our method enables better resolution in the assignment of brain activity resulting from each of two consecutive olfactory stimulations, because the two exposure episodes are separated by a longer time period (1 h). Moreover, because the *c-Fos* gene encodes a transcription factor, the co-detection of its mRNA and protein inside the same subcellular compartment (nucleus) makes it unequivocal to determine if the cell produced mRNA, protein or both.

RNA Probe Design and Validation

For the design of cRNA probes to V2R vomeronasal receptors, we investigated whether different receptor genes harbor specific regions anywhere in the coding or non-coding regions. However, nucleotide and protein similarities among the members of each clade were found to be very high (>80%), though members from different clades usually share less than 70% nucleotide sequence identity. These similarity levels are constant throughout the entire gene sequence, including exons, introns, and untranslated regions. Unlike previously published reports (Isogai et al., 2011), one could not obtain probes that permit safe discrimination between cells expressing different receptors in the same clade, even under highly stringent hybridization conditions. For each clade, we chose probes based on one or two receptors, and each probe exhibited 80% minimum nucleotide similarity with other receptor members in the same clade, but 72% maximum allowed similarity with receptors in other clades. The rate of co-labeling of cells with two differently labeled probes in the same clade was high in prior validation experiments (usually >90% concordance). Each cRNA probe was produced with rNTPs labeled with haptens DNP (Egr1) and/or DIG (V2R) (Roche) from 1 kb suitable fragments cloned into pGEM-T-Easy vector (Promega), using SP6 or T7 RNA polymerases (Roche). VNO neurons in the basal zone co-express receptors in the V2R A/B/D families plus one receptor in the V2R C family (Silvotti et al., 2007). Since C family members are widely expressed in these neurons, we did not include probes for them in our investigation of the molecular identity of neurons activated by each olfactory stimulus.

Statistical Analyses

Statistical analyses were performed using R and Stat packages, and XLSTAT add-on in Excel. For comparing mean behavioral output measurements and numbers of c-Fos positive cells in each brain region, we applied one-way Analysis of Variance (ANOVA), followed by Tukey-Kramer HSD *post-hoc* analysis. In each case, *P*-values indicate the probability that the null hypothesis (the means are equal or were drawn from like populations) is true. $P > 0.05$ led to rejection of the null hypothesis in all tests. For comparing distributions of active cells in the MeA for two stimulus groups in **Figure 11**, we counted cells along the dorsal-ventral axis of the MeA, the position and direction of which were estimated taking the third ventricle in the hypothalamus as a proxy. The axis ventral limit (the origin in all graphs in **Figure 11**) was defined as the most ventral pixel in the MeA in each image. The axis dorsal limit was defined as the point 600 μm from the origin along the dorsal-ventral axis. Data collected at bregma –1.46 mm from all animals in each group being compared were plotted in scale intervals of 60 μm (total of 10 intervals plotted in bar graphs in **Figure 11**) along the dorsal-ventral MeA axis. Comparisons between distributions for any two groups were performed with the Kolmogorov-Smirnov

distribution comparison test (KS test), applied on cumulative ranks for the intervals mentioned above. In this case, *P*-values indicate the probability that the null hypothesis (the distributions are equal or were drawn from like populations) is true.

Results

A Wide Range of Intra- and Interspecies Chemosignals Detected by the VNO Activate the Medial Nucleus of the Amygdala

In order to investigate in detail the patterns of activity in the MeA, we separately exposed C57BL/6 mice to the various intra- and interspecies olfactory stimuli listed in Table S1 (see also Materials and Methods). For all stimuli, the amounts employed induced similarly potent behavioral responses in mouse subjects (**Figure 1A**; see also Chamero et al., 2007; Papes et al., 2010; Isogai et al., 2011). Interestingly, several heterospecific odors were shown to trigger defensive behaviors (**Figure 1A**). Other odors (such as same-strain mouse odors) were unable to elicit defensive behaviors, and instead induced aggressive or reproductive responses (**Figure 1A**).

With this protocol, increases in VNO and brain activity for each stimulus could be evaluated by comparison with appropriate unscented controls (listed in Table S2). Most of the stimuli activated a large number of VNO sensory neurons (**Figure 1B**), as judged by the expression of the marker of VNO activity *Egr1* (Isogai et al., 2011). Some stimuli were presented in different forms (Table S1) and therefore cannot be compared, but we noticed a tendency for intraspecific signals to activate a smaller subpopulation of VNO cells than interspecific stimuli, such as predator odors (**Figure 1B**).

Next, we analyzed whether VNO activation was accompanied by activity in the AOB and MeA, known to have anatomical connections (direct or indirect) with olfactory sensory organs (**Figures 1C,D**). In animals exposed to heterospecific or conspecific odors, induction of the marker of neuronal activity c-Fos was strong in the AOB (**Figures 1D**, **2A**, **3Ai**, **4**; see also Papes et al., 2010) and MeA (**Figure 1D** and Table S2). Rabbit urine, which does not induce defensive or aggressive behaviors in mice, did not activate these nuclei, nor did generally noxious stimuli such as foot shock (**Figure 1D** and Table S2).

Interestingly, we noticed that heterospecific signals tended to activate the AOB and MeA more strongly than conspecific odors (**Figure 1D**), and we found a statistically significant positive correlation between activity induced by each stimulus in these areas and the number of *Egr1*-expressing cells in the VNO (**Figures 2A,B**; see also **Figures 2C,D** for control brain areas).

Investigation of Activity in the AOB and MeA after Exposure to Intra- and Interspecies Pure Stimuli

Next, we intended to confirm that the observed activity in the MeA was due to pheromones and kairomones, by using pure ligands instead of complex odorous mixtures (see Materials and Methods section for details on pure stimuli). Recombinant versions of Mup proteins (rMups) were employed, namely, mouse pheromone rMups, which are able to induce aggressive and territorial behaviors (Chamero et al., 2007; Kaur et al., 2014), and a predator kairomone rMup, which triggers defensive behaviors (Papes et al., 2010).

First, we confirmed that neural activity due to each pure rMup is indeed part of that induced by the corresponding native stimulus, because exposure to a combination of each rMup plus the respective complex odor resulted in c-Fos counts which are not the sum of c-Fos positive cells in animals separately exposed to the pure or to the native stimuli alone (**Figure 3A**). Importantly, we found that equal amounts of predator (cat or rat) or mouse rMups elicited similar activity in the AOB or MeA (**Figures 3A,B**). This first-hand comparison of brain activity due to different pure stimuli revealed that the MeA, the activity of which correlates with VNO activation induced by complex stimuli (**Figures 1**, **2**), is indeed activated by pure pheromones and kairomones.

The Medial Nucleus of the Amygdala Receives Major Functional Inputs from the VNO

In order to determine if the observed MeA activity was dependent on the VNO-mediated detection of pheromones/kairomones, we adopted a genetic strategy, where MeA c-Fos analysis was performed in TrpC2$^{-/-}$ mutant animals without a functional VNO (see Materials and Methods for details on the knockout line).

First, we confirmed that instinctive behaviors toward a large variety of hetero- or conspecific odors were impaired in TrpC2$^{-/-}$ mutants (data not shown; see also Stowers et al., 2002; Chamero et al., 2007; Papes et al., 2010). Next, we found that the behavioral defects in the mutant animals were accompanied by severely reduced or abolished c-Fos expression in the AOB (**Figure 4A** and Table S2; see also **Figure 4B** and (Papes et al., 2010) for representative images of the pAOB activation after exposure to various odors), consistent with the notion that this brain region mainly collects information from the VNO (Wagner et al., 2006). Importantly, in the MeA, the number of c-Fos positive cells is much lower in TrpC2$^{-/-}$ than in TrpC2$^{+/+}$ mice, being comparable to unscented controls (**Figure 5A**, left graph for complex native stimuli and right graph for pure stimuli, and **Figure 5B** for representative images from animals of both genotypes exposed to conspecific or heterospecific odors; see also Table S2), suggesting that the observed increase in c-Fos expression in wild-type animals primarily results from processing of VNO signals.

It is important to note that other regions, such as the piriform cortex, another olfactory area in the brain, do not exhibit changes in activity in TrpC2$^{-/-}$ animals (**Figure 4A**, bottom graph), in keeping with the notion that this brain region does not receive major inputs from the VNO (Stettler and Axel, 2009). It further shows that loss of activity is not widely distributed in the brains of TrpC2$^{-/-}$ mice, strengthening the idea that the decrease in MeA activity in the mutants is specific and due to the lack of a functional VNO.

Olfactory Stimuli Activate Broadly Distributed Ensembles of Neurons in the Medial Amygdala

Next, we investigated and compared how distinct stimuli lead to activation patterns in the MeA, by carefully evaluating the spatial

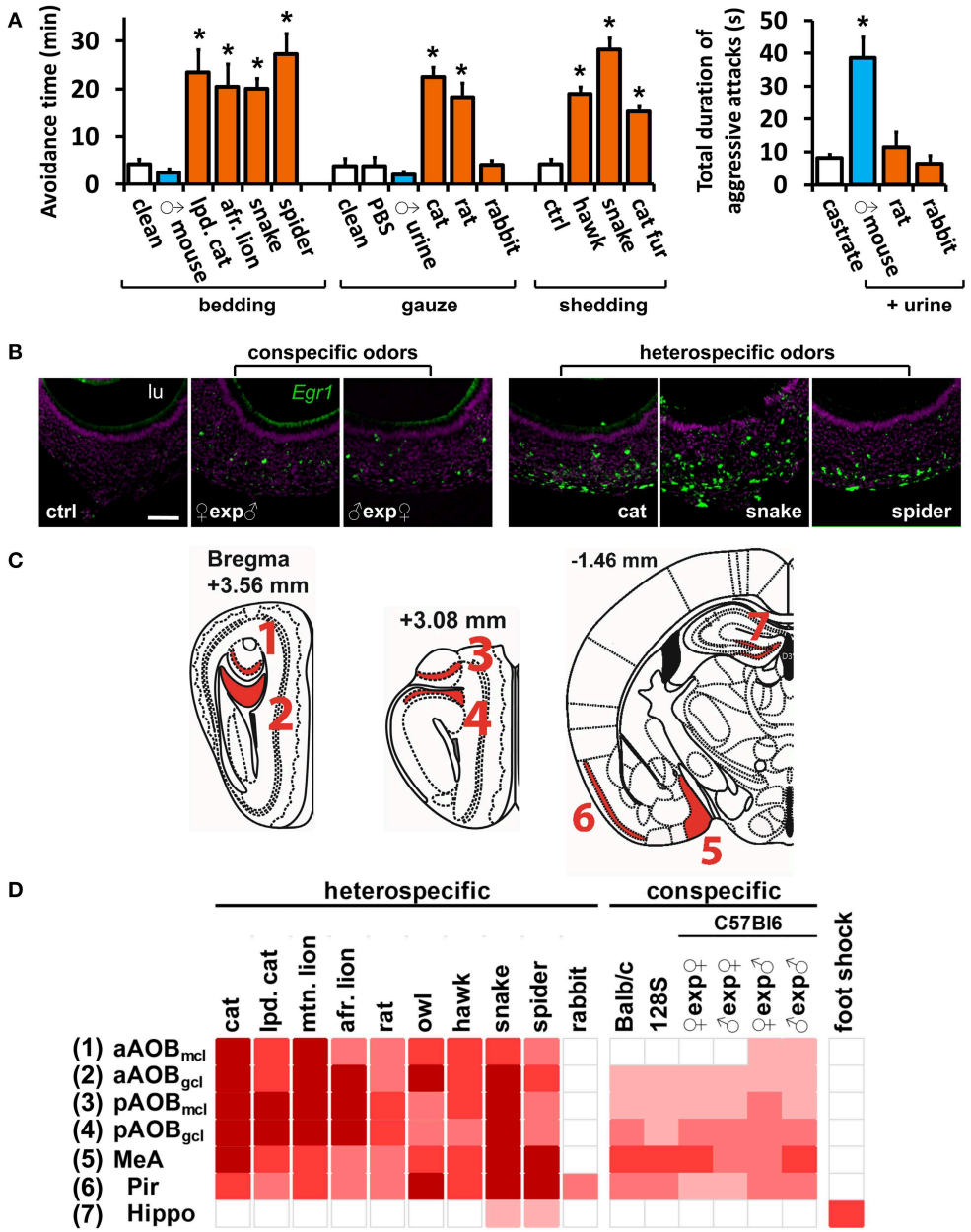


FIGURE 1 | A wide range of olfactory stimuli activates the medial nucleus of the amygdala. (A) Increased avoidance defensive behavior (left) after exposure to various heterospecific mammalian and non-mammalian predator odors. Some heterospecific (rabbit) and conspecific stimuli do not induce behavior. In contrast, inter-male aggression (right) is elicited by conspecific, but not by heterospecific, odors. Heterospecific stimuli are shown in orange and conspecific ones in blue, grouped according to presentation form (bedding, gauze or bodily shedding). Mean ± s.e.m. $^*p < 0.01$; ANOVA followed by Tukey-Kramer HSD *post-hoc* analysis against respective control (white bars). See Tables S1 and S2 for a list of stimuli and controls. **(B)** Exposure of male mice to conspecific or heterospecific stimuli activates VNO neurons, as evidenced by *in situ* hybridization to immediate early gene *Egr1*. Control (ctrl) odor is PBS-soaked gauze for liquid stimuli (similar number of *Egr1*-positive cells were found for other controls). Scale bar represents 100 μm. lu, VNO lumen. Purple labeling, nuclear stain; green, *Egr1 in situ* hybridization signal. **(C)** Representation of coronal sections through the mouse brain at the indicated bregma values, showing the location of analyzed brain nuclei in **(C)**. 3V, third ventricle; Aq, cerebral aqueduct. **(D)** Heat map representing the activation of the medial nucleus of the amygdala (MeA) and accessory olfactory bulb (AOB) sub-regions, in animals exposed to various heterospecific and conspecific olfactory stimuli. As a control, activity in the piriform cortex (Pir), which does not receive major vomeronasal system inputs, is also shown, as well as the hippocampus (hippo), the activity of which is not directly related to olfaction and was used as control. Bright red indicates the largest number of observed c-Fos-positive cells per unit area for each nucleus. White indicates activation comparable to control level. See Table S2 for numbers of c-Fos-expressing cells and a key to heat map colors in each area, for each odor and respective controls. A white gap separating two columns indicates that the corresponding stimuli were presented in different forms and should not be compared. ♀exp♂ denotes a female mouse exposed to male odors. $n = 6–26$. gcl, granule cell layer of the AOB; mcl, mitral cell layer. n.d., not determined.

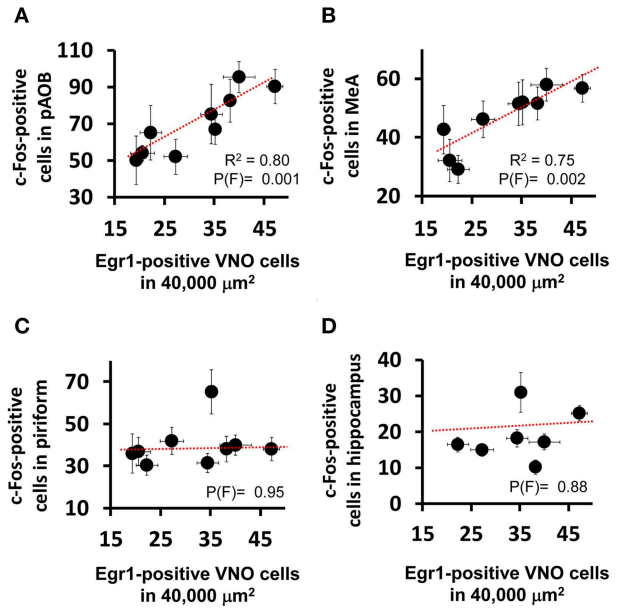


FIGURE 2 | Activity in the medial nucleus of the amygdala is correlated with vomeronasal sensory detection activity. Scatter plots comparing the activation level in the VNO (judged by the number of nuclei expressing the immediate early gene *Egr1* in a 40,000 μm^2 area of sensory epithelium) and the activation level seen in **(A)** AOB (posterior aspect) and **(B)** MeA, as judged by the number of c-Fos positive cells in each brain region per unit area (as denoted in Table S2). Each dot represents a different stimulus. The R^2 and probability values for the F statistics are given in each panel, indicating the amount of variance explained by the linear regression model (dashed red line) and the likelihood that the data fit the model, respectively. Note the positive correlation between AOB and MeA activation and VNO activation, but absence of correlation for the piriform cortex **(C)**, the activation of which reflects events in the main olfactory epithelium, not the VNO. **(D)** The hippocampus is used as a control region not directly affected by either the main olfactory or vomeronasal system activation.

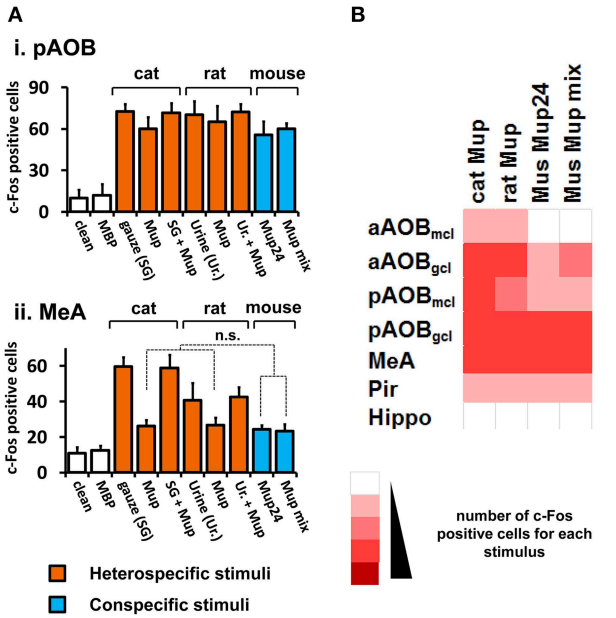


FIGURE 3 | Activity induced by pure VNO stimuli in the medial nucleus of the amygdala and control brain areas. (A) Comparison among activity in the AOB (i) and MeA (ii) in animals separately exposed to purified ligands presented in the same amount (cat, rat or mouse Mups). Mup24 or a mixture of Mup24, Mup3, Mup8, and Mup25 (Mup mix) were used at the same total amount. The number of c-Fos expressing cells is also shown for corresponding native stimuli, which are cat-scented gauze (SG) and rat urine (Ur.). Heterospecific stimuli are indicated in orange and conspecific ones in blue. White bars represent unscented controls (MBP-soaked or clean gauze for liquid or solid stimuli, respectively). $n = 8$–12. n.s., no statistically significant difference. **(B)** Heat map representing the activation of the AOB, MeA and control areas (same as in **Figure 1**) in animals exposed to purified heterospecific and conspecific olfactory stimuli. Abbreviations and color grading are the same as in **Figure 1**. See Table S2 for numbers of c-Fos expressing cells.

distribution of activated cells in animals exposed to our wide set of heterospecific and conspecific stimuli. Overall, an average of 10–25% of MeA cells were activated, depending on the stimulus. In mice exposed to each of the predator odors, c-Fos-expressing cells were found broadly distributed in the MeA (**Figures 6D–H**). For such heterospecific stimuli, the distributive pattern was seen in both the dorsal and ventral aspects of the posterior MeA (MeAp) and in the anterior MeA (MeAa) (examples in **Figure 6**; see also **Figure 7** and quantitation in **Figure 8**).

Strikingly, for most conspecific signals, the same broad distribution pattern was observed (**Figures 6B,C**), in both the dorsal and ventral aspects of the MeAp and in the anterior MeA (MeAa) (**Figures 7**, **8**). In male mice exposed to female odors, the activated cells were found in both the dorsal and ventral MeAp, but with a higher concentration in the dorsal aspect (**Figures 6C**, **7B**).

The Active Ensembles of Neurons in the MeA are not Invariant

Next, we investigated the stereotypy and invariance of the ensemble of activated cells in the MeA. To this end, we developed a dual c-Fos immunostaining/*in situ* hybridization protocol to parse out, in the same animal, the sets of active neurons related to two sequential olfactory exposure events (see Materials and Methods for details). In these experiments, c-Fos protein is indicative of activation during the first exposure, while *c-Fos* mRNA labels cells activated after the second exposure (**Figures 9D–G**).

Exposure of mice to the same stimulus twice leads to largely distinct subsets of activated cells in the MeA, with 20–30% overlap (**Figures 9A–C**; see also Video S1 for an explanation of dual staining visualization). This contrasts with the level of overlap we observed between groups of activated cells in the piriform cortex related to two instances of exposure to the same stimulus: in this area, the same stimulus resulted in the consistent activation of largely overlapping groups of neurons over two subsequent trials (**Figure 9C**, right bars, Pir): 83.7 ± 1.7% of all piriform cells expressing c-Fos protein also express *c-Fos* mRNA; and 77.2 ± 2.1% of all cells expressing *c-Fos* mRNA also express c-Fos protein.

Interestingly, the concordance level between the active MeA ensembles in two exposures to the same stimulus is significantly

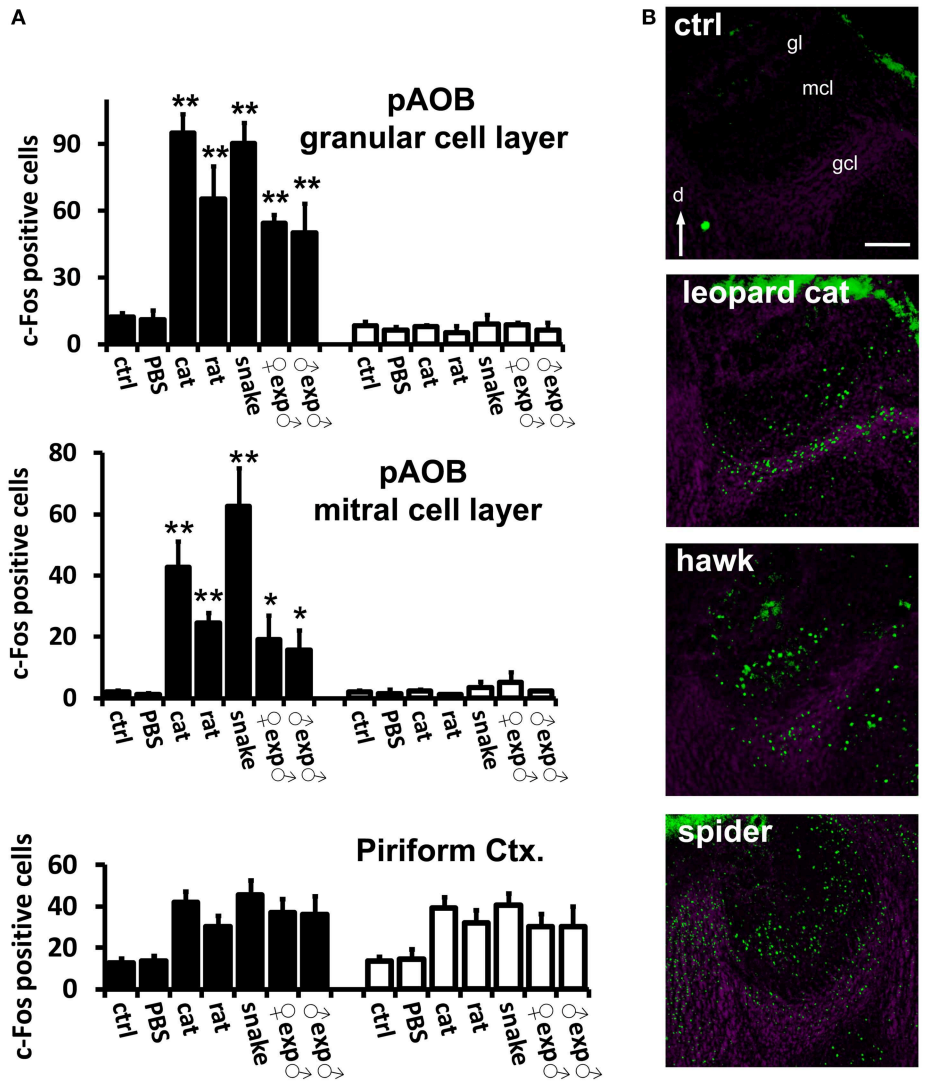


FIGURE 4 | Confirmation of VNO-mediated stimulus detection by investigation of activity in the accessory olfactory bulb in the brain. **(A)** Quantification of odor-evoked c-Fos expression in the AOB in TrpC2$^{+/+}$ (black bars) or TrpC2$^{-/-}$ (white bars) genotypes. pAOB means activity in the posterior AOB. The top bar graph means activity in the granule cell layer and the middle graph represents activity in the mitral cell layer. Control odor is PBS-soaked or clean gauze for liquid or solid stimuli, respectively. $n = 8$–20. $^{*}p < 0.05$; $^{**}p < 0.01$. Mean ± s.e.m. ANOVA followed by Tukey-Kramer HSD *post-hoc* analysis (comparison of TrpC2$^{+/+}$ against TrpC2$^{-/-}$ for each odor). See Table S2 for numbers of c-Fos expressing cells and for other odors, and for a full description of controls, in both genotypes. Activation by each test stimulus and loss of activity in the mutants confirm that the stimuli activate the VNO and connected accessory olfactory pathway in the brain. Activity in the piriform cortex, which is not known to receive massive inputs from the vomeronasal organ, is not affected by the genetic ablation of the VNO in TrpC2$^{-/-}$ animals (bottom graph). **(B)** Examples of immunostaining for c-Fos (green fluorescence) showing activation of the posterior division of the AOB in animals exposed to three taxonomically distantly related species (heterospecific odors). Purple labeling shows nuclear staining. gcl, granule cell layer; mcl, mitral cell layer; gl, glomerular layer; d, dorsal. Scale bar represents 100 μm. See Table S1 for a description of stimuli, and Table S2 for quantification of AOB activation. See also Papes et al. (2010), for images of AOB c-Fos immunostaining for other stimuli.

larger than that expected by chance alone [23.5 ± 0.8% of all cells expressing c-Fos protein that also express *c-Fos* mRNA vs. 1.1 ± 0.2% overlap expected by chance (chi-square test of goodness-of-fit; $P = 0.85$); or 27.8 ± 1.9% of all cells expressing *c-Fos* mRNA that also express c-Fos protein vs. 0.96 ± 0.3% overlap expected by chance ($P = 0.82$)]. Together, the foregoing results suggest that the active ensembles in the MeA, though not random, are not invariant.

Distinct Olfactory Stimuli Activate Intermingled Ensembles of Neurons in the Medial Amygdala

To directly compare the spatial organization of activity in the MeA due to distinct stimuli (**Figure 10**), we used the dual c-Fos immunostaining/*in situ* hybridization protocol described above. When we compared snake and cat stimuli, the overlap seen between the active MeA ensembles during the two exposures was not small [25.2 ± 2.4% of all cells expressing c-Fos protein

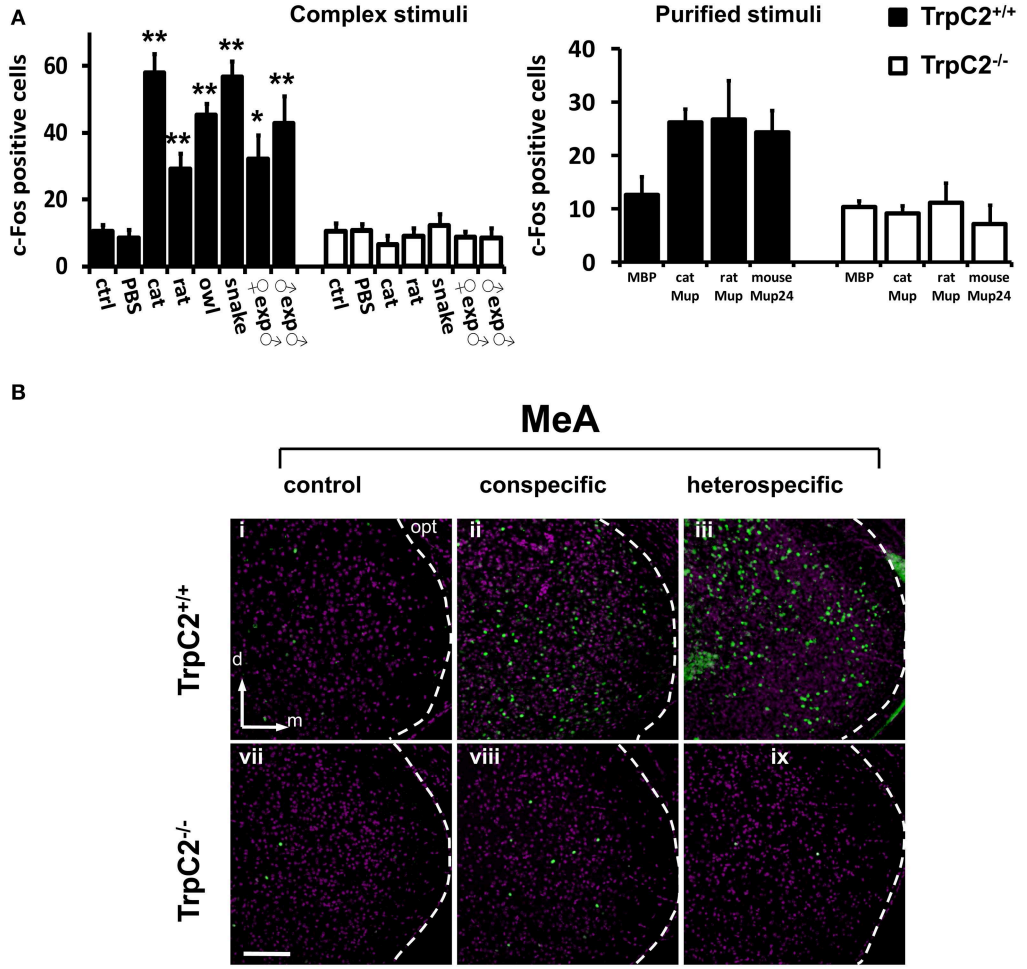


FIGURE 5 | Investigation of activity in the medial nucleus of the amygdala after exposure to odors detected by the VNO. (A) Quantification of odor-evoked c-Fos expression in the MeA in TrpC2$^{+/+}$ (black bars) or TrpC2$^{-/-}$ (white bars) genotypes. The left bar graph shows data for exposures to native (complex) conspecific or heterospecific stimuli; the right bar graph exhibits data for purified stimuli. Control odor is PBS-soaked or clean gauze for liquid or solid stimuli, respectively. $n = 8$–20. $^{*}p < 0.05$; $^{**}p < 0.01$. Mean ± s.e.m. ANOVA followed by Tukey-Kramer HSD *post-hoc* analysis (comparison of TrpC2$^{+/+}$ against TrpC2$^{-/-}$ for each odor). See Table S2 for numbers of c-Fos expressing cells and for other odors, and for a full description of controls, in both genotypes. **(B)** Exposure to conspecific and heterospecific stimuli leads to activation in the MeA, judged by c-Fos expression (green). This effect is significantly impaired in TrpC2$^{-/-}$ animals, where the VNO is non-functional. The white dashed lines mark the boundaries for the MeA. d, dorsal; m, medial; opt, optic tract. Scale bar represents 100 μm.

that also express *c-Fos* mRNA vs. 1.5 ± 0.4% overlap expected by chance (chi-square test of goodness-of-fit; $P = 0.81$); or 23.8 ± 0.9% of all cells expressing *c-Fos* mRNA that also express c-Fos protein vs. 1.4 ± 0.4% overlap expected by chance ($P = 0.90$)]. Similar results were obtained in the comparison between snake (heterospecific) and female mouse (conspecific) odors (not shown). Although significantly larger than that expected by chance alone, this overlap level does not permit us to easily and unequivocally interpret whether different stimuli activate non-overlapping ensembles or whether the active sets of cells have some degree of overlap in the MeA, because the same stimulus induces c-Fos expression in subsets of cells with an equivalent degree of overlap over two subsequent trials (**Figure 9C**).

However, the comparison between multiple stimuli with our dual staining protocol does enable us to determine if there is spatial segregation of activated cells in the MeA. We did not verify any immediately discernible differential distribution of activated cells when one predator stimulus was compared to a different predator stimulus (**Figure 10B**), indicating that the active ensembles related to exposures with these distinct stimuli were intermingled in broad regions of the MeA. Similarly, we could not verify spatial segregation in the MeA when a predator odor was compared in the same animal with odors from conspecific same-sex individuals: in males, the comparison between a predator stimulus and female mouse odors (which activate cells distributed in the entire MeA but concentrated along its dorsal side) also revealed that these two stimuli activate mostly interspersed groups of cells throughout the MeA (**Figure 10C**).

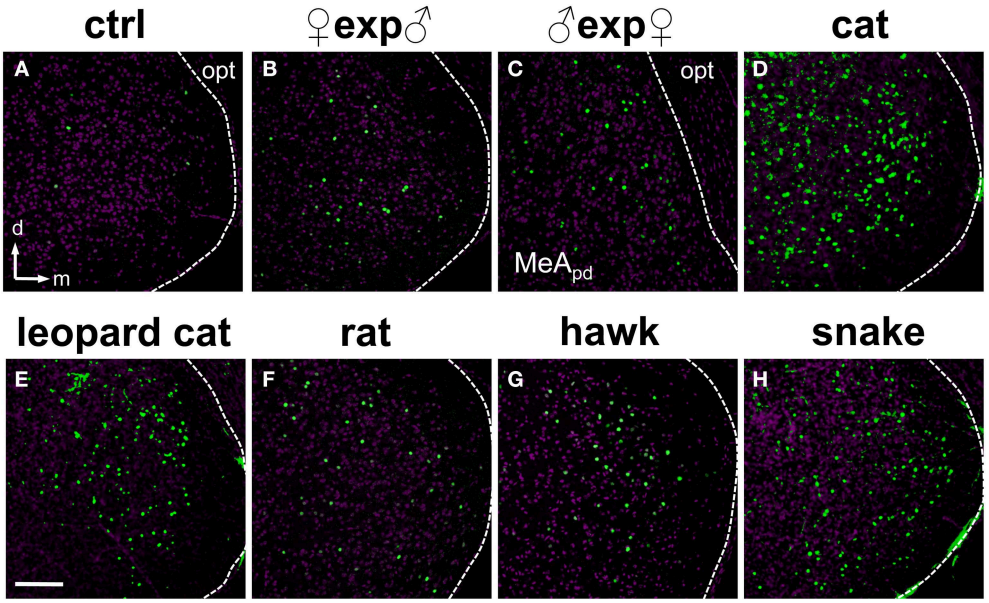


FIGURE 6 | Olfactory stimuli activate dispersed ensembles of cells in the medial nucleus of the amygdala. (A) MeA activity observed in animals exposed to control odor (ctrl). **(B–H)** Distributed patterns of c-Fos expression in the posterior aspect of the MeA in animals exposed to conspecific **(B,C)** or heterospecific **(D–H)** stimuli, including several predator species. Panel **(C)** represents the dorsal portion of the MeA (MeApd), near the optic tract. Remaining panels represent the whole MeA. The white dashed lines mark the boundaries for the MeA. $n = 4$–6. d, dorsal; m, medial; opt, optic tract. Scale bar represents 100 μm.

Organization of Activity in the MeA Does Not Reflect Behavioral Output, Stimulus Valence, Stimulus Origin, Chemical Category or Receptors Activated at the Sensory Organ Level

If distinct olfactory stimuli activate intermingled ensembles of cells in the MeA, without spatial order, how is activity organized in this nucleus? Odors capable of eliciting defensive responses activated both the dorsal and ventral MeA (**Figures 6**–**8**), and therefore activity related to defensive stimuli is not exclusively restricted to or markedly enriched in the ventral MeA, as previously suggested (Swanson, 2000; Canteras, 2002; Choi et al., 2005). The spatial distributions of cells in the active ensembles are not significantly different for heterospecific vs. conspecific stimuli (**Figure 11B**; two sample Kolmogorov-Smirnov distribution comparison (KS) test, $P = 0.26$; see Materials and Methods and **Figure 11A** for details). Therefore, the organization of MeA activity does not seem to reflect the source of stimulus origin (heterospecific vs. conspecific) or the behavioral outputs (reproductive vs. defensive).

Second, we alternatively hypothesized that activity in the MeA could be organized according to the taxonomic groups to which the species tested belong. Even though the numbers of distinct species used in each taxonomic group were limited, our data do not support this model, because the distributions of activated cells for avian predators (**Figures 6G**, **8C**), mammals (**Figures 6D–F**, **7B**, **8A,C**), reptiles (**Figures 6H**, **7B**, **8B**), and even invertebrates (**Figure 8B**) are all dispersed across the nucleus, without preference for dorsal or ventral MeA, or for pMeA or aMeA (**Figure 8**).

Third, we examined whether activity in the MeA is dependent on the chemical nature of the stimuli. For example, small organic molecules could be represented in one MeA sector, while proteins/peptides would evoke activity in another. However, our data show that male mouse urine (containing both Mup proteins and small organic VNO ligands; Chamero et al., 2007) activates a distributed ensemble (**Figures 5B**, **6B,C**, **8B**), whereas cat odor (containing a defensive behavior-inducing Mup; Papes et al., 2010) produces a similar pattern (**Figures 5B**, **6D**, **8B**). Therefore, our data indicate that stimuli of dissimilar chemical nature do not necessarily induce distinct patterns of MeA activity, suggesting that the mapping of olfactory information in this nucleus is not dependent on the chemical class to which the stimulus belongs.

Finally, we verified whether activity in the MeA is dependent on the molecular identity of activated sensory VNO neurons. Previous publications (Isogai et al., 2011) indicate that most stimuli employed in our study are detected by cells in the basal zone of the VNO, characterized by the expression of GPCR receptors in the V2R family (~120 family members, phylogenetically grouped into several clades; Silvotti et al., 2007). Therefore, we decided to focus on defining the V2R receptors expressed in activated VNO neurons in animals individually exposed to each stimulus, using double fluorescent *in situ* hybridization with one probe to detect the expression of the marker of vomeronasal neuron activation *Egr1* and other probes to detect specific clades of V2R receptors (Figure S1A) (see Materials and Methods for probe design and validation).

Stimuli that activate VNO neurons expressing V2R receptors in the A4 clade (nomenclature following Silvotti et al., 2007),

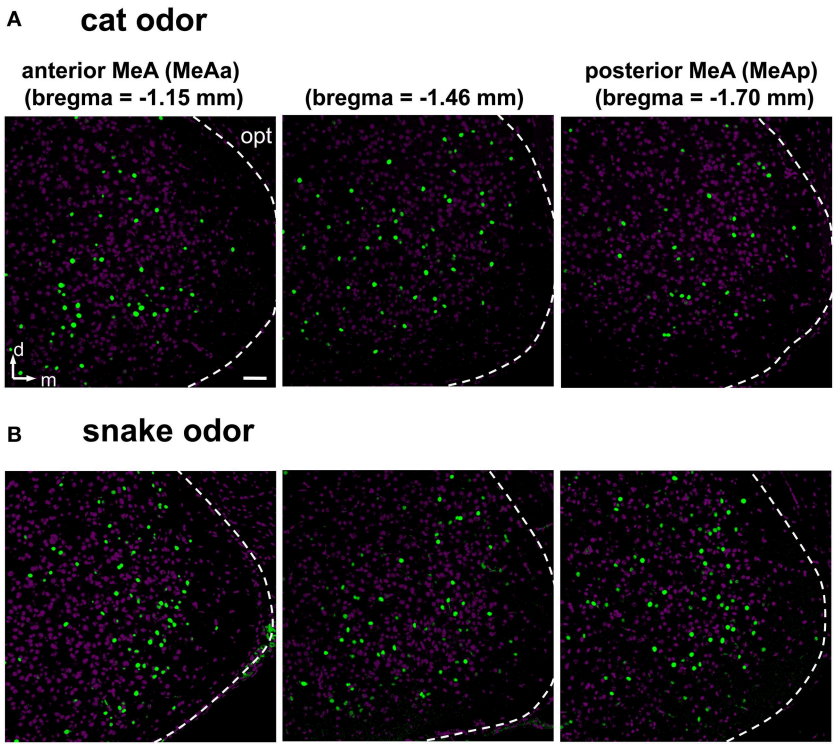


FIGURE 7 | Examples of activity in the medial nucleus of the amygdala along the rostral-caudal axis after exposure to heterospecific stimuli. **(A,B)** Activation in the MeA is distributed across the nucleus and along the rostral-caudal axis (sections in three bregma positions are shown), in animals exposed to cat **(A)** or snake **(B)** odors. Images represent immunostaining for the marker of neuronal activation c-Fos (green fluorescence). Purple labeling, nuclear stain. The white dashed lines mark the boundaries for the MeA. d, dorsal; m, medial; opt, optic tract. Scale bar represents 100 μm.

such as feline odors, all produce distributed c-Fos expression in the MeA (**Figures 6D,E**, Figures S1B, S1C, right panel). Chemosignals detected by VNO cells expressing clades A8, A5, and A1 of V2R receptors, such as same or opposite-sex adult mouse odors (**Figures 6B,C** and Figure S1B), also activate dispersed ensembles in the MeA. The ensembles activated by odors belonging to these two categories are in fact intermingled (**Figures 9**, **10**), and the distributions of activated cells (**Figure 11C**) are not statistically significantly different between the two groups (two-sample Kolmogorov-Smirnov test; $P = 0.40$).

Discussion

Representation of Olfactory Information in the Brain

In this study, we investigated the activity in the mouse medial amygdala induced by odors that elicit instinctive behaviors important for the survival of the individual and the species (pheromones and kairomones). Using a combination of genetic and brain activity analyses, we show that a wide range of different odors activate cells in the MeA, and that this area is functionally involved in the circuit initiated at the VNO. Though the general involvement of the amygdala in processing olfactory information has been appreciated for some time (Swanson, 2000; Takahashi, 2014), its exact place in the neural mapping and representation of odors remains poorly characterized. We investigated the organization of activity in this area and found that there is a lack of spatial segregation for activity related to distinct stimuli, without reflecting output behaviors or stimulus valence, which is in opposition to the current views about how activity in the medial amygdala is laid out.

Activation of the Medial Amygdala by a Large Repertoire of Intra- and Interspecific Odors

In this study, we comparatively analyzed brain activity in the MeA by using a wide range of intra- and interspecies olfactory stimuli (Table S1) which are able to induce instinctive behavioral responses in mouse subjects, including aggression, sexual behavior, or defensive behavior (**Figure 1A**). Curiously, we found that several heterospecific odors trigger defensive behaviors, greatly expanding the previously described list of kairomones (Papes et al., 2010) and evidencing the importance of olfaction in the detection of danger. In animals exposed to most conspecific and heterospecific stimuli, a significant subgroup of VNO sensory neurons was activated. We noticed that heterospecific odors that induce defensive behaviors tended to activate more VNO cells than other types of stimuli (**Figure 1B**), a result that stresses the importance of the mammalian VNO in the detection of odors from other species (Papes et al., 2010; Isogai et al., 2011).

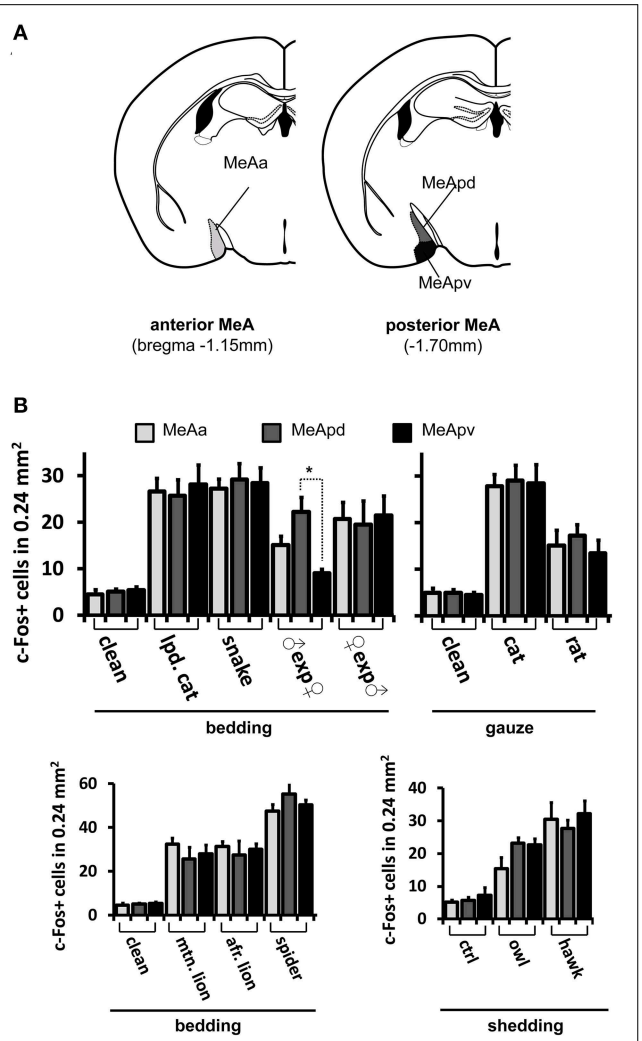


FIGURE 8 | Activity in the medial nucleus of the amygdala after stimulation with pheromones or kairomones is not spatially segregated. (A) Schematic representation of the anatomical divisions of the MeA, showing its anterior (left) and posterior (right) aspects and the different subsectors therein, such as the anterior MeA (MeAa), posterodorsal MeA (MeApd), and posteroventral MeA (MeApv). **(B)** Distribution of activity in MeAa, MeApd, and MeApv parts of the MeA, for several stimuli (grouped by presentation form). *$p < 0.01$. Mean ± s.e.m. ANOVA followed by Tukey-Kramer HSD *post-hoc* analysis. See also **Figures 6**, **8** for representative c-Fos images and other stimuli. lpd. cat, leopard cat; mtn. lion, mountain lion; afr. lion, African lion. ♀exp♂ denotes a female mouse exposed to male odors. In most cases, activity is dispersed in the MeA, without spatial segregation in the three subsectors, with the exception of a conspecific stimulus, namely, in males exposed to female odors.

An investigation of activity in the AOB and MeA in mice exposed to such chemosignals revealed that these two areas are activated by those VNO stimuli (**Figures 1**, **2**). Direct projections of VNO neurons to the AOB are known since the times of Ramon y Cajal, and indirect anatomical links between the MeA and VNO have been known for a while (Canteras et al., 1995, 1997; Petrovich et al., 2001; Blanchard et al., 2005; Mohedano-Moriano et al., 2007). Our data now provide a comprehensive view of how the AOB and MeA are activated by odorous stimuli, revealing that a strikingly large set of behavior-inducing odors activates these areas. Additionally, we found that these nuclei are more strongly activated by heterospecific odors than by conspecific stimuli (**Figure 1D**), revealing the unprecedented fact that the mammalian brain is exquisitely activated by odors from other species in the environment. Finally, we show that activity in the MeA is correlated with the detection of stimuli by the VNO (**Figure 2**), supporting the idea that it belongs to a functional pathway initiated at the VNO to process olfactory information.

Because most stimuli employed in these experiments were complex in nature, we needed to confirm that pheromones and kairomones contained therein could indeed activate the circuit initiated at the VNO, including the AOB and MeA. In fact, when we used recombinant versions of Mup proteins from mouse and cat, which act as pheromones and kairomones, respectively (Chamero et al., 2007; Papes et al., 2010), the VNO, AOB, and MeA were activated (**Figure 3**).

By using a wide variety of behavior-inducing odors, together these experiments (**Figures 1**–**3**) led to the corroboration that the MeA is activated by pheromones and kairomones, and greatly expanded the known repertoire of signals, both complex and pure, able to provide sensory input to this region; moreover, these data suggest that the MeA mostly receives functional inputs from the VNO, because MeA activity is highly correlated with VNO detection of behavior-inducing odors.

Functional Links between MeA and VNO

The activity we observed in the MeA with complex olfactory stimuli (**Figure 1**) may not necessarily be dictated by the VNO, because MeA neurons may also receive inputs from other sensory organs. In this vein, we noted that the piriform cortex, which does not receive major inputs from the VNO, is also activated by our complex stimuli (**Figure 1D**). Therefore, we needed to establish the existence of possible functional links between the observed MeA activity and VNO detection of pheromones/kairomones. In order to do this, we exposed VNO-deficient TrpC2$^{-/-}$ mice to the chemosignals described in the previous section. We observed that the expression of the indirect marker of MeA neuronal activation c-Fos was much lower in TrpC2$^{-/-}$ than in TrpC2$^{+/+}$ mice, similar to the expression seen in animals exposed to unscented controls (**Figure 5**), indicating that MeA activation is functionally linked to the detection of stimuli at the VNO sensory interface.

In combination with the fact that activity in the MeA is highly correlated with VNO activity (**Figures 1**, **2**), these results in TrpC2$^{-/-}$ animals indicate that the MeA is mostly controlled by the vomeronasal system, favoring the model in which it is part of a functional circuit initiated at the VNO to control behavior (Canteras et al., 1997; Swanson, 2000; Blanchard et al., 2005; Takahashi, 2014).

Organization of Odor-induced Activity in the Medial Amygdala

In our study, the use of a wide selection of heterospecific and conspecific signals and of potent stimuli from several species

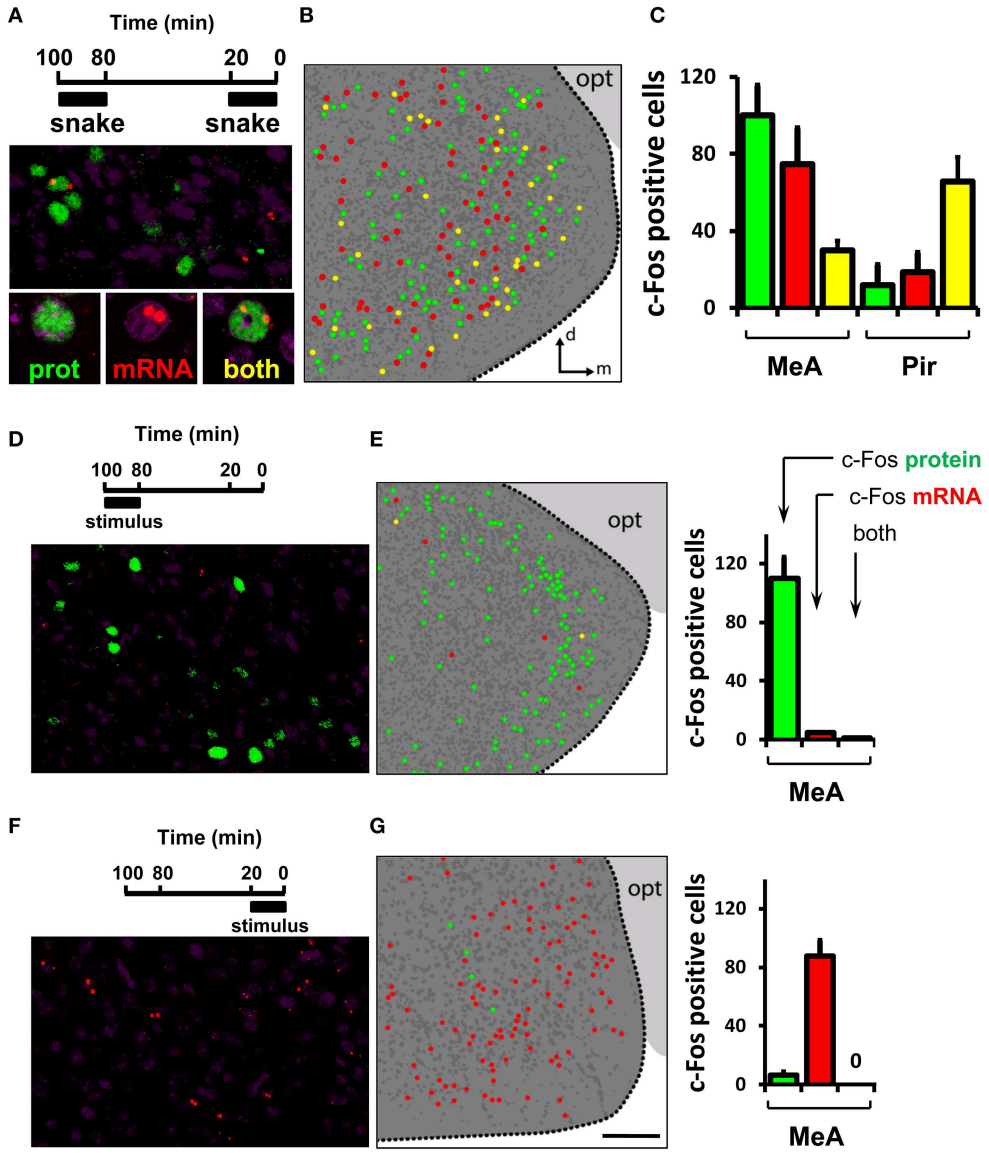


FIGURE 9 | Activity in the medial nucleus of the amygdala after detection of a VNO stimulus is not invariant. (A) Dual immunostaining/*in situ* hybridization to compare activity related to two sequential exposures to the same olfactory stimulus. Top, exposure protocol. Bottom, representative image showing location of cells activated in the first exposure (green nuclear fluorescence after c-Fos immunostaining), in the second exposure (two red nuclear fluorescent foci after *c-Fos* FISH) or both (yellow). **(B)** Schematic drawing of a representative image of activated cells in the MeA after two sequential exposures to the same stimulus, collected across a 30 μm thick z-series. Dark gray dots represent nuclei of non-activated cells inside the MeA. Dark gray background indicates the medial amygdala as depicted in **Figure 8A**; light gray background marks the optic tract (opt). **(C)** Quantification of activated cells related to each of two instances of stimulus exposure. The first bar (green) in each set represents cells activated in the first exposure only, the second (red) bar in each set exhibits activated cells in the second exposure only, and the third bar (yellow) in each set represents cell activated in both exposures. Quantification of cells in the piriform cortex (Pir) is shown for comparison. **(D)** Dual immunostaining/*in situ* hybridization to control for activity related to one exposure to olfactory stimulus during the first application window (100 to 80 min prior to brain fixation). Top, exposure protocol. Bottom, representative image showing location of cells activated in the first exposure (green nuclear fluorescence after c-Fos immunostaining). Rare nuclear red fluorescence foci indicate *c-Fos* mRNA detected by FISH. Yellow cells express both nuclear c-Fos protein and mRNA. **(E)** Left, schematic representation of activated cells in the MeA after one exposure to stimulus during the first application window, collected across a 30 μm thick z-series. Right, quantification of activated cells related to stimulus exposure. The first bar (green) in each set represents cells activated in the first exposure only, the second bar (red) in each set exhibits rare cells expressing *c-Fos* mRNA, and the third bar (yellow) represents very few cells expressing both nuclear c-Fos protein and mRNA, indicating that *c-Fos* mRNA labels cells activated during the second exposure, because virtually no mRNA derived from the first exposure remains at the end of the session. (**F,G**) Same as in (**D,E**), but to examine activated cells related to one exposure to stimulus during the second application window (20 to 0 min before brain fixation), indicating that c-Fos protein is indicative of the first exposure window, because not enough time transpired after the second exposure onset to allow c-Fos protein to be synthesized at any detectable level. $n = 4$–6. d, dorsal; m, medial; opt, optic tract. Scale bar represents 100 μm; for (**B**, **E**), the scale is the same as in **(G)**.

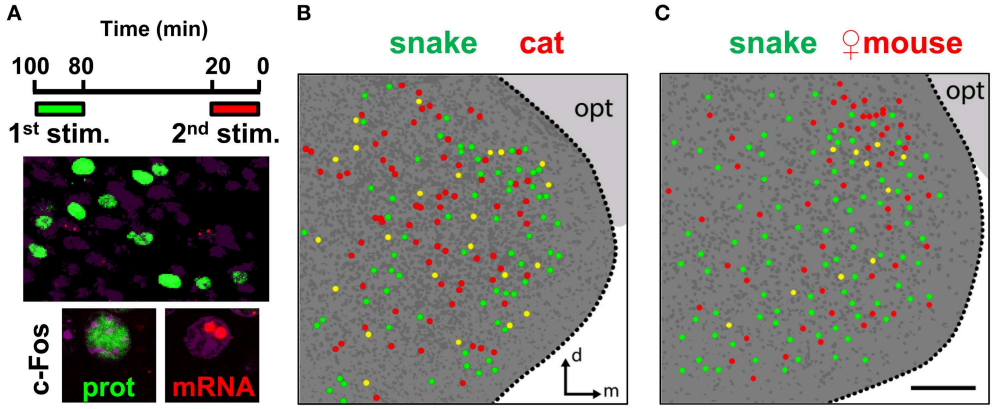


FIGURE 10 | Distinct stimuli activate intermingled ensembles of neurons in the medial nucleus of the amygdala. (A) Dual immunostaining/*in situ* hybridization to compare activity in the MeA related to two sequential exposures to distinct olfactory stimuli. Top, exposure protocol. Bottom, representative images showing cells activated in the first exposure (green nuclear fluorescence after c-Fos immunostaining) and in the second exposure (two red nuclear fluorescent foci after c-Fos FISH). **(B)** Schematic drawing of a representative image of activated cells in the MeA after two sequential exposures to distinct predatory olfactory stimuli, collected across a 30 μm thick z-series. Dark gray dots represent nuclei of non-activated cells inside the MeA. Dark gray background indicates the medial amygdala; light gray background marks the optic tract (opt). **(C)** Same as in **(B)**, but to examine activated cells related to sequential exposures to a predator odor and conspecific odor. $n = 4$–6. d, dorsal; m, medial; opt, optic tract. Scale bar represents 100 μm; for **(B)**, the scale is the same as in **(C)**.

enabled us to conclude that each tested stimulus induces c-Fos expression in cells broadly distributed throughout the MeA, with no apparent spatial segregation (**Figures 6**–**8**). Such data suggest that different stimuli do not result in grossly spatially segregated ensembles of active neurons in this nucleus of the amygdala. We also investigated activity in the granule and mitral cell layers of the AOB, and, similarly to the MeA, we found it not to be organized in a spatially segregated fashion (defensive and social stimuli produced similarly distributed activity), in keeping with previous publications (Luo et al., 2003; Hendrickson et al., 2008; Ben-Shaul et al., 2010). These data suggest that the distributive activity in the AOB is maintained in the MeA, the first information processing station after the olfactory bulb in the brain.

We further showed that different VNO stimuli activate intermingled ensembles of cells in the MeA, without evident spatial separation (**Figures 6**, **10**). The apparent lack of segregation of activated ensembles for different olfactory stimuli indicates that a spatial map to internally represent VNO ligands is not formed in the MeA (or that its underlying organization is undiscernible at this resolution).

These results put into question previous ideas according to which the MeA would be functionally divided into two sectors, dorsal and ventral, and stimuli related to distinct behavioral outputs (namely, reproductive or defensive) would activate spatially segregated groups of neurons in those two sectors, respectively (Swanson, 2000; Canteras, 2002; Choi et al., 2005). Although previous anatomical and functional evidences suggested that some stimuli may induce coherent activity in the MeA consistent with this notion (Choi et al., 2005), the use of a wide range of stimuli in our study enabled us to rule out this model by showing that distinct stimuli activate an equivalent percentage of MeA neurons and that they are mostly intermingled, without apparent spatial segregation.

Though stimuli that induce distinct behavioral outputs (defensive vs. reproductive) were not found to be spatially segregated in a consistent fashion, it remains possible that these two sets of cells send information to different downstream areas, resulting in distinct coding and processing pathways to trigger opposing behaviors. In fact, the active MeA ensembles related to cat and female odors were found to be projection neurons with largely distinct projection sites in the brain (Choi et al., 2005); moreover, tracing studies indicated, at a grosser level, that amygdala projection neurons target several brain areas, with some degree of overlap (Canteras et al., 1995; Dong et al., 2001; Petrovich et al., 2001; Canteras, 2002).

The distributive organization we observed in the MeA is also noteworthy in view of the fact that this nucleus is heterogeneous and contains cells selectively activated by particular ligand types: for example, some cells are active in animals exposed to predator odors, others are active in males exposed to females, others are activated by same-sex odors, while some are responsive to all kinds of stimulation (Bergan et al., 2014). It is therefore possible that the distinct subtypes of MeA neurons are intermingled, but projecting to distinct target output regions.

Interestingly, the distributive organization of MeA activity resembles the piriform cortex, where volatile odorants detected by the main olfactory epithelium are internally represented by distributed ensembles of active neurons, without any discernible spatial organization, irrespective of the stimulus' chemical nature, concentration or valence (Stettler and Axel, 2009). In both the piriform and MeA, each stimulus activates a percentage of the neurons, and the activated neurons are distributed, with no apparent spatial segregation or stereotypy. Moreover, different odors activate distinct, but partially overlapping, ensembles of

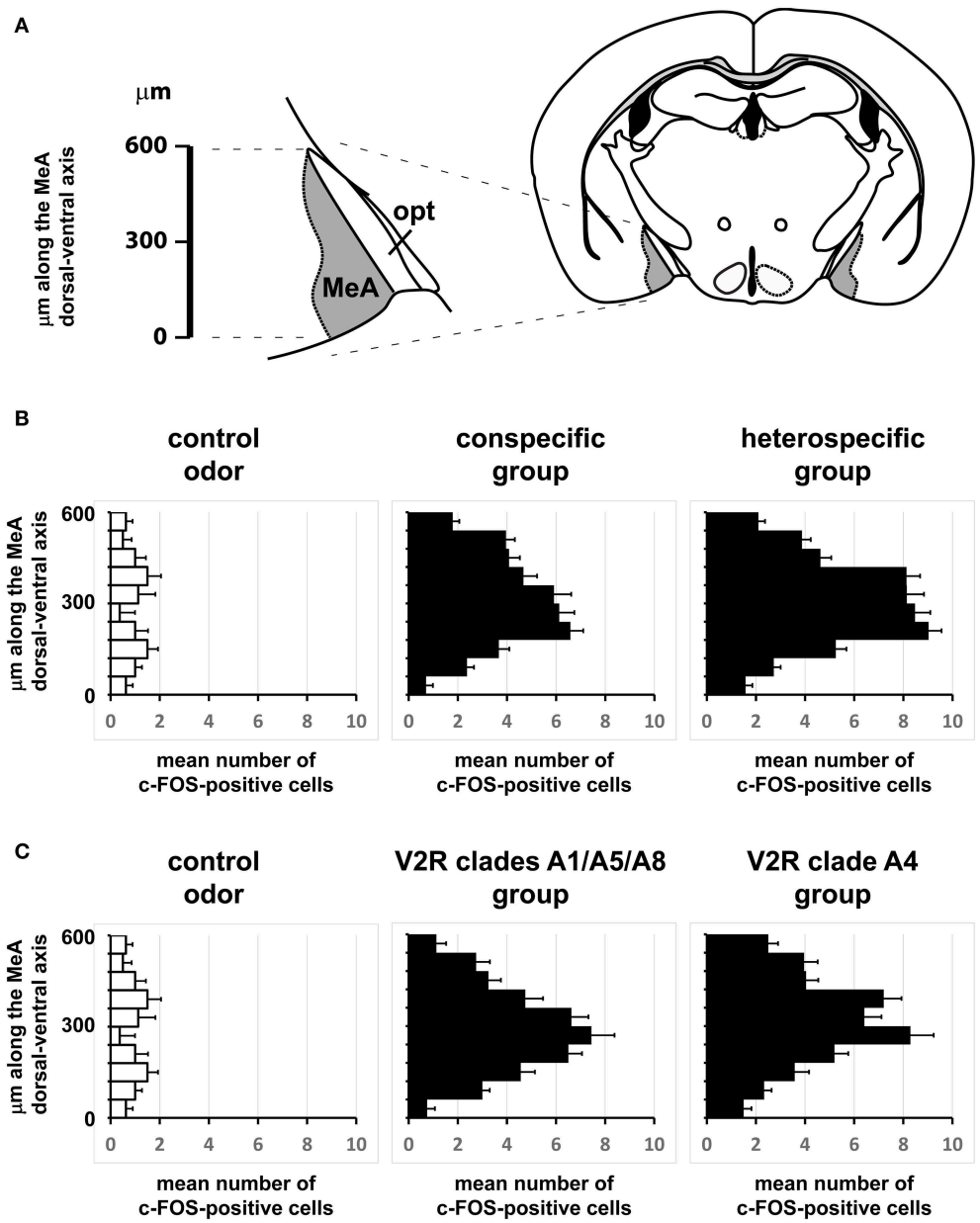


FIGURE 11 | Comparisons of the spatial distributions of activated cells along the dorsal-ventral axis of the medial nucleus of the amygdala. (A) Schematic diagram showing how the dorsal-ventral axis was positioned in the MeA. The axis origin (0) was defined as the most ventral pixel in the MeA in each scored image, while the upper extreme at the dorsal side lies 600 μm from the origin along the dorsal-ventral axis. This segment was further subdivided into 10 intervals and activated cells were counted for each interval in each image. **(B)** Horizontal bars represent the mean number of activated cells in each of the 10 intervals along the MeA axis (600 μm in total length), leading to a representation of the spatial distribution of the active ensemble. Black bars in the middle graph were obtained with data from mice exposed to conspecific stimuli (♂exp♀, ♀exp♂, ♀exp♀, and ♂exp♂ groups); black bars in the right graph are for mice exposed to heterospecific odors (cat, leopard cat, mountain lion, rat, owl, hawk, and snake); white bars in the left graph represent basal level activation with control odors (see Table S2). $n = 8$ for each species. Error bars are s.e.m. **(C)** Similar to **(B)**, but comparing MeA activation after exposure to odors that activate VNO neurons expressing A1/A5/A8 V2R receptors (♂exp♀, ♀exp♂, and ♂exp♂ groups) against the group of animals exposed to stimuli related to A4 V2R receptors (cat, leopard cat, and mountain lion). $n = 8$ for each species. Error bars are s.e.m.

neurons. The similarity between the representation of odors in the piriform and in the MeA concurs with the suggested cortical-like nature of some amygdalar areas.

Curiously, the cortical amygdala, which receives olfactory information collected by the main olfactory epithelium, seems to be organized in a spatially segregated fashion (Miyamichi

et al., 2011; Sosulski et al., 2011). Moreover, volatile odorants that elicit innate behaviors of different valence (appetitive or aversive) activate different populations of neurons within the cortical amygdala (Root et al., 2014), suggesting that the representation in such brain region is different from the representation of pheromones/kairomones in the MeA.

We have also investigated the stereotypy of the active cells in the MeA, and found that neurons activated by exposure to one stimulus are mostly distinct from those activated by a later exposure to the same stimulus (**Figure 9**). These data show little concordance for the sets of MeA cells activated in response to the same stimulus in two trials, suggesting that determined or immutable ensembles of cells related to each VNO stimulus do not exist in this brain area; however, because the concordance is higher than that expected by chance alone, the active ensemble to one stimulus is probably not random.

The little concordance between the two groups of neurons activated by the same odor in the MeA is not due to restrictions imposed by the use of c-Fos as a marker for neuronal activation in our dual immunostaining/*in situ* hybridization technique, because a high level of overlap was seen for two sequential exposures to the same stimulus in another brain area, the piriform cortex, using the same procedure (**Figure 9**). Our data on the piriform cortex is in agreement with a previous publication, which verified that piriform neurons that respond to an odorant have a high chance of responding at least once again to the same odorant over subsequent trials (Stettler and Axel, 2009). It is interesting to note, however, that we observed higher levels of concordance in the piriform cortex than another study (Shakhawat et al., 2014), which used a similar dual staining procedure to evaluate responses in the primary olfactory cortex, but found that the same odor activates ensembles with $<30\%$ overlap when given repeatedly; a possible reason for such difference is the fact that Shakhawat and collaborators employed purified odorants while we used complex stimuli, which activate a larger ensemble of piriform neurons, increasing the chances of overlap in a subsequent exposure trial. Further studies will be necessary to explore the difference between stereotypy in the MeA and piriform. If confirmed by additional methods (e.g., electrophysiology or functional imaging), the representation of odorants in the piriform and of pheromones/kairomones in the MeA will be different in one significant aspect: in the piriform, each odor stimulates activity in a distributed and sparse ensemble of neurons, but according to our data that ensemble is mostly defined (**Figure 9**); in contrast, our results show that the active ensembles in the MeA in response to conspecific/heterospecific stimuli are non-immutable (**Figure 9**).

Lastly, we show that the distributive pattern of activity in the MeA is not organized to reflect the type of stimulus employed, because no discernible differential distribution of activated cells is observed between animals exposed to heterospecific or to conspecific stimuli (**Figure 10**). Moreover, we show that MeA activity is not organized to reflect the different behavioral consequences of the detected stimuli (reproductive vs. defensive). These data are contrary to previous notions established for the organization of the medial amygdala (Swanson, 2000; Canteras, 2002; Choi et al., 2005) and call for alternative models to explain how pheromones and kairomones produce coherent activity in this brain region. We further showed that the MeA activity does not indicate the stimulus chemical nature or the repertoire of receptors expressed in activated neurons at the sensory interface (**Figures 10**, **11**).

Further anatomical and functional studies will be needed to confirm that the MeA does not internally represent the ensuing behaviors after detection of chemosignals by the VNO. If confirmed, these findings will pose an exciting final question: where are the olfaction-mediated instinctive behaviors represented in the brain? The hypothalamus, particularly its ventromedial nucleus, the dorsal premammillary nucleus, and the periaqueductal gray, anatomically positioned downstream to the MeA (Motta et al., 2009), are likely candidates, since they are activated by pheromones/kairomones (Dielenberg et al., 2001; Meredith and Westberry, 2004; Choi et al., 2005; Lin et al., 2011) and have been causally implicated in numerous behaviors. In the future, functional mapping of the flow of olfactory information along this brain circuit will be needed to understand the transition between the dispersed amygdala activity we describe here and the representation and generation of adaptive behaviors in yet uncharacterized higher brain sites.

Acknowledgments

We thank GAG Pereira and JA Yunes for resources, AR Castro, GL Castro and DM Mendes for providing snake and spider odors, NDM Villanueva for help with statistical analyses, ML Justino for help with artwork, the Life Sciences Core Facility (LaCTAD-UNICAMP) staff for help with confocal microscopy. VC, TN, and LC performed *Egr1 in situ* hybridization experiments and c-Fos immunostaining, TN and MS performed dual staining experiments, and other authors contributed to remaining data. VC, TN, AC, and FP wrote the manuscript. This work was supported by a Young Investigator award from the Sao Paulo Research Foundation—FAPESP (grant # 09/00473-0) and by a PRP/UNICAMP grant to FP, and by FAPESP fellowships to VC, TN, MS, and LC.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2015.00283

References

Ben-Shaul, Y., Katz, L. C., Mooney, R., and Dulac, C. (2010). *In vivo* vomeronasal stimulation reveals sensory encoding of conspecific and allospecific cues by the mouse accessory olfactory bulb. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5172–5177. doi: 10.1073/pnas.0915147107

Bergan, J. F., Ben-Shaul, Y., and Dulac, C. (2014). Sex-specific processing of social cues in the medial amygdala. *Elife* 3:e02743. doi: 10.7554/eLife.02743

Blanchard, D. C., Canteras, N. S., Markham, C. M., Pentkowski, N. S., and Blanchard, R. J. (2005). Lesions of structures showing FOS expression to cat presentation: effects on responsivity to a Cat, Cat odor, and nonpredator threat. *Neurosci. Biobehav. Rev.* 29, 1243–1253. doi: 10.1016/j.neubiorev.2005.04.019

Canteras, N. S. (2002). The medial hypothalamic defensive system: hodological organization and functional implications. *Pharmacol. Biochem. Behav.* 71, 481–491. doi: 10.1016/S0091-3057(01)00685-2

Canteras, N. S., Chiavegatto, S., Ribeiro do Valle, L. E., and Swanson, L. W. (1997). Severe reduction of rat defensive behavior to a predator by discrete hypothalamic chemical lesions. *Brain Res. Bull.* 44, 297–305. doi: 10.1016/S0361-9230(97)00141-X

Canteras, N. S., Simerly, R. B., and Swanson, L. W. (1995). Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. *J. Comp. Neurol.* 360, 213–245. doi: 10.1002/cne.903600203

Chamero, P., Marton, T. F., Logan, D. W., Flanagan, K., Cruz, J. R., Saghatelian, A., et al. (2007). Identification of protein pheromones that promote aggressive behaviour. *Nature* 450, 899–902. doi: 10.1038/nature05997

Chen, X., Gabitto, M., Peng, Y., Ryba, N. J. P., and Zuker, C. S. (2011). A gustotopic map of taste qualities in the mammalian brain. *Science* 333, 1262–1266. doi: 10.1126/science.1204076

Choi, G. B., Dong, H. W., Murphy, A. J., Valenzuela, D. M., Yancopoulos, G. D., Swanson, L. W., et al. (2005). Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus. *Neuron* 46, 647–660. doi: 10.1016/j.neuron.2005.04.011

Dey, S., Chamero, P., Pru, J. K., Chien, M.-S., Ibarra-Soria, X., Spencer, K. R., et al. (2015). Cyclic regulation of sensory perception by a female hormone alters behavior. *Cell* 161, 1334–1344. doi: 10.1016/j.cell.2015.04.052

Dielenberg, R. A., Hunt, G. E., and McGregor, I. S. (2001). "When a rat smells a cat": the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor. *Neuroscience* 104, 1085–1097. doi: 10.1016/S0306-4522(01)00150-6

Dong, H. W., Petrovich, G. D., and Swanson, L. W. (2001). Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res. Rev.* 38, 192–246. doi: 10.1016/S0165-0173(01)00079-0

Fernandez-Fewell, G. D., and Meredith, M. (1994). c-fos expression in vomeronasal pathways of mated or pheromone-stimulated male golden hamsters: contributions from vomeronasal sensory input and expression related to mating performance. *J. Neurosci.* 14, 3643–3654.

Ferrero, D. M., Moeller, L. M., Osakada, T., Horio, N., Li, Q., Roy, D. S., et al. (2013). A juvenile mouse pheromone inhibits sexual behaviour through the vomeronasal system. *Nature* 502, 368–371. doi: 10.1038/nature12579

Guzowski, J. F., McNaughton, B. L., Barnes, C. A., and Worley, P. F. (1999). Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. *Nat. Neurosci.* 2, 1120–1124. doi: 10.1038/16046

Guzowski, J. F., McNaughton, B. L., Barnes, C. A., and Worley, P. F. (2001). Imaging neural activity with temporal and cellular resolution using FISH. *Curr. Opin. Neurobiol.* 11, 579–584. doi: 10.1016/S0959-4388(00)00252-X

Haga, S., Hattori, T., Sato, T., Sato, K., Matsuda, S., Kobayakawa, R., et al. (2010). The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. *Nature* 466, 118–122. doi: 10.1038/nature09142

Hendrickson, R. C., Krauthamer, S., Essenberg, J. M., and Holy, T. E. (2008). Inhibition shapes sex selectivity in the mouse accessory olfactory bulb. *J. Neurosci.* 28, 12523–12534. doi: 10.1523/JNEUROSCI.2715-08.2008

Isogai, Y., Si, S., Pont-Lezica, L., Tan, T., Kapoor, V., Murthy, V. N., et al. (2011). Molecular organization of vomeronasal chemoreception. *Nature* 478, 241–245. doi: 10.1038/nature10437

Kaur, A. W., Ackels, T., Kuo, T. H., Cichy, A., Dey, S., Hays, C., et al. (2014). Murine pheromone proteins constitute a context-dependent combinatorial code governing multiple social behaviors. *Cell* 157, 676–688. doi: 10.1016/j.cell.2014.02.025

Kimchi, T., Xu, J., and Dulac, C. (2007). A functional circuit underlying male sexual behaviour in the female mouse brain. *Nature* 448, 1009–1014. doi: 10.1038/nature06089

Kollack-Walker, S., and Newman, S. W. (1997). Mating-induced expression of c-fos in the male Syrian hamster brain: role of experience, pheromones, and ejaculations. *J. Neurobiol.* 32, 481–501.

Leinders-Zufall, T., Brennan, P., Widmayer, P., Chandramani, S. P., Maul-Pavicic, A., Jäger, M., et al. (2004). MHC class I peptides as chemosensory signals in the vomeronasal organ. *Science* 306, 1033–1037. doi: 10.1126/science.1102818

Lin, D., Boyle, M. P., Dollar, P., Lee, H., Lein, E. S., Perona, P., et al. (2011). Functional identification of an aggression locus in the mouse hypothalamus. *Nature* 470, 221–226. doi: 10.1038/nature09736

Logan, D. W., Marton, T. F., and Stowers, L. (2008). Species specificity in major urinary proteins by parallel evolution. *PLoS ONE* 3:e3280. doi: 10.1371/journal.pone.0003280

Luo, L., and Flanagan, J. G. (2007). Development of continuous and discrete neural maps. *Neuron* 56, 284–300. doi: 10.1016/j.neuron.2007.10.014

Luo, M., Fee, M. S., and Katz, L. C. (2003). Encoding pheromonal signals in the accessory olfactory bulb of behaving mice. *Science* 299, 1196–1201. doi: 10.1126/science.1082133

Meredith, M., and Westberry, J. M. (2004). Distinctive responses in the medial amygdala to same-species and different-species pheromones. *J. Neurosci.* 24, 5719–5725. doi: 10.1523/JNEUROSCI.1139-04.2004

Miyamichi, K., Amat, F., Moussavi, F., Wang, C., Wickersham, I., Wall, N. R., et al. (2011). Cortical representations of olfactory input by trans-synaptic tracing. *Nature* 472, 191–196. doi: 10.1038/nature09714

Mohedano-Moriano, A., Pro-Sistiaga, P., Úbeda-Bañón, I., Crespo, C., Insausti, R., and Martinez-Marcos, A. (2007). Segregated pathways to the vomeronasal amygdala: Differential projections from the anterior and posterior divisions of the accessory olfactory bulb. *Eur. J. Neurosci.* 25, 2065–2080. doi: 10.1111/j.1460-9568.2007.05472.x

Motta, S. C., Goto, M., Gouveia, F. V., Baldo, M. V. C., Canteras, N. S., and Swanson, L. W. (2009). Dissecting the brain's fear system reveals the hypothalamus is critical for responding in subordinate conspecific intruders. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4870–4875. doi: 10.1073/pnas.0900939106

Munger, S. D., Leinders-Zufall, T., and Zufall, F. (2009). Subsystem organization of the mammalian sense of smell. *Annu. Rev. Physiol.* 71, 115–140. doi: 10.1146/annurev.physiol.70.113006.100608

Nodari, F., Hsu, F.-F., Fu, X., Holekamp, T. F., Kao, L.-F., Turk, J., et al. (2008). Sulfated steroids as natural ligands of mouse pheromone-sensing neurons. *J. Neurosci.* 28, 6407–6418. doi: 10.1523/JNEUROSCI.1425-08.2008

Papes, F., Logan, D. W., and Stowers, L. (2010). The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. *Cell* 141, 692–703. doi: 10.1016/j.cell.2010.03.037

Paxinos, G., and Franklin, K. B. J. (2004). *The Mouse Brain in Stereotaxic Coordinates*. Waltham, MA: Academic Press.

Petrovich, G. D., Canteras, N. S., and Swanson, L. W. (2001). Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. *Brain Res. Rev.* 38, 247–289. doi: 10.1016/S0165-0173(01)00080-7

Root, C. M., Denny, C. A., Hen, R., and Axel, R. (2014). The participation of cortical amygdala in innate, odour-driven behaviour. *Nature* 515, 269–273. doi: 10.1038/nature13897

Sam, M., Vora, S., Malnic, B., Ma, W., Novotny, M. V., and Buck, L. B. (2001). Neuropharmacology. Odorants may arouse instinctive behaviours. *Nature* 412, 142. doi: 10.1038/35084137

Shakhawat, A. M., Harley, C. W., and Yuan, Q. (2014). Arc visualization of odor objects reveals experience-dependent ensemble sharpening, separation, and merging in anterior piriform cortex in adult rat. *J. Neurosci.* 34, 10206–10210. doi: 10.1523/JNEUROSCI.1942-14.2014

Silva, B. A, Mattucci, C., Krzywkowski, P., Murana, E., Illarionova, A., Grinevich, V., et al. (2013). Independent hypothalamic circuits for social and predator fear. *Nat. Neurosci.* 16, 1731–1733. doi: 10.1038/nn.3573

Silvotti, L., Moiani, A., Gatti, R., and Tirindelli, R. (2007). Combinatorial co-expression of pheromone receptors, V2Rs. *J. Neurochem.* 103, 1753–1763. doi: 10.1111/j.1471-4159.2007.04877.x

Smith, W. A., Butler, A. J. L., Hazell, L. A., Chapman, M. D., Pomés, A., Nickels, D. G., et al. (2004). Fel d 4, a cat lipocalin allergen. *Clin. Exp. Allergy* 34, 1732–1738.

Sosulski, D. L., Bloom, M. L., Cutforth, T., Axel, R., and Datta, S. R. (2011). Distinct representations of olfactory information in different cortical centres. *Nature* 472, 213–216. doi: 10.1038/nature09868

Stettler, D. D., and Axel, R. (2009). Representations of odor in the piriform cortex. *Neuron* 63, 854–864. doi: 10.1016/j.neuron.2009.09.005

Stowers, L., Holy, T. E., Meister, M., Dulac, C., and Koentges, G. (2002). Loss of sex discrimination and male-male aggression in mice deficient for TRP2. *Science* 295, 1493–1500. doi: 10.1126/science.1069259

Swanson, L. W. (2000). Cerebral hemisphere regulation of motivated behavior. *Brain Res.* 886, 113–164. doi: 10.1016/S0006-8993(00)02905-X

Takahashi, L. K. (2014). Olfactory systems and neural circuits that modulate predator odor fear. *Front. Behav. Neurosci.* 8:72. doi: 10.3389/fnbeh.2014.00072

Trinh, K., and Storm, D. R. (2003). Vomeronasal organ detects odorants in absence of signaling through main olfactory epithelium. *Nat. Neurosci.* 6, 519–525. doi: 10.1038/nn1039

Wagner, S., Gresser, A. L., Torello, A. T., and Dulac, C. (2006). A multireceptor genetic approach uncovers an ordered integration of VNO sensory inputs in the accessory olfactory bulb. *Neuron* 50, 697–709. doi: 10.1016/j.neuron.2006.04.033

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 23 February 2015
doi: 10.3389/fnins.2015.00052

Neural correlates underlying naloxone-induced amelioration of sexual behavior deterioration due to an alarm pheromone

Tatsuya Kobayashi†, Yasushi Kiyokawa*, Yukari Takeuchi and Yuji Mori

Laboratory of Veterinary Ethology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo, Japan

Edited by:
Xiaogang Wu, Institute for Systems Biology, USA

Reviewed by:
Tianxiao Huan, Framingham Heart Study, USA
Socorro Retana-Marquez, Universidad Autónoma Metropolitana, Mexico

****Correspondence:***
Yasushi Kiyokawa, Laboratory of Veterinary Ethology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
e-mail: akiyo@mail.ecc.u-tokyo.ac.jp

†Present address:
Tatsuya Kobayashi, Product Science Division, Japan Tobacco Inc., 6-2, Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan

Sexual behavior is suppressed by various types of stressors. We previously demonstrated that an alarm pheromone released by stressed male Wistar rats is a stressor to other rats, increases the number of mounts needed for ejaculation, and decreases the hit rate (described as the number of intromissions/sum of the mounts and intromissions). This deterioration in sexual behavior was ameliorated by pretreatment with the opioid receptor antagonist naloxone. However, the neural mechanism underlying this remains to be elucidated. Here, we examined Fos expression in 31 brain regions of pheromone-exposed rats and naloxone-pretreated pheromone-exposed rats 60 min after 10 intromissions. As previously reported, the alarm pheromone increased the number of mounts and decreased the hit rate. In addition, Fos expression was increases in the anterior medial division (BNSTam), anterior lateral division (BNSTal) and posterior division (BNSTp) of the bed nucleus of the stria terminalis, parvocellular part of the paraventricular nucleus of the hypothalamus, arcuate nucleus, dorsolateral and ventrolateral periaqueductal gray, and nucleus paragigantocellularis (nPGi). Fos expression was decreased in the magnocellular part of the paraventricular nucleus of the hypothalamus. Pretreatment with naloxone blocked the pheromone-induced changes in Fos expression in the magnocellular part of the paraventricular nucleus of the hypothalamus, ventrolateral periaqueductal gray, and nPGi. Based on these results, we hypothesize that the alarm pheromone deteriorated sexual behavior by activating the ventrolateral periaqueductal gray-nucleus paragigantocellularis cluster and suppressing the magnocellular part of the paraventricular nucleus of the hypothalamus (PVN) via the opioidergic pathway.

Keywords: opioid, alarm pheromone, sexual behavior, nucleus paragigantocellularis, periaqueductal gray, paraventricular nucleus of hypothalamus

INTRODUCTION

Sexual behavior is very important for reproduction in animals. It can be suppressed by various types of manipulations; for example, social isolation (Barrot et al., 2005), maternal separation (Rhees et al., 2001), prenatal stress (Wang et al., 2006), parasitic infection (Lin et al., 1990; Klein, 2003), immobilization, foot shock, and water immersion (Retana-Marquez et al., 1996) suppress male sexual behavior in rodents. In addition, we have found that an alarm pheromone released from stressed conspecifics deteriorates male sexual behavior in rats (Kobayashi et al., 2011).

It is well-known that stressed animals release specific odors, possibly to warn or alarm nearby conspecifics, some of which are defined as alarm pheromones (Karlson and Luscher, 1959; Inagaki et al., 2014). We previously investigated an alarm pheromone that causes an increase in body temperature in rats placed in a box, which recently placed two male rats that had received foot shocks (Kikusui et al., 2001). This phenomenon suggests that the rats that received the foot shocks served as pheromone donors and released an alarm pheromone when they received the shocks. It is possible to induce pheromone release by applying electrical stimulation to the perianal region of an anesthetized donor (Kiyokawa et al., 2004); therefore, we established a method that traps this pheromone in water (Kiyokawa et al., 2005a). We found that this pheromone-containing water can evoke a variety of stress responses, depending on the experimental model (Kiyokawa et al., 2006; Inagaki et al., 2008; Kobayashi et al., 2011), through the vomeronasal system (Kiyokawa et al., 2007, 2013a) and main olfactory system (Inagaki et al., 2014).

This alarm pheromone is a suitable candidate to analyze the effects of stress on sexual behavior because the natural stress responses were induced through the olfactory system. We observed the sexual behavior of pairs of rats in the presence of this pheromone and found that it increases the number of mounts and decreases the hit rate in male rats (Kobayashi et al., 2011). Subsequently, we found that this pheromone-induced deterioration in sexual behavior is gender-specific because it occurs when the pheromone was presented to the male, but not the female, before a sexual encounter (Kobayashi et al., 2011). We conducted Fos mapping in a variety of brain regions and found that the nucleus paragigantocellularis (nPGi) is a key region

associated with the pheromone-induced deterioration. We have found increased Fos expression in the anterior medial division (BNSTam), anterior lateral division (BNSTal), and posterior division (BNSTp) of the bed nucleus of the stria terminalis, medial and basal amygdala, and corticotropin-releasing hormone (CRH)-containing neurons in the paraventricular nucleus of the hypothalamus (PVN), which are in the parvocellular part of the PVN (pPVN) (Kobayashi et al., 2013a). However, we did not find any other candidate nuclei that were directly involved in this deterioration or that activated the nPGi. This failure might be attributed to neural activation from ejaculation, which obscured Fos expression associated with pheromone-induced sexual behavior deterioration.

In parallel with the immunohistochemical analyses, we pharmacologically analyzed the neural mechanism of deterioration. We found that systemic pretreatment with the opioid receptor antagonist naloxone attenuated sexual deterioration in male rats. This suggests that opioids are involved in the neural mechanism (Kobayashi et al., 2013b). However, regions in which we found increased Fos expression during deterioration (Kobayashi et al., 2013a), such as the nPGi and several other nuclei, to our knowledge, do not express opioid receptors. Therefore, it remains unclear how opioids are involved in the deterioration of sexual behavior by the alarm pheromone.

To address these issues, in this study, we measured Fos expression in 31 brain regions in pheromone-exposed rats that showed a deterioration of sexual behavior but did not achieve ejaculation. We also assessed pheromone-exposed rats that were pretreated with naloxone in order to obtain further information about the role of opioids in deterioration.

MATERIAL AND METHODS

ANIMALS

Twenty-five sexually naïve male (aged 7.5 weeks) and female (aged 8.5 weeks) Wistar Imamichi rats were used in this study (Institute for Animal Reproduction, Ibaraki, Japan). All animals were provided with food and water *ad libitum* and kept on a 12-h light-dark cycle (lights turned off at 2000). The colony room was maintained at a constant temperature (24 ± 1°C) and humidity (40–45%). Animals of the same sex were housed in pairs in wire-topped, transparent cages (410 × 250 × 180 mm) with wood shavings for bedding. All procedures were approved by the Animal Care and Use Committee of the Faculty of Agriculture of The University of Tokyo.

PREPARATION OF THE ALARM PHEROMONE

Before the sexual behavior test, water samples that contained either the alarm pheromone or a control odor were prepared according to a previously described method (Kiyokawa et al., 2005a; Kobayashi et al., 2013b). Adult male Wistar Imamichi rats (aged 12–16 weeks) were anesthetized and 2 intradermal needles (27G) were attached at either the neck or perianal region. After spraying purified water (5 mL) on the ceiling of an acrylic box (200 × 200 × 100 mm), the anesthetized donor was placed in the box and given 15 electrical stimuli (10 V for 1 s) through the needles for 5 min at 20 s intervals. Care was taken that water droplets did not fall from the ceiling and that the donor was kept under anesthesia during the stimulations. The electrical stimulation of the perianal region induced the release of alarm pheromones and stimulation of the neck region was conducted to provide a similar amount of control olfactory stimuli. Following this, the donor was removed and the water droplets containing the alarm pheromone or control odor were collected. The samples were stored at 4°C until use.

SEXUAL BEHAVIOR TEST

Sexual behavior tests were conducted as described in previous studies (Kobayashi et al., 2011, 2013a,b). Briefly, 1 day before the test, male rats were housed individually and acclimatized for 30 min to the experimental room and devices. Female rats were also acclimatized to the experimental room for 30 min. On the test day, the test was conducted in the home cage of the male subject that had been placed in the experimental room under a dim red light. Saline or naloxone (40 mg·kg^{-1} dissolved in saline; Sigma-Aldrich, St. Louis, MO) was administered intraperitoneally to the male 60 min before the test. At the beginning of the test, pheromone or control samples (750 μL each) were dropped on two sheets of filter paper (5 × 5 cm) that were placed in acrylic plates (12 × 6 cm, 3 mm thickness), one of which had 18 holes, 9 mm in diameter. The plates were then placed in the home cage for 5 min. Following this, the plates were removed and the female was introduced. Sexual behavior was video recorded and observed in an adjacent room during the test. When the tenth intromission was observed, the female was removed. Twenty-five male rats were divided into three groups according to the type of sample and injection: control odor and saline injection (Control-SAL; $n = 8$), alarm pheromone and saline injection (Pheromone-SAL; $n = 8$), and alarm pheromone and naloxone injection (Pheromone-NAL; $n = 9$).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was conducted as described in previous studies (Kiyokawa et al., 2013b, 2014; Kobayashi et al., 2013a). Briefly, 60 min after the tenth intromission, male rats were deeply anesthetized with sodium pentobarbital and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed and immersed overnight in the same fixative and then placed in 30% sucrose in phosphate buffer for cryoprotection. The brain was cut into 25-μm-thick coronal sections. Six successive sections containing some of the target regions were collected, and the second and fifth sections were stained with Cresyl violet to confirm localization in the brain. The remaining sections were incubated in H_2O_2, then primary antibody targeting the Fos protein (PC38, Merck Millipore, Billerica, MA) for 65 h, and biotinylated anti-rabbit secondary antibody (BA-1000, Vector Laboratories, Burlingame, CA) for 2 h. Following this, the sections were processed using the ABC kit (Vector Laboratories) and developed using a 3,3′-diaminobenzidine solution with nickel intensification.

QUANTIFICATION

Fos expression was analyzed as described in previous studies (Kiyokawa et al., 2005b, 2013b; Kobayashi et al., 2013a). Four

sections showing each of the 31 regions were imaged using a microscope equipped with a digital camera (DP30BW; Olympus, Tokyo, Japan). The number of c-Fos-immunoreactive cells in a 0.5-mm square was counted bilaterally using Image J software (version 1.41) by an experimenter who was blind to the experimental groups. The raphe nuclei are located in the center of the brain; therefore, Fos expression could not be counted bilaterally. In some cases, the designated area to be counted was smaller than the boundaries of the 0.5-mm square; therefore, only the cells in the brain region of interest, and not in other regions within the square, were counted.

DATA ANALYSIS AND STATISTICAL PROCEDURES

Male sexual behavior was analyzed as described in previous studies by a researcher who was blind to the experimental conditions (Kobayashi et al., 2011, 2013a,b). The following measures of sexual behavior were recorded: mount latency (pelvic thrusting from the rear of the female rat without penile insertion), intromission latency (deeper pelvic thrusting from the rear of the female rat with penile insertion), latency for tenth intromission (time from the first intromission to tenth intromission), and number of mounts (number of mounts needed for 10 intromissions). In addition, the hit rate was calculated. Behavioral and immunohistochemical data are expressed as mean ± the standard error of the mean (SEM). The significance was set at $P < 0.05$ for all statistical tests. All data were analyzed by one-way analysis of variance followed by Fisher's PLSD *post-hoc* test.

RESULTS

The number of mounts [$F_{(2, 22)} = 5.47$, $P = 0.012$; **Figure 1A**] and hit rate [$F_{(2, 22)} = 5.40$, $P = 0.012$; **Figure 1B**] were significantly different between the groups, whereas the mount latency [$F_{(2, 22)} = 0.11$, $P = 0.892$; **Figure 1C**], intromission latency [$F_{(2, 22)} = 0.68$, $P = 0.519$; **Figure 1D**], and latency to tenth intromission [$F_{(2, 22)} = 0.30$, $P = 0.746$; **Figure 1E**] were not. *Post-hoc* tests revealed that the Pheromone-SAL group showed an increased number of mounts ($P = 0.017$) and decreased hit rate ($P = 0.022$) when compared with the Control-SAL group. However, these measures were not different between the Control-SAL and Pheromone-NAL groups (number of mounts, $P = 0.653$; hit rate, $P = 0.565$). A *post-hoc* test also confirmed that the Pheromone-SAL group showed an increased number of mounts ($P = 0.005$) and decreased hit rate ($P = 0.005$) when compared with the Pheromone-NAL group.

We measured Fos expression in 31 brain regions (**Figure 2**). The mean number of c-Fos-immunoreactive cells is summarized

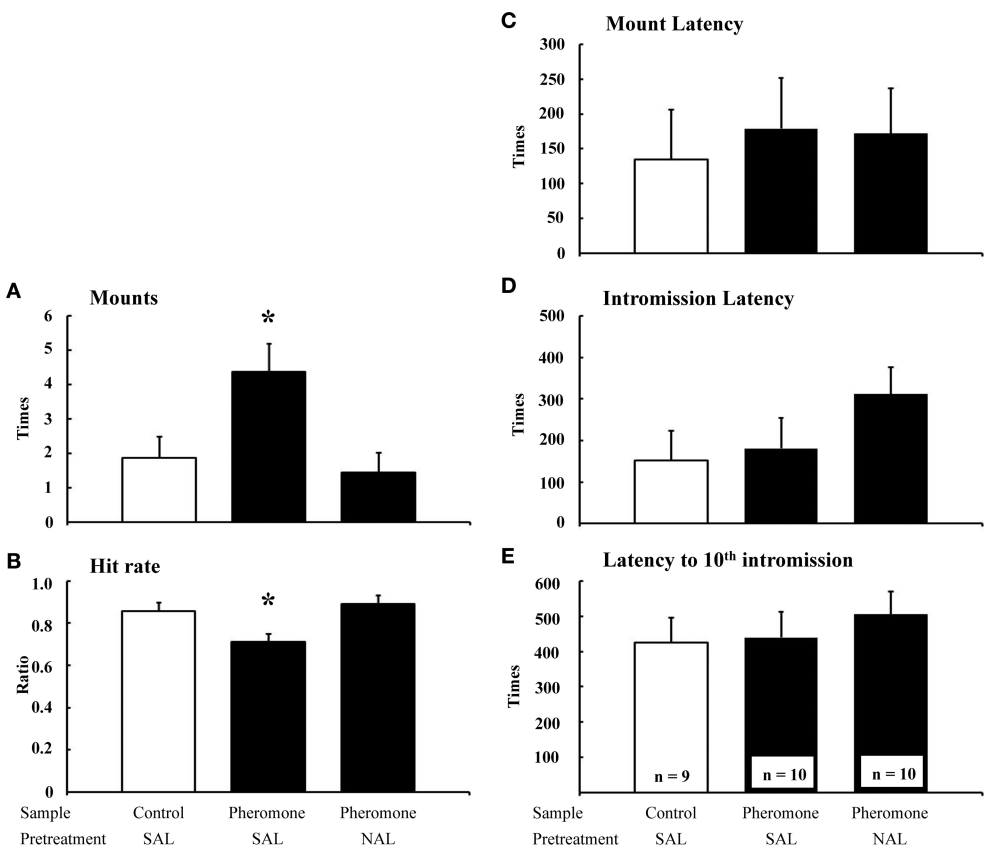


FIGURE 1 | The effects of naloxone on the pheromone-induced deterioration of sexual behavior. Number of mounts **(A)**, hit rate **(B)**, mount latency **(C)**, intromission latency **(D)**, and latency to 10th intromission **(E)** of the subjects (mean + SEM). Subjects were pretreated with either saline (SAL) or naloxone (40 mg·kg^{-1}; NAL) and exposed to a control odor (Control) or an alarm pheromone (Pheromone). *$P < 0.05$ compared with the Control-SAL group, ANOVA followed by Fisher's PLSD *post-hoc* test.

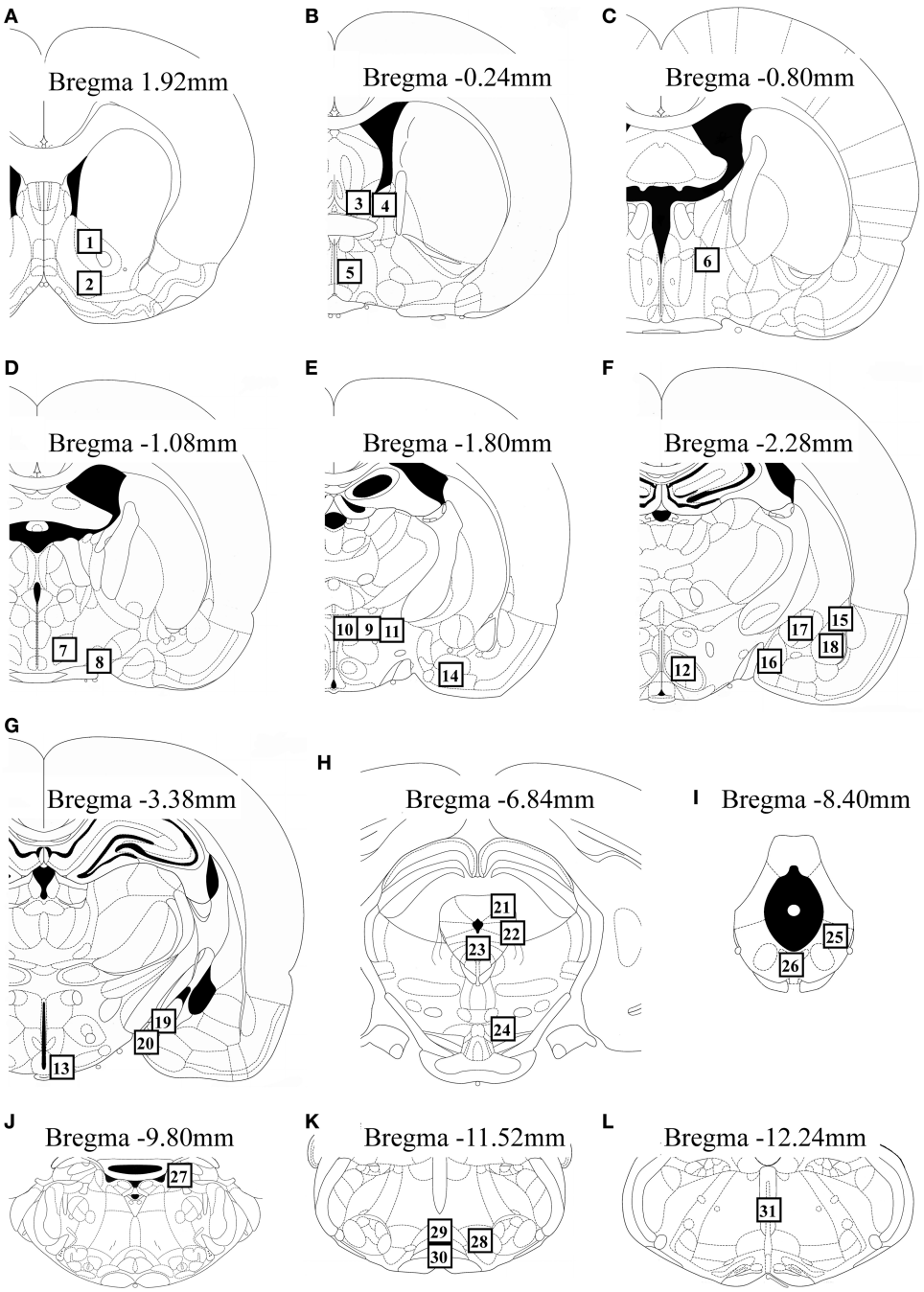


FIGURE 2 | Schematic diagrams showing the location of brain regions (open square containing numbers) in which c-Fos-immunoreactive cells were counted. The regions analyzed were: the nucleus accumbens core and shell **(A)**, BNSTam, BNSTal, and medial preoptic area **(B)**, BNSTp **(C)**, lateral hypothalamic area and supraoptic nucleus **(D)**, mPVN, pPVN, peduncular lateral hypothalamus, and anterior cortical amygdala **(E)**, ventromedial nucleus of hypothalamus and lateral, anterior medial, central, and basolateral amygdala **(F)**, Arc and posterodorsal and posteroventral medial amygdala **(G)**, dlPAG, lateral periaqueductal gray, dorsal raphe nucleus, and ventral tegmental area **(H)**, vlPAG and dorsal region of the dorsal raphe nucleus **(I)**, locus coeruleus **(J)**, nPGi and raphe magnus and pallidus **(K)**, and raphe obscurus **(L)**. This figure was adapted from a rat brain atlas (Paxinos and Watson, 2007). For an explanation of the abbreviations, see the corresponding numbers in **Table 1**.

in **Table 1**. Fos expression was significantly different among the groups in the nucleus accumbens core [$F_{(2,\ 22)} = 8.21$, $P = 0.002$] and shell [$F_{(2,\ 22)} = 6.07$, $P = 0.008$], BNSTam [$F_{(2,\ 22)} = 4.81, P = 0.019$], BNSTal [$F_{(2,\ 22)} = 12.9, P < 0.001$], BNSTp [$F_{(2,\ 22)} = 6.66$, $P = 0.006$], magnocellular part of the PVN [mPVN; $F_{(2,\ 22)} = 8.09$, $P = 0.002$], pPVN [$F_{(2,\ 22)} = 8.28$, $P = 0.002$], ventromedial nucleus of hypothalamus [$F_{(2,\ 22)} = 5.55$, $P = 0.011$], arcuate nucleus [Arc;

Table 1 | The number of c-Fos-immunoreactive cells per 0.25 mm^2 in various brain regions.

Regions	Control-SAL (8)	Pheromone-SAL (8)		Pheromone-NAL (9)	
THE REGIONS WHERE PRETREATMENT WITH NALOXONE BLOCKED THE CHANGE BY THE PHEROMONE					
9. Magnocellular part of the paraventricular nucleus of hypothalamus (mPVN)	17.4 ± 2.0	9.8 ± 1.0*	$P = 0.003$	17.9 ± 1.6	$P = 0.795$
25. Ventrolateral periaqueductal gray (vlPAG)	33.2 ± 2.8	71.9 ± 6.7*	$P < 0.001$	32.9 ± 2.1	$P = 0.969$
28. Nucleus paragigantocellularis (nPGi)	7.3 ± 0.8	21.5 ± 2.6*	$P < 0.001$	5.7 ± 0.7	$P = 0.226$
THE REGIONS WHERE PRETREATMENT WITH NALOXONE DID NOT BLOCK THE ACTIVATION BY THE PHEROMONE					
3. Anterior medial division of the bed nucleus of the stria terminalis (BNSTam)	18.0 ± 1.4	33.2 ± 4.3*	$P = 0.009$	30.9 ± 4.3*	$P = 0.020$
4. Anterior lateral division of the bed nucleus of the stria terminalis (BNSTal)	16.5 ± 4.5	34.0 ± 4.0*	$P = 0.012$	48.1 ± 4.7*	$P < 0.001$
6. Posterior division of the bed nucleus of the stria terminalis (BNSTp)	19.9 ± 2.3	37.7 ± 5.9*	$P = 0.004$	36.3 ± 2.3*	$P = 0.005$
10. Parvocellular part of the paraventricular nucleus of hypothalamus (pPVN)	34.6 ± 3.0	51.6 ± 4.8*	$P = 0.036$	64.7 ± 6.7*	$P < 0.001$
13. Arcuate nucleus (Arc)	9.4 ± 1.9	30.3 ± 4.7*	$P = 0.001$	34.6 ± 4.1*	$P < 0.001$
21. Dorsolateral periaqueductal gray (dlPAG)	16.2 ± 1.6	23.6 ± 1.8*	$P = 0.007$	22.9 ± 1.7*	$P = 0.011$
THE REGIONS WHERE PRETREATMENT WITH NALOXONE, BUT NOT PHEROMONE EXPOSURE, CAUSED ACTIVATION					
1. Nucleus accumbens core	31.5 ± 6.7	39.0 ± 7.6	$P = 0.470$	69.2 ± 6.9*	$P = 0.001$
2. Nucleus accumbens shell	7.4 ± 2.4	5.0 ± 1.4	$P = 0.436$	15.0 ± 2.4*	$P = 0.020$
12. Ventromedial nucleus of hypothalamus	8.0 ± 1.0	9.2 ± 1.4	$P = 0.568$	14.4 ± 1.8*	$P = 0.005$
17. Central amygdala	8.7 ± 1.3	7.6 ± 2.2	$P = 0.939$	74.9 ± 15.5*	$P < 0.001$
23. Dorsal raphe nucleus	6.0 ± 0.8	6.3 ± 1.1	$P = 0.813$	10.0 ± 0.8*	$P = 0.005$
THE REGIONS WHERE NEITHER THE PHEROMONE NOR PRETREATMENT WITH NALOXONE CAUSED ACTIVATION					
5. Medial preoptic area	81.9 ± 7.7	90.6 ± 6.2		88.8 ± 3.9	
7. Lateral hypothalamic area	13.9 ± 2.9	17.8 ± 2.5		19.6 ± 2.8	
8. Supraoptic nucleus	13.8 ± 3.2	13.5 ± 2.6		8.9 ± 0.9	
11. Peduncular lateral hypothalamus	16.1 ± 3.1	20.2 ± 2.4		25.6 ± 2.9	
14. Anterior cortical amygdala	36.5 ± 4.1	34.6 ± 5.2		34.2 ± 4.0	
15. Lateral amygdala	6.5 ± 1.0	6.8 ± 1.1		7.7 ± 0.5	
16. Anterior medial amygdala	24.0 ± 3.1	32.4 ± 3.3		31.9 ± 2.8	
18. Basal amygdala	13.6 ± 2.0	12.3 ± 2.6		14.0 ± 2.3	
19. Posterodorsal medial amygdala	39.0 ± 3.8	42.7 ± 2.1		40.1 ± 3.0	
20. Posteroventral medial amygdala	28.5 ± 5.8	45.6 ± 5.4		44.0 ± 5.9	
22. Lateral periaqueductal gray	16.8 ± 2.0	21.9 ± 1.6		25.8 ± 3.5	
24. Ventral tegmental area	4.2 ± 0.8	4.1 ± 0.9		4.4 ± 0.7	
26. Dorsal raphe nucleus dorsal part	11.7 ± 1.8	11.2 ± 2.0		13.3 ± 1.6	
27. Locus coeruleus	13.3 ± 2.5	20.0 ± 3.1		15.8 ± 2.6	
29. Raphe magnus	6.0 ± 0.8	7.0 ± 1.6		6.4 ± 1.2	
30. Raphe pallidus	4.9 ± 0.5	5.0 ± 0.4		5.5 ± 1.0	
31. Raphe obscurus	11.9 ± 2.7	12.2 ± 2.8		18.1 ± 2.1	

Data are expressed as means ± SEM. For the location of each region, see the corresponding numbers in ***Figure 2****. The number of rats per group is given in parentheses. * P < 0.05 compared to the Control-SAL group, One-Way ANOVA followed by Fisher's PLSD post-hoc test.*

$F(2,\ 22) = 12.3$, $P < 0.001$], central amygdala [$F_{(2,\ 22)} = 16.0$, $P < 0.001$], dorsolateral [dlPAG; $F_{(2,\ 22)} = 5.53$, $P = 0.011$] and ventrolateral periaqueductal gray [vlPAG; $F_{(2,\ 22)} = 28.0$, $P < 0.001$], dorsal raphe nucleus [$F_{(2,\ 22)} = 6.11$, $P = 0.008$], and nPGi [$F_{(2,\ 22)} = 21.9$, $P < 0.001$].

Post-hoc tests revealed that these brain regions could be divided into the following 3 categories. The first category is composed of the mPVN (**Figures 3A–C**), vlPAG (**Figures 3D–F**), and nPGi (**Figures 3G–I**), in which pretreatment with naloxone blocked the pheromone-induced change in Fos expression. Compared with the Control-SAL group, the Pheromone-SAL group ($P = 0.003$), but not the Pheromone-NAL group ($P = 0.795$), showed decreased Fos expression in the mPVN. Fos expression in the vlPAG and nPGi in the Pheromone-SAL group (vlPAG, $P < 0.001$; nPGi, $P < 0.001$), but not the Pheromone-NAL group (vlPAG, $P = 0.969$; nPGi, $P = 0.226$) was increased compared with that in the Control-SAL group. The second category is composed of the BNSTam, BNSTal, BNSTp, pPVN, Arc, and dlPAG, in which pretreatment with naloxone did not block the pheromone-induced increase Fos expression. Both the Pheromone-SAL (BNSTam, $P = 0.009$; BNSTal, $P = 0.012$; BNSTp, $P = 0.004$; pPVN, $P = 0.036$; Arc, $P = 0.001$; dlPAG, $P = 0.007$) and Pheromone-NAL groups (BNSTam, $P = 0.020$; BNSTal, $P < 0.001$; BNSTp, $P = 0.005$; pPVN, $P < 0.001$; Arc, $P < 0.001$; dlPAG, $P = 0.011$) showed increased Fos expression when compared with the Control-SAL group in these regions. The last category is composed of the nucleus accumbens core and shell, ventromedial nucleus of hypothalamus, central amygdala, and dorsal raphe nucleus, in which pretreatment with naloxone increased Fos expression but the alarm pheromone

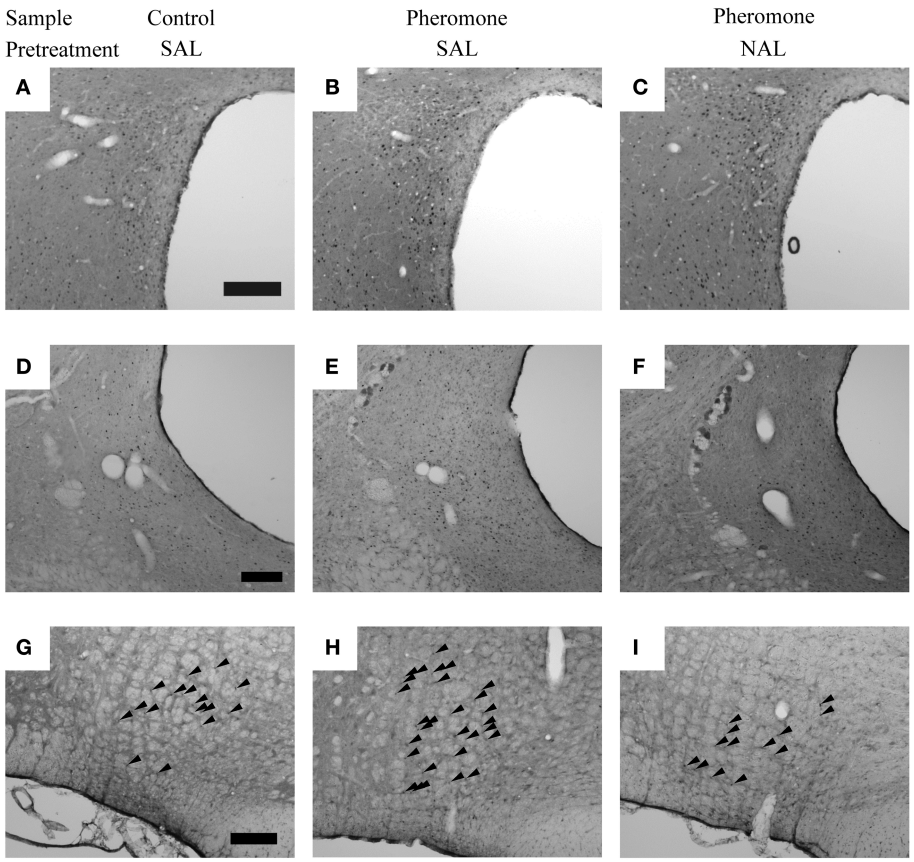


FIGURE 3 | The nuclei in which pretreatment with naloxone attenuated the pheromone-induced changes in Fos expression. Photomicrographs showing Fos expression in the paraventricular nucleus of the hypothalamus **(A–C)**, ventrolateral periaqueductal gray **(D–F)**, and nucleus paragigantocellularis of rats **(G–I)** that were pretreated with either saline (SAL) or naloxone (40 mg·kg^{-1}; NAL) and exposed to a control odor (Control) or an alarm pheromone (Pheromone). Arrowheads indicate Fos expression. The scale bar represents 200 μm.

did not. In these regions, the Pheromone-NAL group (nucleus accumbens core, $P = 0.001$; nucleus accumbens shell, $P = 0.020$; ventromedial nucleus of hypothalamus, $P = 0.005$; central amygdala, $P < 0.001$; dorsal raphe nucleus, $P = 0.005$), but not the Pheromone-SAL group (nucleus accumbens core, $P = 0.470$; nucleus accumbens shell, $P = 0.436$; ventromedial nucleus of hypothalamus, $P = 0.568$; central amygdala, $P = 0.939$; dorsal raphe nucleus, $P = 0.813$), showed increased Fos expression compared with the Control-SAL group.

Post-hoc tests confirmed a significant difference between the Pheromone-SAL and Pheromone-NAL groups in nuclei in the first (mPVN, $P = 0.001$; vlPAG, $P < 0.001$; nPGi, $P < 0.001$) and third categories (nucleus accumbens core, $P = 0.006$; nucleus accumbens shell, $P = 0.003$; ventromedial nucleus of hypothalamus, $P = 0.020$; central amygdala, $P < 0.001$; dorsal raphe nucleus, $P = 0.009$). Similarly, Fos expression was not different between the Pheromone-SAL and Pheromone-NAL groups in nuclei in the second category (BNSTam, $P = 0.665$; BNSTp, $P = 0.787$; pPVN, $P = 0.090$; Arc, $P = 0.437$; dlPAG, $P = 0.774$), except in the BNSTal, where the Pheromone-NAL group showed increased Fos expression compared with the Pheromone-SAL group ($P = 0.034$).

DISCUSSION

In the present study, we attempted to clarify the neural mechanisms underlying the alarm pheromone-induced deterioration in sexual behavior in male rats and assessed how opioids were involved. We observed that the pheromone affected Fos expression in the BNSTam, BNSTal, BNSTp, mPVN, pPVN, Arc, dlPAG, vlPAG, and nPGi. In addition, pretreatment with naloxone blocked these effects in the mPVN, vlPAG, and nPGi. Based on these results, we have revised our hypothesis regarding the neural mechanisms underlying the alarm pheromone-induced deterioration in sexual behavior (Kobayashi et al., 2013a,b) as follows: when a male rat detects the alarm pheromone, the vomeronasal system, including the BNSTp, and the main olfactory system (Inagaki et al., 2014) receive information about the pheromone. Information from these 2 olfactory systems activates the pPVN, which subsequently activates opioidergic neurons in the Arc. The opioidergic neurons suppress the mPVN and indirectly activate the vlPAG-nPGi cluster. As a result, sexual behavior deteriorates (**Figure 4**).

In addition to the nuclei found in our previous study, and excluding the neural responses related to ejaculation, we found activation in the Arc, dlPAG, and vlPAG and suppression in the

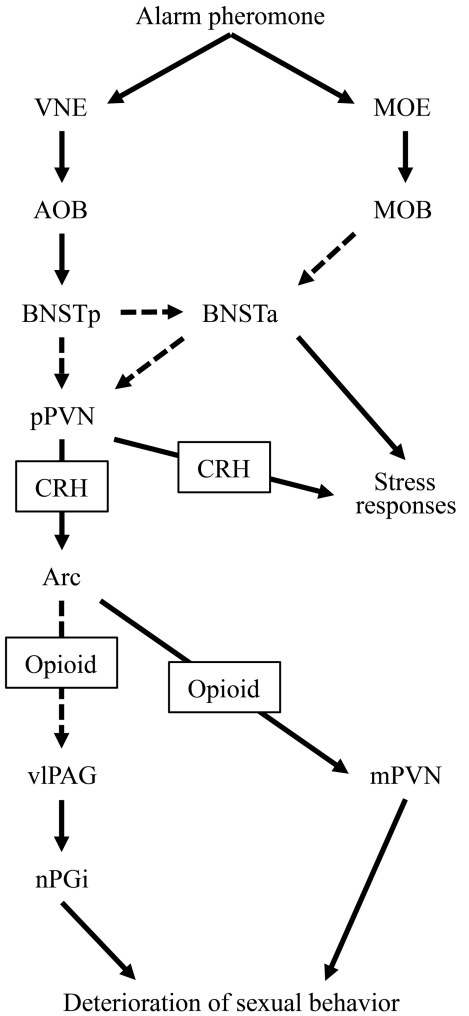


FIGURE 4 | A diagram of the hypothesized neural circuit inducing deterioration in sexual behavior by the alarm pheromone. Solid arrows indicate direct projections while dotted arrows indicate indirect or unclear projections. Abbreviations: AOB, accessory olfactory bulb; Arc, arcuate nucleus; BNSTa, anterior division of the bed nucleus of the stria terminalis; BNSTp, posterior division of the bed nucleus of the stria terminalis; CRH, corticotropin-releasing hormone; MOB, main olfactory bulb; MOE, main olfactory epithelium; mPVN, magnocellular part of the paraventricular nucleus of hypothalamus; nPGi, nucleus paragigantocellularis; pPVN, parvocellular part of the paraventricular nucleus of hypothalamus; vlPAG, ventrolateral periaqueductal gray; VNE, vomeronasal epithelium.

mPVN during deterioration in sexual behavior. Among these brain regions, the vlPAG and mPVN might be directly involved in the deterioration. It has been reported that lesioning the vlPAG reduces the number of mounts (Clark, 1975), suggesting that vlPAG activation deteriorates sexual behavior. The modulatory effects of the vlPAG might be exerted through the nPGi because lesioning the vlPAG (Clark, 1975) and nPGi (Yells et al., 1992) evokes the same pattern of deterioration. In addition, the vlPAG sends dense projections to the nPGi, which are activated during sexual behavior (Normandin and Murphy, 2008). Therefore, the vlPAG and nPGi might compose a cluster that modulates sexual behavior. Similarly, the mPVN might be involved in the deterioration; it has been reported that lesions in both the mPVN and pPVN, but not in the pPVN, increase the number of mounts and decrease the hit rate (Liu et al., 1997a). Therefore, it has been suggested that suppression of the mPVN deteriorates male sexual behavior. However, the mPVN might deteriorate sexual behavior in parallel with the vlPAG-nPGi cluster because, to the best of our knowledge, the mPVN and vlPAG-nPGi cluster are not anatomically connected. In contrast, activation of the dlPAG seems to be less important for the deterioration of sexual behavior. Anatomical evidence suggests that this region receives sensory information regarding sexual behavior from genital organs in the female (Klop et al., 2005). This information might also activate the dlPAG of the male in the present study. The Arc might be indirectly involved in the deterioration as discussed below.

Based on the present findings, we suggest that naloxone attenuated, rather than compensated for, the alarm pheromone-induced deterioration in sexual behavior. In the present and previous studies, rats that were pretreated with naloxone did not show a pheromone-induced deterioration in sexual behavior (Kobayashi et al., 2013b). One possible interpretation of this is that naloxone worked as an antagonist to the pheromone. Naloxone facilitates sexual behavior (McIntosh et al., 1980) and an opioid receptor antagonist increases Fos expression in nuclei that are not related to the nPGi, such as the nucleus accumbens core and shell, BNSTal, and central amygdala (Park and Carr, 1998; Carr et al., 1999). Therefore, an alternative interpretation could be that naloxone compensated the pheromone effects by facilitating sexual behavior through a separate neural mechanism. However, in the present study, we found that pretreatment with naloxone attenuated the pheromone-induced activation and suppression of the vlPAG-nPGi cluster and mPVN, respectively. Therefore, these results support the former interpretation.

Alarm pheromone-induced activation and suppression of the vlPAG-nPGi cluster and mPVN, respectively, were blocked by pretreatment with naloxone; therefore, these changes might be mediated by opioids. One possible source of opioids might be the Arc. Although its direct role in the deterioration of sexual behavior remains to be studied, the Arc contains abundant opioid peptides (Le Merrer et al., 2009), which are released by several stressors (Marinelli et al., 2004). In addition, the Arc sends direct projections to the mPVN (Sawchenko and Swanson, 1983); this suggests that opioid neurons in the Arc could directly suppress the mPVN. Indeed, an injection of opioids into the PVN inhibits male sexual behavior (Melis et al., 1999). The Arc could also affect the vlPAG-nPGi cluster because it sends projections to the medial preoptic area (MPOA) (Horvath et al., 1992). Electrical stimulation of the MPOA activates neurons in the vlPAG that project to the nPGi (Rizvi et al., 1996). Taken together, we propose that the Arc is the source of opioids that deteriorated the sexual behavior in the present study by suppressing the mPVN and activating the vlPAG-nPGi cluster.

In contrast to the suggested potential role of the MPOA, in our study, Fos expression in this region did not show any difference attributable to either the alarm pheromone or the pretreatment with naloxone. It is possible that the neural activation related to the expression of sexual behavior and/or intromissions obscured

the alarm pheromone-induced difference in Fos expression. In this study, all the animals expressed 10 intromissions with equivalent latencies to mount, intromission, and tenth intromission, even when the alarm pheromone increased the number of mounts and decreased hit rate. It is known that the MPOA plays an important role in the expression of sexual behavior (Liu et al., 1997b). In addition, Fos expression in the MPOA is increased in subjects after 5 intromissions (Baum and Everitt, 1992). Therefore, high levels of Fos expression induced by the expression of sexual behavior and/or 10 intromissions might prevent us from observing any alarm pheromone-induced change of Fos expression in this region.

In the present study, we found that alarm pheromone-induced activation of the BNSTam, BNSTal, BNSTp, pPVN, and Arc was not blocked by pretreatment with naloxone. In the context of our hypothesized neural circuit that has been discussed above and in our previous study (Kobayashi et al., 2013a,b),these nuclei might be upstream of opioidergic neurons in the Arc. The alarm pheromone is perceived by the vomeronasal epithelium (Kiyokawa et al., 2007, 2013a), which transmits information to the accessory olfactory bulb, and subsequently, the BNSTp. This information is transmitted to the anterior BNST (BNSTa), composed of BNSTal and BNSTam, and the pPVN. It is currently unclear whether the information is transmitted from the BNSTp to the BNSTa and pPVN directly or indirectly. The BNSTa is only activated when it receives simultaneous information from the main olfactory system (Inagaki et al., 2014), which enhances the responsiveness of the pPVN to the stimuli (Inagaki et al., 2014). The activated pPVN sends CRHergic output to the Arc and induces opioid secretion. Therefore, pretreatment with naloxone may not affect the activity in these nuclei (**Figure 4**).

In summary, an alarm pheromone increased the number of mounts and decreased the hit rate in male rats. This was accompanied by an increase in Fos expression in the BNSTam, BNSTal, BNSTp, pPVN, Arc, dlPAG, vlPAG, and nPGi and a decrease in the mPVN. Naloxone attenuated the pheromone-induced deterioration in sexual behavior and modification of Fos expression in the mPVN, vlPAG, and nPGi. Based on these results, we suggest that the alarm pheromone activates opioidergic neurons in the Arc, which deteriorate male sexual behavior by activating the vlPAG-nPGi cluster and suppressing the mPVN.

ACKNOWLEDGMENTS

This study was supported by JSPS KAKENHI Grant Numbers 21228006 and 23688035.

REFERENCES

Barrot, M., Wallace, D. L., Bolanos, C. A., Graham, D. L., Perrotti, L. I., Neve, R. L., et al. (2005). Regulation of anxiety and initiation of sexual behavior by CREB in the nucleus accumbens. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8357–8362. doi: 10.1073/pnas.0500587102

Baum, M. J., and Everitt, B. J. (1992). Increased expression of c-fos in the medial preoptic area after mating in male rats: role of afferent inputs from the medial amygdala and midbrain central tegmental field. *Neuroscience* 50, 627–646. doi: 10.1016/0306-4522(92)90452-8

Carr, K. D., Kutchukhidze, N., and Park, T. H. (1999). Differential effects of mu and kappa opioid antagonists on Fos-like immunoreactivity in extended amygdala. *Brain Res.* 822, 34–42. doi: 10.1016/S0006-8993(99)01088-4

Clark, T. K. (1975). Sexual inhibition is reduced by rostral midbrain lesions in the male rat. *Science* 190, 169–171. doi: 10.1126/science.1166311

Horvath, T. L., Naftolin, F., and Leranth, C. (1992). GABAergic and catecholaminergic innervation of mediobasal hypothalamic beta-endorphin cells projecting to the medial preoptic area. *Neuroscience* 51, 391–399. doi: 10.1016/0306-4522(92)90323-T

Inagaki, H., Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2008). Enhancement of the acoustic startle reflex by an alarm pheromone in male rats. *Physiol. Behav.* 93, 606–611. doi: 10.1016/j.physbeh.2007.10.021

Inagaki, H., Kiyokawa, Y., Tamogami, S., Watanabe, H., Takeuchi, Y., and Mori, Y. (2014). Identification of a pheromone that increases anxiety in rats. *Proc. Natl. Acad. Sci. U.S.A.* 111, 18751–18756. doi: 10.1073/pnas.1414710112

Karlson, P., and Luscher, M. (1959). "Pheromones": a new term for a class of biologically active substances. *Nature* 183, 55–56. doi: 10.1038/183055a0

Kikusui, T., Takigami, S., Takeuchi, Y., and Mori, Y. (2001). Alarm pheromone enhances stress-induced hyperthermia in rats. *Physiol. Behav.* 72, 45–50. doi: 10.1016/S0031-9384(00)00370-X

Kiyokawa, Y., Honda, A., Takeuchi, Y., and Mori, Y. (2014). A familiar conspecific is more effective than an unfamiliar conspecific for social buffering of conditioned fear responses in male rats. *Behav. Brain Res.* 267, 189–193. doi: 10.1016/j.bbr.2014.03.043

Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2004). Alarm pheromones with different functions are released from different regions of the body surface of male rats. *Chem. Senses* 29, 35–40. doi: 10.1093/chemse/bjh004

Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2005a). Alarm pheromone that aggravates stress-induced hyperthermia is soluble in water. *Chem. Senses* 30, 513–519. doi: 10.1093/chemse/bji044

Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2005b). Mapping the neural circuit activated by alarm pheromone perception by c-Fos immunohistochemistry. *Brain Res.* 1043, 145–154. doi: 10.1016/j.brainres.2005.02.061

Kiyokawa, Y., Kikusui, T., Takeuchi, Y., and Mori, Y. (2007). Removal of the vomeronasal organ blocks the stress-induced hyperthermia response to alarm pheromone in male rats. *Chem. Senses* 32, 57–64. doi: 10.1093/chemse/bjl036

Kiyokawa, Y., Kodama, Y., Kubota, T., Takeuchi, Y., and Mori, Y. (2013a). Alarm pheromone is detected by the vomeronasal organ in male rats. *Chem. Senses* 38, 661–668. doi: 10.1093/chemse/bjt030

Kiyokawa, Y., Kodama, Y., Takeuchi, Y., and Mori, Y. (2013b). Physical interaction is not necessary for the induction of housing-type social buffering of conditioned hyperthermia in male rats. *Behav. Brain Res.* 256, 414–419. doi: 10.1016/j.bbr.2013.08.037

Kiyokawa, Y., Shimozuru, M., Kikusui, T., Takeuchi, Y., and Mori, Y. (2006). Alarm pheromone increases defensive and risk assessment behaviors in male rats. *Physiol. Behav.* 87, 383–387. doi: 10.1016/j.physbeh.2005.11.003

Klein, S. L. (2003). Parasite manipulation of the proximate mechanisms that mediate social behavior in vertebrates. *Physiol. Behav.* 79, 441–449. doi: 10.1016/S0031-9384(03)00163-X

Klop, E. M., Mouton, L. J., Kuipers, R., and Holstege, G. (2005). Neurons in the lateral sacral cord of the cat project to periaqueductal grey, but not to thalamus. *Eur. J. Neurosci.* 21, 2159–2166. doi: 10.1111/j.1460-9568.2005.04039.x

Kobayashi, T., Kiyokawa, Y., Arata, S., Takeuchi, Y., and Mori, Y. (2013a). c-Fos expression during the modulation of sexual behavior by an alarm pheromone. *Behav. Brain Res.* 237, 230–237. doi: 10.1016/j.bbr.2012.09.042

Kobayashi, T., Kiyokawa, Y., Arata, S., Takeuchi, Y., and Mori, Y. (2013b). Naloxone blocks the deterioration of male sexual behavior induced by alarm pheromone in rats. *J. Vet. Med. Sci.* 75, 761–765. doi: 10.1292/jvms.12-0454

Kobayashi, T., Kiyokawa, Y., Takeuchi, Y., and Mori, Y. (2011). Pretreatment with CP-154526 blocks the modifying effects of alarm pheromone on components of sexual behavior in male, but not in female, rats. *Chem. Senses* 36, 623–632. doi: 10.1093/chemse/bjr017

Le Merrer, J., Becker, J. A., Befort, K., and Kieffer, B. L. (2009). Reward processing by the opioid system in the brain. *Physiol. Rev.* 89, 1379–1412. doi: 10.1152/physrev.00005.2009

Lin, Y. C., Rikihisa, Y., Kono, H., and Gu, Y. (1990). Effects of larval tapeworm (Taenia taeniaeformis) infection on reproductive functions in male and female host rats. *Exp. Parasitol.* 70, 344–352. doi: 10.1016/0014-4894(90)90116-T

Liu, Y. C., Salamone, J. D., and Sachs, B. D. (1997a). Impaired sexual response after lesions of the paraventricular nucleus of the hypothalamus in male rats. *Behav. Neurosci.* 111, 1361–1367. doi: 10.1037/0735-7044.111.6.1361

Liu, Y. C., Salamone, J. D., and Sachs, B. D. (1997b). Lesions in medial preoptic area and bed nucleus of stria terminalis: differential effects on copulatory behavior and noncontact erection in male rats. *J. Neurosci.* 17, 5245–5253.

Marinelli, P. W., Quirion, R., and Gianoulakis, C. (2004). An *in vivo* profile of beta-endorphin release in the arcuate nucleus and nucleus accumbens following exposure to stress or alcohol. *Neuroscience* 127, 777–784. doi: 10.1016/j.neuroscience.2004.05.047

McIntosh, T. K., Vallano, M. L., and Barfield, R. J. (1980). Effects of morphine, beta-endorphin and naloxone on catecholamine levels and sexual behavior in the male rat. *Pharmacol. Biochem. Behav.* 13, 435–441. doi: 10.1016/0091-3057(80)90251-8

Melis, M. R., Succu, S., Spano, M. S., and Argiolas, A. (1999). Morphine injected into the paraventricular nucleus of the hypothalamus prevents noncontact penile erections and impairs copulation: involvement of nitric oxide. *Eur. J. Neurosci.* 11, 1857–1864. doi: 10.1046/j.1460-9568.1999.00603.x

Normandin, J. J., and Murphy, A. Z. (2008). Nucleus paragigantocellularis afferents in male and female rats: organization, gonadal steroid receptor expression, and activation during sexual behavior. *J. Comp. Neurol.* 508, 771–794. doi: 10.1002/cne.21704

Park, T. H., and Carr, K. D. (1998). Neuroanatomical patterns of fos-like immunoreactivity induced by a palatable meal and meal-paired environment in saline- and naltrexone-treated rats. *Brain Res.* 805, 169–180. doi: 10.1016/S0006-8993(98)00719-7

Paxinos, G., and Watson, C. (2007). *The Rat Brain in Stereotaxic Coordinates.* Tokyo: Elsevier.

Retana-Marquez, S., Salazar, E. D., and Velazquez-Moctezuma, J. (1996). Effect of acute and chronic stress on masculine sexual behavior in the rat. *Psychoneuroendocrinology* 21, 39–50. doi: 10.1016/0306-4530(95)00029-1

Rhees, R. W., Lephart, E. D., and Eliason, D. (2001). Effects of maternal separation during early postnatal development on male sexual behavior and female reproductive function. *Behav. Brain Res.* 123, 1–10. doi: 10.1016/S0166-4328(00)00381-8

Rizvi, T. A., Murphy, A. Z., Ennis, M., Behbehani, M. M., and Shipley, M. T. (1996). Medial preoptic area afferents to periaqueductal gray medullo-output neurons: a combined Fos and tract tracing study. *J. Neurosci.* 16, 333–344.

Sawchenko, P. E., and Swanson, L. W. (1983). The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat. *J. Comp. Neurol.* 218, 121–144. doi: 10.1002/cne.902180202

Wang, C. T., Shui, H. A., Huang, R. L., Tai, M. Y., Peng, M. T., and Tsai, Y. F. (2006). Sexual motivation is demasculinized, but not feminized, in prenatally stressed male rats. *Neuroscience* 138, 357–364. doi: 10.1016/j.neuroscience.2005.11.026

Yells, D. P., Hendricks, S. E., and Prendergast, M. A. (1992). Lesions of the nucleus paragigantocellularis: effects on mating behavior in male rats. *Brain Res.* 596, 73–79. doi: 10.1016/0006-8993(92)91534-L

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 November 2014; accepted: 06 February 2015; published online: 23 February 2015.

*Citation: Kobayashi T, Kiyokawa Y, Takeuchi Y and Mori Y (2015) Neural correlates underlying naloxone-induced amelioration of sexual behavior deterioration due to an alarm pheromone. Front. Neurosci. **9**:52. doi: 10.3389/fnins.2015.00052*

This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 28 July 2015
doi: 10.3389/fnins.2015.00262

Chemostimuli for guanylyl cyclase-D-expressing olfactory sensory neurons promote the acquisition of preferences for foods adulterated with the rodenticide warfarin

Kevin R. Kelliher[1] *and Steven D. Munger*[2, 3, 4]*

[1] College of Chiropractic, University of Bridgeport, Bridgeport, CT, USA, [2] Department of Pharmacology and Therapeutics, University of Florida, Gainesville, FL, USA, [3] Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, University of Florida, Gainesville, FL, USA, [4] Center for Smell and Taste, University of Florida, Gainesville, FL, USA

Many animals have the ability to acquire food preferences from conspecifics via social signals. For example, the coincident detection of a food odor by canonical olfactory sensory neurons (OSNs) and agonists of the specialized OSNs expressing the receptor guanylyl cyclase GC-D (GC-D+ OSNs) will promote a preference in recipient rodents for similarly odored foods. It has been hypothesized that these preferences are acquired and maintained regardless of the palatability or quality of the food. We assessed whether mice could acquire and maintain preferences for food that had been adulterated with the anticoagulant rodenticide warfarin. After olfactory investigation of a saline droplet containing either cocoa (2%, w/w) or cinnamon (1%, w/w) along with a GC-D+ OSN-specific chemostimulus (either of the guanylin-family peptides uroguanylin and guanylin; 1–50 nM), C57BL/6J mice exhibited robust preferences for unadulterated food containing the demonstrated odor. The peptide-dependent preference was observed even when the food contained warfarin (0.025% w/w). Repeated ingestion of warfarin-containing food over four days did not disrupt the preference, even though mice were not re-exposed to the peptide stimulus. Surprisingly, mice continued to prefer warfarin-adulterated food containing the demonstrated odor when presented with a choice of warfarin-free food containing a novel odor. Our results indicate that olfactory-mediated food preferences can be acquired and maintained for warfarin-containing foods and suggest that guanylin peptides may be effective stimuli for promoting the ingestion of foods or other edibles with low palatability or potential toxicity.

Keywords: uroguanylin, guanylin, GC-D, learning, mouse

OPEN ACCESS

Edited by:
Markus Fendt, Otto-von-Guericke University Magdeburg, Germany

Reviewed by:
Hiro Matsunami, Duke University, USA
Marc Spehr, RWTH-Aachen University, Germany

***Correspondence:**
Steven D. Munger, Department of Pharmacology and Therapeutics, University of Florida, PO Box 100267, Gainesville, FL 32610, USA
steven.munger@ufl.edu

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *16 June 2015*
Accepted: *13 July 2015*
Published: *28 July 2015*

Citation:
Kelliher KR and Munger SD (2015) Chemostimuli for guanylyl cyclase-D-expressing olfactory sensory neurons promote the acquisition of preferences for foods adulterated with the rodenticide warfarin. Front. Neurosci. 9:262. doi: 10.3389/fnins.2015.00262

Introduction

Rodents use chemostimuli found on the breath and in urine and feces to communicate information about food (Galef, 2012). When a conspecific detects these semiochemicals simultaneously with a specific food odor it acquires a long-lasting preference for foods containing that same odor. These socially transmitted food preferences (STFPs) may result from the direct interaction of a novice rodent (observer) with an experienced one (demonstrator) (e.g., Galef et al., 1988; Crawley, 2007; Galef, 2012) or may be learned through pairing of the social odor with a particular feeding site (Arakawa et al., 2013). We have previously reported that both CS_2 (found in rodent breath; Munger et al., 2010) and the guanylin family peptide uroguanylin (UG, a gut peptide excreted in urine and feces; Leinders-Zufall et al., 2007; Arakawa et al., 2013) mediate the formation of food preferences through activation of a specialized olfactory subsystem, the hallmark of which is a population of olfactory sensory neurons (OSNs) expressing the type D receptor guanylyl cyclase (GC-D). GC-D-expressing (GC-D+) OSNs detect both CS_2 and guanylin-family peptides and are required for the acquisition of STFPs initiated by those semiochemicals.

Social interactions can lead to increased preferences to previously avoided foods in birds (Mason et al., 1984), rats (Galef et al., 1990), sheep (Thorhallsdottir et al., 1990), and cattle (Ralphs and Olsen, 1990). However, socially mediated flavor aversion has only been clearly shown to occur in birds (Mason et al., 1984); attempts in rats (Galef et al., 1990; Jing et al., 2014), sheep (Pfister and Price, 1996), and cattle (Cibils et al., 2008) have been unsuccessful in demonstrating a socially mediated avoidance of demonstrated foods or feeding sites. While chemostimuli that activate GC-D+ OSNs can promote the acquisition of preferences for laboratory chow containing innocuous flavorings such as cocoa or cinnamon (Galef et al., 1988; Munger et al., 2010; Arakawa et al., 2013), it is less clear whether these same mechanisms can promote preferences for foods containing toxic substances. In this study we investigated whether the guanylin-family peptides UG and guanylin, which specifically activate GC-D+ OSNs (Leinders-Zufall et al., 2007), can elicit a preference for odored food when that food has been tainted with the anticoagulant rodenticide warfarin. We further tested whether this preference was maintained over time or in the presence of unadulterated food. Together, our findings suggest that food preference acquisition mediated by guanylin-family peptides is robust, prolonged, and unaffected by aversive cues in foods.

Materials and Methods

Animals

All experimental procedures were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. Mice were housed in an AAALAC accredited laboratory facility. Male C57BL/6J (B6) mice were obtained from the Jackson Laboratory (Bar harbor, MN). Mice were initially group housed (4–5 per cage) in standard cages ($28 \times 17 \times 12.5$ cm) with filter-top lids. All mice received water and standard rodent chow *ad libitum* prior to the experiments. The room in which the mice resided was environmentally controlled on a 12:12 h light:dark cycle (0600–1800 h lighting) at a temperature of 21°C, relative humidity of 50–60%.

Food Preference Testing

Food preference assays were modified from those used previously for testing the social transmission of food preference in rats and mice (Galef et al., 1983; Posadas-Andrews and Roper, 1983; Valsecchi and Galef, 1989; Crawley, 2007; Ryan et al., 2008; Munger et al., 2010; Arakawa et al., 2013). In all experiments, subject mice were housed in groups of two or three for 3 days in standard cages with the food container placed on the cage floor. Mice were fed a crushed rodent diet (2018SX, Harlan) to habituate them to powdered food. The amount of food was restricted to 2 g/mouse/day to facilitate feeding during the food preference tests. Throughout the experiment mice were monitored for weight loss and overall health. Food deprived mice were removed from the experiment if their body weight dropped more than 25%. Mice fed food containing warfarin were also removed from the experiment and euthanized if they began showing significant signs of sickness. Food preference was quantified by computing the ratio of the demonstrated food consumed vs. the total food consumed by the subject mice (preference ratio, PR). All data were expressed as mean ± S.E.M. Differences were accepted as significant if $p < 0.05$ (see below for details of statistical tests).

Concentration-response to Uroguanylin (UG) and Guanylin (Experiment 1)

B6 mice were randomly assigned into three groups, with each group receiving different concentrations of UG. On the first test day, each mouse was moved to individual cages and presented with a petri dish containing a drop of saline (150 µl) with either cocoa (2%, Hershey's) or cinnamon (1%, McCormick) plus UG at a concentration of 50 nM ($n = 8$), 1 nM ($n = 9$), or 0 nM ($n = 13$). After 1 h of exposure [during which time mice would physically interact with the saline drop, allowing them to aspirate the peptide solution into the nasal cavity (Spehr et al., 2006)] mice were moved to clean cages. After 3 h, mice were presented with two food trays (3 g of food per tray): one odored with 1% cinnamon and one with 2% cocoa. Both trays were also adulterated with warfarin (0.025%, the concentration found in many commercial rodenticides). Food trays included a weighted base that captured spilled food (which was routinely minimal). After 1 h of feeding the food trays were removed and weighed to calculate the amount of food consumed. Experiments testing the efficacy of different concentrations of guanylin were performed identically to those testing UG except for the replacement of that peptide with guanylin at concentrations of 50 nM ($n = 7$), 1 nM ($n = 7$), or 0 nM ($n = 13$).

Duration of Preference Maintenance (Experiment 2)

B6 mice were randomly assigned into two groups, one to be exposed to saline containing UG (50 nM) plus odor ($n = 8$) and the other to saline plus odor alone ($n = 8$). Testing was done over a 5-day period. On Day 1 of this experiment we

used the same testing paradigm as in Experiment 1 except that all mice were placed back into the home cages at the end of testing. On day 2 mice were transferred from their home cages to testing cages 3 h prior to testing. Mice were then given the choice to feed on warfarin-adulterated food odored with either cocoa or cinnamon. After 1 h trays were removed and weighed to determine the amount of each food consumed. Mice were placed back into their home cages until retesting on day 5. Mice were maintained on a restricted diet of powdered normal rat chow (2 g/mouse/day) during the non-testing days (days 3, 4). Mice typically exhibited signs of warfarin effects on later days, including lethargy, hunched posture and/or a dull coat, and were removed from the study if these signs were moderate or severe.

Preference Maintenance Given a Novel Odor Choice (Experiment 3)

B6 mice were randomly assigned into three groups, with one group exposed to saline containing an odor (1% cinnamon or 2% cocoa) plus UG (50 nM; $n = 10$) and the second saline plus odor alone ($n = 10$). Testing was done over a 2-day period. On Day 1 of this experiment we used the same testing paradigm as in Experiment 1 except that all mice were placed back into the home cages at the end of testing. On Day 2, mice were transferred from their home cages to their testing cages 3 h prior to testing. Mice were then given the choice to feed on food containing the demonstrated odor and adulterated with warfarin (0.025%) or food containing a completely novel food odor (1% ginger) and no warfarin. After 1 h of feeding, food trays were removed and weighed to determine the amount of each food consumed.

Data Analysis

Food preferences were calculated as a ratio of demonstrated food consumed/ non-demonstrated food consumed, where the "demonstrated food" contained the demonstrated odor and the "non-demonstrated food" contained the novel odor. Data for Experiments 1 and 3 were each analyzed using a One-Way ANOVA followed by Tukeys *post-hoc* tests. Data for Experiment 2 was analyzed using a Two-Way, repeated measures ANOVA with presented odor and test day as the independent variables. Tukeys *post-hoc* tests were used for multiple comparisons of significant results.

Results

Nanomolar Concentrations of Guanylin-family Peptides Can Induce Preferences for Warfarin-adulterated Foods

We previously showed that mice form food preferences to odored food when the odor is first paired with uroguanylin (UG) at concentrations as low as 50 nM (Arakawa et al., 2013). However, electroolfactogram studies in the mouse olfactory epithelium showed significant responses at even lower concentrations of UG ($EC_{50} < 1$ nM) (Leinders-Zufall et al., 2007). Here, we found that mice exposed to a food odor plus 50 nM UG formed a preference for food adulterated with 0.025% warfarin and containing the demonstrated odor [PR = 0.76 ± 0.04 SEM; One-Way ANOVA: $F_{(1, 29)} = 4.31$, $p < 0.05$; Tukey's *post-hoc* vs. 0 nM UG control, $p < 0.05$] **(Figure 1, Table 1)**. This preference was absent in mice exposed to food odor alone (PR = 0.52 ± 0.06 SEM). Mice exposed to food odor plus 1 nM UG exhibited a lesser preference (PR = 0.65 ± 0.05 SEM) that was not significantly greater than the 0 nM UG control (Tukeys's *post-hoc*, $p > 0.05$). Together, these results show that UG is similarly effective at eliciting a food preference in unadulterated (Arakawa et al., 2013) or warfarin-adulterated food (**Figure 1**).

Another guanylin-family peptide, guanylin, is the most effective chemostimulus ($EC_{50} < 200$ pM) yet identifed for GC-D+ OSNs (Leinders-Zufall et al., 2007). Here we observe

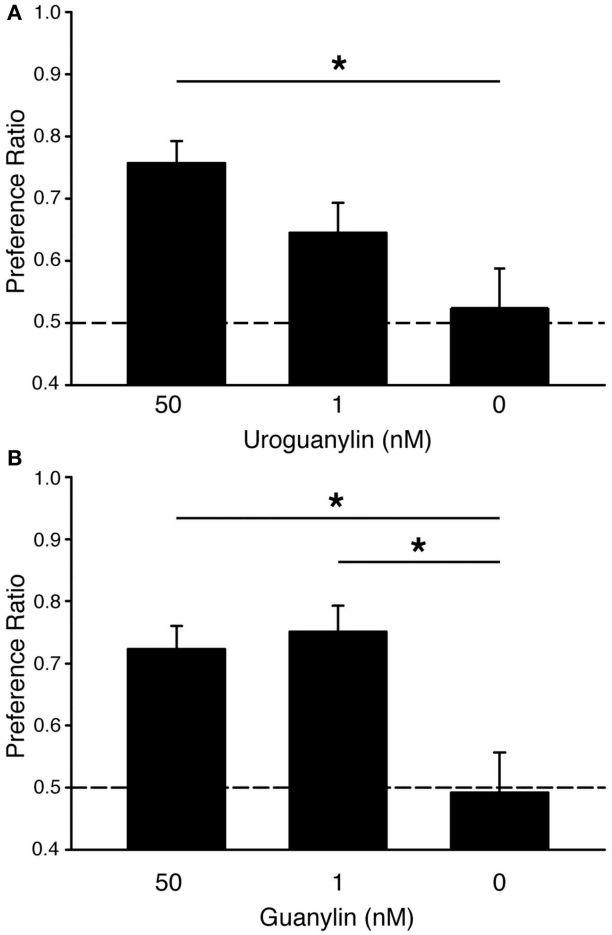


FIGURE 1 | Guanylin family peptides promote preferences for odored food adulterated with warfarin. (A) C57BL/6J mice acquired preferences for food containing a demonstrated odor when that odor was paired with 50 nM uroguanylin (UG) even when both food choices had been adulterated with the rodenticide warfarin (0.025%). Mice were demonstrated a food odor paired with 50 nM UG ($n = 8$), 1 nM UG ($n = 9$), or 0 nM UG ($n = 13$). One-Way ANOVA [$F_{(1, 29)} = 4.31$, $P < 0.05$] followed by Tukeys *post-hoc* test (*$P < 0.05$). **(B)** C57BL/6J mice acquired preferences for food containing a demonstrated odor when that odor was paired with either 50 nM or 1 nM guanylin even when both food choices had been adulterated with warfarin (0.025%). Mice were demonstrated a food odor paired with 50 nM guanylin ($n = 7$), 1 nM guanylin ($n = 7$), and 0 nM guanylin ($n = 14$). One-Way ANOVA [$F_{(1, 26)} = 6.29$, $P < 0.01$] followed by Tukeys *post-hoc* test (*$P < 0.05$). Dashed lines, no preference. Error bars, standard error of the mean.

TABLE 1 | Food consumed by observer mice in food preference assays (mean ± s.e.m.).

Stimulus		Food consumed (g)		
		Total food	w/demonstrated odor	w/novel odor
EXPERIMENT 1				
Odor alone		2.25 ± 0.10	1.15 ± 0.10	1.10 ± 0.11
Odor + UG (50 nM)		2.19 ± 0.20	1.62 ± 0.21	0.57 ± 0.12
Odor + UG (1 nM)		1.97 ± 0.15	1.37 ± 0.1	0.60 ± 0.09
EXPERIMENT 2				
Odor alone		2.89 ± 0.15	1.42 ± 0.11	1.47 ± 0.13
Odor + G (50 nM)		2.00 ± 0.25	1.47 ± 0.13	0.54 ± 0.09
Odor + G (1 nM)		2.10 ± 0.36	1.48 ± 0.2	0.7 ± 0.23
EXPERIMENT 3				
Odor alone	*Day 1*	2.19 ± 0.31	1.18 ± 0.31	1.01 ± 0.12
	Day 2	1.98 ± 0.31	0.94 ± 0.16	1.04 ± 0.17
	Day 5	0.55 ± 0.12	0.30 ± 0.08	0.25 ± 0.16
Odor + UG (50 nM)	*Day 1*	2.00 ± 0.25	1.47 ± 0.13	0.53 ± 0.09
	Day 2	2.10 ± 0.36	1.48 ± 0.2	0.62 ± 0.23
	Day 5	0.63 ± 0.06	0.04 ± 0.01	0.02 ± 0.01
EXPERIMENT 4				
Odor alone		2.21 ± 0.15	1.26 ± 0.14	0.95 ± 0.15
Odor + UG		2.51 ± 0.15	1.62 ± 0.12	0.89 ± 0.11
Odor alone w/novel choice		1.82 ± 0.22	0.96 ± 0.19	1.04 ± 0.19
Odor + UG w/novel choice		1.45 ± 0.19	1.15 ± 0.09	0.3 ± 0.18

UG, uroguanylin; G, guanylin; Odor: either 2% cocoa or 1% cinnamon (counterbalanced).

that guanylin is also an effective social cue in the acquisition of food preferences in mice **(Figure 1, Table 1)**. Mice showed significant preferences for food containing a demonstrated odor after exposure to that odor plus either 50 nM guanylin (PR = 0.72 ± 0.04 SEM) or 1 nM guanylin (PR = 0.75 ± 0.04 SEM) but not in controls containing odor only [PR = 0.49 ± 0.06 SEM; One-Way ANOVA, $F_{(1, 26)} = 6.29$, $p < 0.01$; Tukeys's *post-hoc* vs. 0 nM G control, $p < 0.05$].

Mice Maintain a Preference for Several Days after a Single Uroguanylin Exposure

Food preferences acquired after exposure to social cues can last for days or weeks. We tested whether preferences acquired after exposure to UG could be maintained for food adulterated with warfarin. As before, we found that mice exposed to a food odor plus 50 nM UG acquire a preference for food adulterated with 0.025% warfarin and containing the demonstrated odor (PR = 0.76 ± 0.04 SEM) when tested on the same day as the UG exposure; mice exposed to food odor alone did not demonstrate a preference (PR = 0.49 ± 0.05 SEM) **(Figure 2, Table 1)**. These mice maintained a preference for the demonstrated odored food when retested 1 and 4 days after the UG exposure (Day 2: PR = 0.72 ± 0.06 SEM; Day 5: PR = 0.74 ± 0.08) despite having no additional exposure to UG (**Figure 3**). A Two-Way repeated measures ANOVA showed a significant effect of UG exposure [$F_{(1, 46)} = 19.55$, $p < 0.001$; Tukey's *post-hoc* vs. 0 nM UG, $p < 0.01$ on each test day] on the preference measures across the 5 day period.

Mice Maintain a Preference for Food Containing Warfarin Even in the Presence of Unadulterated Food

Warfarin can cause significant distress in rodents that ingest it even before reaching lethal levels. We next tested whether UG-dependent preferences for food adulterated with warfarin are maintained when mice are given a choice of unadulterated (i.e., warfarin-free) food that contains a novel odor. As before, mice were exposed to 50 nM UG plus a food odor or to food odor alone and then given a choice of foods containing warfarin and either the demonstrated or novel odor. These mice were then tested again the next day; however, on this second day of testing mice were given a choice of food containing warfarin plus the demonstrated odor or food containing a new odor (ginger) and no warfarin. Mice that had been exposed to UG maintained a strong preference for food containing the demonstrated odor [PR = 0.74 ± 0.06 SEM; One-Way ANOVA, $F_{(1, 19)} = 10.1$, $p < 0.005$] **(Figure 3, Table 1)**.

Discussion

The sensory cues that influence food choices include general odors, semiochemicals, tastes, and post-ingestive signals. In rodents, the social chemostimuli CS_2, UG, and guanylin activate specialized GC-D+ OSNs to elicit the acquisition of food preferences (Leinders-Zufall et al., 2007; Munger et al., 2010; Zufall and Munger, 2010; Arakawa et al., 2013). Here, we found that UG and guanylin are highly effective stimuli for promoting the acquisition of preferences for foods, even if those foods

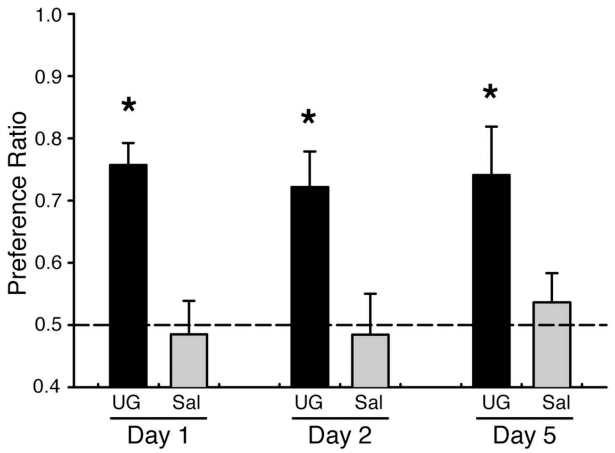


FIGURE 2 | Mice maintain UG-mediated food preferences even after ingestion of food adulterated with warfarin. C57BL/6J mice were demonstrated a food odor paired with 50 nM UG (black, $n = 8$) or saline (gray, $n = 8$). Mice exposed to UG, but not saline controls, showed a significant preference for food containing the demonstrated odor and warfarin (0.025%); this preference was maintained after 5 days without additional exposure to UG. Two-Way repeated measures ANOVA on stimulus and day [$F_{(1, 46)} = 19.55$, $P < 0.001$] followed by Tukeys *post-hoc* test (*$P < 0.01$). Dashed lines, no preference. Error bars, standard error of the mean. One control mouse was removed from the study prior to Day 5 due to apparent significant distress.

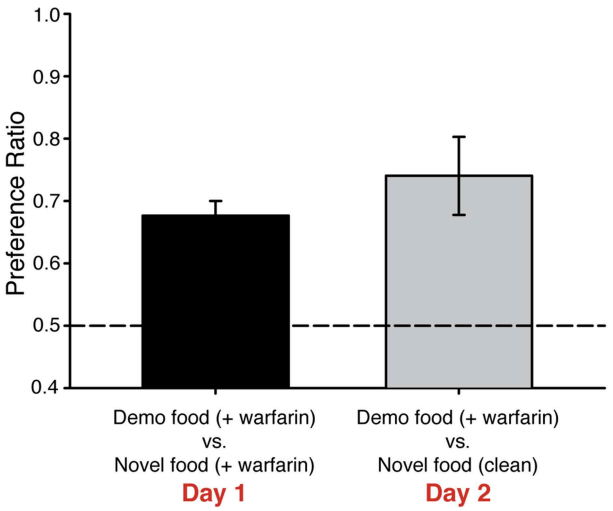


FIGURE 3 | Mice prefer demonstrated food containing warfarin to novel food without the rodenticide. C57BL/6J mice ($n = 10$) were demonstrated a food odor paired with 50 nM UG. On Day 1, mice preferred food containing the demonstrated over food with a novel odor, although both foods contained warfarin (0.025%). On Day 2, these mice continued to prefer food containing both the demonstrated odor and warfarin even though the other food choice contained a novel odor but no warfarin. One-Way ANOVA [$F_{(1, 19)} = 10.1$, $P < 0.005$], followed by Tukeys *post-hoc* test (*$P < 0.01$). Dashed lines, no preference. Error bars, standard error of the mean.

contain the poison warfarin. These preferences remain robust over several days and are retained even when the mouse is given an alternative, unadulterated food choice.

GC-D+ OSNs are exquisitely sensitive to UG and guanylin, with responses seen upon stimulation with picomolar concentrations of the peptides (Leinders-Zufall et al., 2007); CS_2 is also highly effective, activating these cells at submicromolar concentrations (Munger et al., 2010). Electroolfactrogram recordings from the main olfactory epithelium found guanylin ($K_{1/2} = 66.1$ pM) to be a somewhat more effective stimulus than UG ($K_{1/2} = 247$ pM) (Leinders-Zufall et al., 2007). Thus, we expected guanylin to be able to elicit a food preference at lower concentrations than UG. Indeed, this was the case. While 50 nM of either peptide was sufficient to elicit a significant preference in mice, only guanylin could do so at 1 nM (**Figure 1**). This sensitivity is reminiscent of other olfactory subsystems that couple the detection of semiochemicals to defined behavioral outputs. For example, threshold responses of vomeronasal sensory neurons to the exocrine gland-secreting peptides ESP1 (which elicits female sexual behaviors) and ESP22 (which inhibits male sexual behaviors) are each found in the low nanomolar range (Kimoto et al., 2007; Haga et al., 2010; Ferrero et al., 2013). OSNs expressing the trace amine-associate receptor TAAR4, which are important for predator avoidance, respond to carnivore odor β-phenylethylamine with an EC_{50} of ∼1 pM (Zhang et al., 2013). The high value placed on the detection of semiochemicals may favor sensory cells that are exceedingly sensitive to specific stimuli.

The acquisition of STFPs involves the formation of short and long-term memories for food odors that can last weeks or possibly even months (Galef and Whiskin, 2003). There is good evidence that the acquisition and retrieval of short-term memories for STFPs requires the ventral hippocampus (Countryman et al., 2005; Ross and Eichenbaum, 2006). Damage to the ventral hippocampus 1–2 days after training eliminates memory for food odors, but retrieval is not inhibited if the lesion is performed 21 days after training (Ross and Eichenbaum, 2006). Long-term memory for STFPs involves consolidation of memories in neocortical areas and the amygdala (Smith et al., 2007). In our experiments we found that food preferences were maintained for at least four days (**Figure 2**). This preference, and thus the memory of the pairing of the demonstrated odor with the social cue, persists even when the food is paired with concentrations of warfarin that will elicit significant distress. Although we predict that these preferences would have been maintained for even longer periods of time, longer timepoints were not tested as repeated exposure to the warfarin would have lead to unacceptable distress and death.

We found that food preferences induced by guanylin-family peptides were not noticeably impacted by the presence of a potentially dangerous food, containing the rodenticide warfarin, even when an unadulterated food alternative was available (**Figure 3**). This is consistent with the results of experiments in which observer rats do not acquire aversions to foods if the demonstrator rat was made ill (Galef et al., 1983). It has been suggested that rodents do not require socially transmitted taste avoidance since rodents, particularly rats, are neophobic and will generally avoid foods with a novel odor (Galef, 1985; Galef and Beck, 1985). This finding was recently confirmed by Jing et al. (2014), who found that observer rats acquired food preferences from anesthetized or partially anesthetized

demonstrators that were made sick from LiCl injections. These authors suggest that rats do not lack the ability to detect the health status of conspecifics but instead lack the ability to detect potential danger from novel food. Therefore, at least in rodent species, it appears that negative outcomes regarding food are not socially transmitted. Conversely, social learning may be able to override learned avoidance. For example, rats will learn to avoid a dark chamber when entrance to that chamber is paired with foot shocks. This avoidance is inhibited if the experienced rat is paired with another rat in a "safe" chamber where no foot shocks are given (Masuda and Aou, 2009). Furthermore, devaluation of the odor used as a social cue (e.g., CS_2) by pairing it with an aversive taste cue does not eliminate that odor's ability to elicit the acquisition of food preferences (Maier et al., 2014).

Mice, rats, and many other rodents are prevalent and costly pests. They damage crops, consume and contaminate human food and animal feed, damage infrastructure, and transmit human and animal diseases. The economic cost of damage by rats in the U.S. is estimated to exceed $20 billion, while worldwide food losses attributed to rats alone exceed $30 billion (Pimentel et al., 2000; Almeida et al., 2013). Because of this immense economic impact, over $1.3 billion is spent yearly on rodent control strategies, including rodenticide baits that contain lethal compounds such as. While in many cases rodenticides, including warfarin or similar anticoagulants, are an inexpensive and fairly efficient tool for pest rodent control, they have significant limitations. For example, many rodents exhibit significant neophobia, reducing the likelihood that a rodent will approach and consume baits (Baker et al., 2007). Also, some rodenticides must be consumed repeatedly over several days in order to reach lethal levels; if rodents become ill after the initial bait consumption, they may associate this feeling with the bait and not return for subsequent feedings (Baker et al., 2007). Unfortunately, the effectiveness of natural and biological attractants to aid the return of rodents to bait stations has been difficult to determine. For example, studies showing the use of natural odors (lemon and ginger) showed promise when rats were tested in enclosures, but failed to work in field studies (Witmer et al., 2008). Therefore, new strategies are needed to enhance the acceptance and ingestion of edible baits in a species-specific manner, thus increasing bait effectiveness and reducing the potential for baits to be ingested by non-target species. The ability of semiochemicals such as guanylin-family peptides to engage innate preference mechanisms in target rodents may offer an opportunity to safely and efficiently promote bait ingestion by these animals, thus reducing the huge economic and health costs associated with rodent pests.

Acknowledgments

These studies were supported by the National Institute on Deafness and Other Communication Disorders (DC005633) and by the TEDCO Maryland Innovators Initiative.

References

Almeida, A., Corrigan, R., and Sarno, R. (2013). The economic impact of commensal rodent on small businesses in Manhattan's Chinatown: trens and possible causes. *Surburban Sustainability* 1, 2. doi: 10.5038/2164-0866.1.1.2

Arakawa, H., Kelliher, K. R., Zufall, F., and Munger, S. D. (2013). The receptor Guanylyl Cyclase type D (GC-D) ligand uroguanylin promotes the acquisition of food preferences in mice. *Chem. Senses* 38, 391–397. doi: 10.1093/chemse/bjt015

Baker, S., Singleton, G., and Smith, R. (2007). *The Nature of the Beast: Using Biological Processes in Vertebrate Pest Management*. Oxford: Blackwell Publishing.

Cibils, A. F., Howery, L. D., and Ruyle, G. B. (2008). Social models fail to induce diet and feeding site avoidance in naive yearling steers. *Animal* 2, 255–264. doi: 10.1017/S1751731107001437

Countryman, R. A., Orlowski, J. D., Brightwell, J. J., Oskowitz, A. Z., and Colombo, P. J. (2005). CREB phosphorylation and c-Fos expression in the hippocampus of rats during acquisition and recall of a socially transmitted food preference. *Hippocampus* 15, 56–67. doi: 10.1002/hipo.20030

Crawley, J. N. (2007). Mouse behavioral assays relevant to the symptoms of autism. *Brain Pathol.* 17, 448–459. doi: 10.1111/j.1750-3639.2007.00096.x

Ferrero, D. M., Moeller, L. M., Osakada, T., Horio, N., Li, Q., Roy, D. S., et al. (2013). A juvenile mouse pheromone inhibits sexual behaviour through the vomeronasal system. *Nature* 502, 368–371. doi: 10.1038/nature12579

Galef, B. G. Jr. (1985). Direct and indirect behavioral pathways to the social transmission of food avoidance. *Ann. N.Y. Acad. Sci.* 443, 203–215. doi: 10.1111/j.1749-6632.1985.tb27074.x

Galef, B. G. (2012). A case study in behavioral analysis, synthesis and attention to detail: social learning of food preferences. *Behav. Brain Res.* 231, 266–271. doi: 10.1016/j.bbr.2011.07.021

Galef, B. G. Jr., Attenborough, K. S., and Whiskin, E. E. (1990). Responses of observer rats (*Rattus norvegicus*) to complex, diet-related signals emitted by demonstrator rats. *J. Comp Psychol.* 104, 11–19. doi: 10.1037/0735-7036.104.1.11

Galef, B. G. Jr., and Beck, M. (1985). Aversive and attractive marking of toxic and safe foods by Norway rats. *Behav. Neural Biol.* 43, 298–310. doi: 10.1016/S0163-1047(85)91645-0

Galef, B. G. Jr., Mason, J. R., Preti, G., and Bean, N. J. (1988). Carbon disulfide: a semiochemical mediating socially-induced diet choice in rats. *Physiol. Behav.* 42, 119–124. doi: 10.1016/0031-9384(88)90285-5

Galef, B. G. Jr., and Whiskin, E. E. (2003). Socially transmitted food preferences can be used to study long-term memory in rats. *Learn. Behav.* 31, 160–164. doi: 10.3758/BF03195978

Galef, B. G. Jr., Wigmore, S. W., and Kennett, D. J. (1983). A failure to find socially mediated taste aversion learning in Norway rats (*R. norvegicus*). *J. Comp. Psychol.* 97, 358–363. doi: 10.1037/0735-7036.97.4.358

Haga, S., Hattori, T., Sato, T., Sato, K., Matsuda, S., Kobayakawa, R., et al. (2010). The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. *Nature* 466, 118–122. doi: 10.1038/nature09142

Jing, L., Zhou, Q. X., and Xu, L. (2014). Avoidance of potentially harmful food cannot be socially transmitted between rats. *Zool. Res.* 35, 256–261. doi: 10.13918/j.issn.2095-8137.2014.4.256

Kimoto, H., Sato, K., Nodari, F., Haga, S., Holy, T. E., and Touhara, K. (2007). Sex- and strain-specific expression and vomeronasal activity of mouse ESP family peptides. *Curr. Biol.* 17, 1879–1884. doi: 10.1016/j.cub.2007.09.042

Leinders-Zufall, T., Cockerham, R. E., Michalakis, S., Biel, M., Garbers, D. L., Reed, R. R., et al. (2007). Contribution of the receptor guanylyl cyclase GC-D to chemosensory function in the olfactory epithelium. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14507–14512. doi: 10.1073/pnas.0704965104

Maier, J. X., Blankenship, M. L., Barry, N. C., Richards, S. E., and Katz, D. B. (2014). Stability and flexibility of the message carried by semiochemical stimuli, as revealed by devaluation of carbon disulfide followed by social transmission of food preference. *Behav. Neurosci.* 128, 413–418. doi: 10.1037/bne0000002

Mason, J. R., Arzt, A. H., and Reidinger, R. F. (1984). Comparative-assessment of food preferences and aversions acquired by blackbirds via observational-learning. *Auk* 101, 796–803. doi: 10.2307/4086906

Masuda, A., and Aou, S. (2009). Social transmission of avoidance behavior under situational change in learned and unlearned rats. *PLoS ONE* 4:e6794. doi: 10.1371/journal.pone.0006794

Munger, S. D., Leinders-Zufall, T., McDougall, L. M., Cockerham, R. E., Schmid, A., Wandernoth, P., et al. (2010). An olfactory subsystem that detects carbon disulfide and mediates food-related social learning. *Curr. Biol.* 20, 1438–1444. doi: 10.1016/j.cub.2010.06.021

Pfister, J. A., and Price, K. W. (1996). Lack of maternal influence of lamb consumption of locoweed (*Oxytropis sericea*). *J. Anim. Sci.* 74, 340–344.

Pimentel, D., Lach, L., Zuniga, R., and Morrison, D. (2000). Environmental and economic costs of nonindigenous species in the United States. *Bioscience* 50, 53–65. doi: 10.1641/0006-3568(2000)050[0053:EAECON]2.3.CO;2

Posadas-Andrews, A., and Roper, T. J. (1983). Social transmission of food preference in adult rats. *Anim. Behav.* 31, 265–271. doi: 10.1016/S0003-3472(83)80196-1

Ralphs, M. H., and Olsen, J. D. (1990). Adverse influence of social facilitation and learning context in training cattle to avoid eating larkspur. *J. Anim. Sci.* 68, 1944–1952.

Ross, R. S., and Eichenbaum, H. (2006). Dynamics of hippocampal and cortical activation during consolidation of a nonspatial memory. *J. Neurosci.* 26, 4852–4859. doi: 10.1523/JNEUROSCI.0659-06.2006

Ryan, B. C., Young, N. B., Moy, S. S., and Crawley, J. N. (2008). Olfactory cues are sufficient to elicit social approach behaviors but not social transmission of food preference in C57BL/6J mice. *Behav. Brain Res.* 193, 235–242. doi: 10.1016/j.bbr.2008.06.002

Smith, C. A., Countryman, R. A., Sahuque, L. L., and Colombo, P. J. (2007). Time-courses of Fos expression in rat hippocampus and neocortex following acquisition and recall of a socially transmitted food preference. *Neurobiol. Learn. Mem.* 88, 65–74. doi: 10.1016/j.nlm.2007.03.001

Spehr, M., Kelliher, K. R., Li, X. H., Boehm, T., Leinders-Zufall, T., and Zufall, F. (2006). Essential role of the main olfactory system in social recognition of major histocompatibility complex peptide ligands. *J. Neurosci.* 26, 1961–1970. doi: 10.1523/JNEUROSCI.4939-05.2006

Thorhallsdottir, A. G., Provenza, F. D., and Balph, D. F. (1990). Ability of lambs to learn about novel foods while observing or participating with social models. *Appl. Anim. Behav. Sci.* 25, 25–33. doi: 10.1016/0168-1591(90)90066-M

Valsecchi, P., and Galef, B. G. Jr. (1989). Social influences of the food preferences of house mice (*Mus musculus*). *Int. J. Comp. Psychol.* 2, 245–256.

Witmer, G. W., Burke, P. W., and Jojola, S. (2008). *An Evaluation of the Effectiveness of Potential Norway Rat Attractants*. Davis, CA: University of California.

Zhang, J., Pacifico, R., Cawley, D., Feinstein, P., and Bozza, T. (2013). Ultrasensitive detection of amines by a trace amine-associated receptor. *J. Neurosci.* 33, 3228–3239. doi: 10.1523/JNEUROSCI.4299-12.2013

Zufall, F., and Munger, S. D. (2010). Receptor guanylyl cyclases in mammalian olfactory function. *Mol. Cell. Biochem.* 334, 191–197. doi: 10.1007/s11010-009-0325-9

Conflict of Interest Statement: Steven D. Munger is an author of a PCT/US patent application describing the use of guanylin peptides to promote edible ingestion by rodents. Neither author receives or has received personal financial benefit (e.g., royalties) associated with this patent or these studies. Neither institution or laboratory has received any corporate or private funding related to these studies; these studies were entirely supported by the National Institutes of Health and the State of Maryland. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 02 October 2015
doi: 10.3389/fnins.2015.00342

The entorhinal cortex is involved in conditioned odor and context aversions

Barbara Ferry[1], Karine Herbeaux[2], Hervé Javelot[3] and Monique Majchrzak[2]*

[1] Centre of Research in Neuroscience, CNRS UMR 5292 - INSERM U1028 - UCBL1, Lyon, France, [2] Laboratoire de Neurosciences Cognitives et Adaptatives, UMR 7364, Faculté de Psychologie, CNRS, Université de Strasbourg, Neuropôle de Strasbourg, GDR 2905 du CNRS, Strasbourg, France, [3] Etablissement Public de Santé Alsace Nord Brumath – Service Pharmacie - CHU de Strasbourg, Hôpital Civil, Clinique de Psychiatrie – Service de Psychiatrie II, Brumath, France

In a natural environment, avoidance of a particular food source is mostly determined by a previous intake experience during which sensory stimuli such as food odor, become aversive through a simple associative conditioned learning. Conditioned odor aversion learning (COA) is a food conditioning paradigm that results from the association between a tasteless scented solution (conditioned stimulus, CS) and a gastric malaise (unconditioned stimulus, US) that followed its ingestion. In the present experimental conditions, acquisition of COA also led to acquisition of aversion toward the context in which the CS was presented (conditioned context aversion, CCA). Previous data have shown that the entorhinal cortex (EC) is involved in the memory processes underlying COA acquisition and context fear conditioning, but whether EC lesion modulates CCA acquisition has never be investigated. To this aim, male Long-Evans rats with bilateral EC lesion received CS-US pairings in a particular context with different interstimulus intervals (ISI). The results showed that the establishment of COA with long ISI obtained in EC-lesioned rats is associated with altered CCA learning. Since ISI has been suggested to be the determining factor in the odor- and context-US association, our results show that the EC is involved in the processes that control both associations relative to ISI duration.

Keywords: entorhinal cortex, odor aversion, context aversion, food conditioning, odors

OPEN ACCESS

Edited by:
Markus Fendt,
Otto-von-Guericke University
Magdeburg, Germany

Reviewed by:
Jimmy Stehberg,
Universidad Andres Bello, Chile
Yasushi Kiyokawa,
The University of Tokyo, Japan

****Correspondence:***
Barbara Ferry,
CRNL, CNRS UMR 5292 - INSERM U1028 - UCBL1, 50 Avenue Tony Garnier, 69366 Lyon Cedex 07, France
barbara.ferry@cnrs.fr

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *02 July 2015*
Accepted: *09 September 2015*
Published: *02 October 2015*

Citation:
Ferry B, Herbeaux K, Javelot H and Majchrzak M (2015) The entorhinal cortex is involved in conditioned odor and context aversions. Front. Neurosci. 9:342. doi: 10.3389/fnins.2015.00342

Introduction

Finding adequate food sources', including water, is one of the most fundamental aspects of animal life. Guided by various kinds of stimuli present in the environment, animals move through space toward a resource goal, avoiding unsafe environments and the risk of themselves becoming food. This behavior involves the animal finding and recognizing particular cues, whether contingent, in a specific environment, or coming directly from the food source, that indicate the nearness and direction of the goal. Although innate, a lot of encounters with cue stimuli result in learned approaches or avoidances. In this situation, the capacity to anticipate the future and differentiate between safe or unsafe food items depends, at least in part, on previous experience in which the sensory stimuli characterizing a particular food (odor and taste) become associated with the positive (energy input) or negative (gastric malaise, poisoning)

consequences of ingestion through Pavlovian associative learning (e.g., Mehiel and Bolles, 1984; Capaldi et al., 1987; Fedorchak and Bolles, 1987; Harris et al., 2000). These kinds of association have been largely studied (Slotnick and Katz, 1974; Nigrosh et al., 1975; Slotnick, 1984) and the use of conditioned food aversion paradigms in research has provided fundamental insights into the brain mechanisms and structures involved in food-reward/food-poisoning associations (see Miranda, 2012 for a review). One such paradigm, conditioned odor aversion (COA), consists in avoidance of an odorized-tasteless solution (conditioned stimulus, CS) the ingestion of which precedes toxicosis (unconditioned stimulus, US). COA is a robust and long-lasting learned association that can be obtained with a single CS-US pairing (Lorden et al., 1970; Hankins et al., 1973; Taukulis, 1974), and with CS-US delays (interstimulus interval, ISI) ranging from minutes to hours depending on whether the CS is mixed (proximal presentation, inducing orthonasal, and retronasal stimulations: Slotnick et al., 1997; Chapuis et al., 2007) or presented close to the solution (distal presentation, inducing orthonasal stimulation: Garcia et al., 1966; Andrews and Braveman, 1975; Palmerino et al., 1980; Bouton et al., 1986; Ferry et al., 1996, 2006).

Many studies of the mechanisms and systems involved in the memory processes underlying COA learning have focused exclusively on conditioned responses toward the CS. In particular, some showed that the entorhinal cortex (EC) plays an important role in task acquisition. The EC is a parahippocampal structure that receives important olfactory projections (Krettek and Price, 1977; Haberly and Price, 1978; Burwell and Amaral, 1998) and is reciprocally connected to the hippocampus and amygdala (e.g., Amaral and Witter, 1995; Ferry et al., 1997; Pitkänen et al., 2000). In addition, electrophysiological data suggest that the processing of olfactory information in both these regions is modulated by the EC (e.g., Biella and de Curtis, 2000; Gnatkovsky et al., 2004; Mouly and Di Scala, 2006). Interestingly, we found that rats with EC lesions were able to learn to avoid an odor paired with toxicosis, using both distal and proximal CS presentation, even when the ISI was too long (120 min) for such learning to be observed in control animals (Ferry et al., 1996, 1999, 2006, 2007). Further experiments suggested that this facilitation of COA with long ISI may be the consequence of lesion-induced suppression of an inhibitory influence of the EC on brain areas involved in olfactory information processing, such as the basolateral amygdala (BLA; Ferry and Di Scala, 1997; Ferry et al., 1999; Mouly and Di Scala, 2006). Also and non-exclusively, persistence of the odor memory trace supporting COA with long ISI in EC-lesioned rats may involve altered processing of other cues present during conditioning. In concordance with this, several studies have shown that the EC is involved in the processing of information related to the context in which the CS-US association was established, mainly during fear conditioning (Maren and Fanselow, 1997; Ji and Maren, 2008; Majchrzak et al., 2006; but: Phillips and LeDoux, 1995; Good and Honey, 1997; Bannerman et al., 2001; Hales et al., 2014). However, although contextual cues contingent to a specific environment are indicators that also contribute to the behavioral response toward a food source, the relationship between contextual and odor cues during conditioned food aversion learning has been little studied. Context aversion has be-12mmen shown to occur concurrently to COA (Hatfield et al., 1992), but whether EC lesion modulates context aversion has never be investigated during COA.

The aim of the present experiment was to assess whether the establishment of COA with long ISI in EC-lesioned rats was associated with altered contextual information processing. To this end, acquisition of COA and conditioned context aversion (CCA) were tested in EC-lesioned animals using two different procedures. The first (COA), consisted in presenting an olfactory cue (odorized water, CS) in a particular context that was followed by LiCl-induced gastric malaise (US), with short, or long ISI. This forward arrangement between CS and context has previously been shown to result in odor and context aversion (Hatfield et al., 1992). The second (CCA), consisted in administering the US after the animals had been placed in a particular context, with the same short and long ISIs. In this procedure, the CS was presented after the US at the end of the session. This backward arrangement between the two stimuli resulted in acquisition of context aversion but not in COA.

Materials and Methods

Subjects

One hundred and two male Long-Evans rats (supplied from Janvier Labs, Le Genest-St-Isles France; weighing 250–275 g) were used. They were housed two per cage in transparent Makrolon cages (42 × 26 × 15 cm) under controlled temperature (22°C ± 2) and standard 12 h light/dark cycle (lights from 7:00 a.m. to 7:00 p.m.) in a colony room. The animals were provided with *ad libitum* access to food and water. After arrival, the animals were allowed to acclimate to the laboratory conditions for a period of 1 week before surgery.

All procedures involving animals and their care conformed to the institutional guidelines, which comply with international laws and policies (directive 2010/63/European Community) and have been approved by the ethics committee of the Université Claude Bernard Lyon 1 (CE2A-55). Permission references were 69–387517 for BF and 67–289 for MM. All other co-authors were under the responsibility of the former.

Surgery

All surgical procedures were conducted under optimal aseptic, analgesic, and ethical animal care conditions (see Ferry et al., 2014) by those authorized to do so. Rats were anesthetized by i.p. injection of a mix of ketamine (100 mg/kg)/xylasine (10 mg/kg). Following a prophylactic antibiotic treatment (penicillin 0.12 M.U./0.3 ml, i.m.) the rats were given bilateral lesions of the EC by aspiration ($n = 56$) as previously described (Ferry et al., 1999). Sham-lesioned animals were operated similarly but no aspiration was carried out ($n = 46$). Four animals died after surgery (three EC-lesioned and one sham-lesioned). All subjects recovered for 7–11 days after surgery with *ad libitum* access to food and water, and were singly housed until the end of the experiment.

Test Chambers

Habituation Chambers

Eight chambers (25 × 30 × 35 cm) located in a room adjacent to the vivarium were used for habituation to water consumption. They were made of clear perspex and had a wire mesh floor. The spout of a 25-ml glass tube (Richter tube, Strasbourg, France) could be introduced into the cage through a circular hole on the anterior wall of the chamber, located 2 cm above the floor. Intake was measured by reading to the nearest 0.5 ml, the level of liquid of the tube before and after each session.

Conditioning and Testing Chambers

Four place preference boxes located in a room adjacent to the vivarium were used. Each box was constituted by two large compartments of similar size (45 × 45 × 30 cm, compartments A and B) with distinctive visual and tactile features and a third smaller ship-wooden gray painted transit compartment (36 × 18 × 30 cm) that allowed animals to move between compartments A and B when the sliding door of their back wall was open. Compartment A had three wooden black walls and a floor made of tight and flexible wire mesh. Compartment B had three wooden walls with vertical black and white stripes and a floor made of large and rigid wire mesh. The front wall of both compartments was constituted by clear perspex. The spout of the Richter tube could be introduced into each compartment through a circular hole on the back wall of the chamber, located 2 cm above the floor.

Behavioral Procedure

All experimental sessions were carried out during the light portion of the cycle between 11:00 a.m. and 1:00 p.m. After post-surgical recovery, animals were handled (3 min/day) for 3 days and weighed daily to verify their adaptation to the deprivation schedule.

On the first day (Day 1), each animal was placed in the transit compartment of the place preference box with sliding doors open, and allowed to move freely in all compartments during a 15 min session. The time spent in each compartment was recorded. The compartment in which the animals spent more time was chosen as the conditioning context, and the other one was chosen as the neutral context.

Water bottles were removed from the home cage in the evening of Day 1 and a 23 h 45 min water deprivation schedule was initiated. During the water drinking habituation sessions (Day 2 to Day 6), rats had access to water once a day according to the following procedure: On Day 2 and 3 the animals had access to water in their home cage for 30 min on Day 2 and for 15 min on Day 3. From Day 4 to Day 6, animals had access to water for 15 min in the habituation chamber.

On Day 7, each animal was placed in the neutral context equipped with a Richter tube and had access to water for 15 min. Then, the Richter tube was removed and the animal received an i.p. injection of 0.9% NaCl (10 ml/kg). Animals spent an additional period of 60 min in the neutral context.

Conditioning session took place on Day 8 (**Figure 1**). Animals were divided in two experimental groups according to the conditioning procedure (adapted from Desmedt et al., 2003). Animals of COA groups were placed in the conditioning context where they had access for 15 min to odorized water in the Richter tube (CS; 0.01% isoamyl acetate solution). At the end of the 15 min, the Richter tube was removed and animals received an i.p. injection of a lithium chloride (LiCl) inducing gastric malaise (US; 0.15 M; 10 ml/kg) either 5 min (short ISI; COA 5 group) or 120 min (long ISI; COA 120 group) after the removal of the Richter tube. Animals spent the rest of the session in the conditioning context (total duration = 215 min).

Animals of the CCA groups were placed in the conditioning context and received an i.p. injection of LiCl at a timing similar to groups COA (either 20 or 140 min after the beginning of the session, referred successively as CCA 5 and CCA 120 groups for convenience). They spent the rest of the 215 min session in the same compartment and had access to the Richter tube filled with

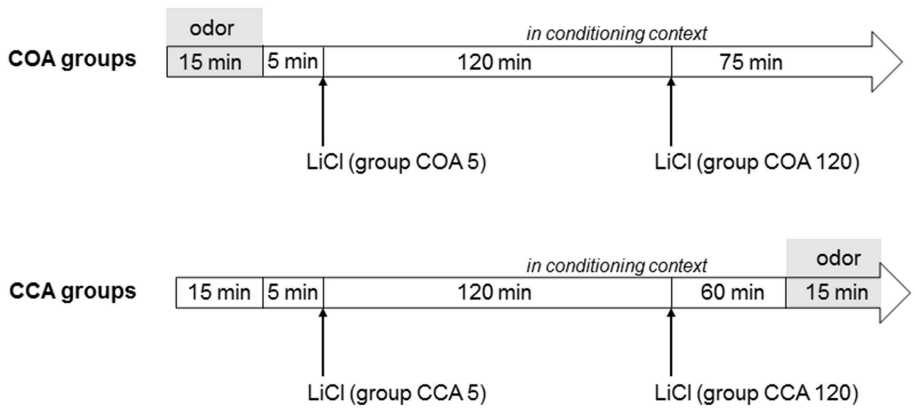


FIGURE 1 | Time schedule of the conditioning session for COA and CCA groups. In COA groups, the animals were subjected to presentation of the odor (symbolized by the gray area) in the conditioning context followed by the US with a short (5 min) or a long (120 min) interstimulus interval (COA 5 and COA 120 groups, respectively). The animals spent the rest of the session in the conditioning context. In CCA groups, the animals were placed in the conditioning context for 15 min and then received the US with a short (5 min) or long (120 min) interstimulus interval (CCA 5 and CCA 120 groups, respectively). The animals spent the rest of the session in the conditioning context in which the odor was presented during the last 15 min of the session (symbolized by the gray area). The total duration of the session was 215 min.

0.01% isoamyl acetate only during the last 15 min of the session. Intake of odorized water was measured by reading the level of liquid on the tube before and after the session in both COA and CCA groups.

EC-lesioned (EC) and sham-lesioned (Sham) rats were randomly assigned to one of these four conditioning procedures. Experimental groups were constituted as follows: COA 5 (EC: $n = 13$; Sham: $n = 11$), COA 120 (EC: $n = 14$; Sham: $n = 10$), CCA 5 (EC: $n = 13$; Sham: $n = 13$), and CCA 120 min (EC: $n = 13$; Sham: $n = 11$).

Conditioned aversions were assessed on Day 9 in the place preference box. To do this, animals were confined in the neutral context with access to the Richter tube filled with 0.01% isoamyl acetate for 15 min. At the end of the session, they returned to their home cages and the amount of liquid consumed during testing was measured. One hour after, animals were placed in the transit compartment with sliding doors open giving access to the two compartments A and B. Time spent in each compartment was measured during 30 min.

Histology

Ten days after completion of behavioral testing, each rat was given an overdose of sodium pentobarbital (100 mg/kg) and was transcardially perfused with 60 ml of saline (4°C) followed by phosphate-buffered 4% paraformaldehyde (pH 7.4; 4°C). The brain was then extracted, post-fixed for 4 h in the same fixative (4°C) and transferred into a 0.1 M phosphate-buffered 20% sucrose solution for about 36–40 h (4°C). All brains were frozen using isopentane (−40°C). Coronal sections, 30 μm, were cut on a freezing microtome (−23°C), and collected onto gelatine-coated slides. These sections were dried at room temperature and stained with cresyl violet. A microscopic inspection was then performed to determine the location and the extent of the lesions.

Data Analysis

Odor aversion was assessed by comparing the volume of odorized solution intake during the conditioning and testing sessions. Context aversion was assessed by comparing the proportion of time spent in the conditioning context before (pre-conditioning session) and after (testing session) conditioning. For each ISI, the data corresponding to the volumes (for COA) and the ratios (time spent in conditioning context/time of the session) were analyzed with a Three-Way repeated measures ANOVA with Lesion (EC vs. sham), Type of procedure (COA vs. CCA) as between subject factors and Session (conditioning vs. testing for COA assessment, pre-conditioning vs. testing for CCA assessment) as within subject factor. *Post-hoc* Newman-Keuls multiple range test (NK) was used to determine the source of detected significances in the ANOVAs. A probability level of <0.05 was accepted as statistically significant throughout.

Results

Histology

Seventeen EC-lesioned animals (with unilateral damage in the EC or extensive lesions of surrounding structures such as the perirhinal cortex, subiculum, or dentate gyrus) and four sham-lesioned animals with damage to the lateral part of the EC (resulting from the insertion of the curved needle) were excluded from the analysis. Final groups were constituted as follows: COA 5 (EC: $n = 9$; Sham: $n = 9$), COA 120 (EC: $n = 10$; Sham: $n = 9$), CCA 5 (EC: $n = 9$; Sham: $n = 13$), and CCA 120 min (EC: $n = 8$; Sham: $n = 10$).

Lesions were drawn from transversal sections stained with cresyl violet. Typical (i.e., smallest and largest) lesion extents observed in COA and CCA groups are illustrated in **Figure 2**. Histological analysis revealed that all the lesions included the lateral EC at the levels −5.60 to −8.30 from Bregma and a part of the medial EC at Bregma −7.80. The largest lesions were estimated to damage a part of the perirhinal cortex at the levels −6.30 to −7.80 mm.

Behavior

Short ISI-induced Conditioned Odor and Context Aversions

The results obtained in the experimental groups conditioned with the short ISI are shown in **Figure 3**. **Figure 3A** represents the mean odorized water intakes (± S.E.M.) measured during the conditioning and testing sessions whereas **Figure 3B** represents the mean proportion of time (± S.E.M.) spent in the conditioning context during the pre-conditioning and testing sessions. As shown in **Figure 3A**, the amount of odorized water intake during conditioning was similar between groups indicating that neither the lesion nor the early intoxication in CCA 5 groups (i.e., 180 min before) had an impact on intake. During testing the amount of odorized water intake was lower in animals exposed to the CS before intoxication, whatever the type of lesion. This indicated that short ISI lead to a COA when the odor is encountered before but not after intoxication. Statistical analyses confirmed these observations and revealed no significant effect of Lesion [$F_{(1, 36)} = 0.14$, p n.s.], a significant effect of Type of procedure [$F_{(1, 36)} = 55.37$, $p < 0.0001$], a significant effect of Session [$F_{(1, 36)} = 39.39$, $p < 0.0001$], and a significant interaction between Session and Type of procedure [$F_{(1, 36)} = 36.54$, $p < 0.0001$]. *Post-hoc* comparisons confirmed that, in both sham- and EC-lesioned COA 5 groups, the odorized water intake during testing was significantly lower than during conditioning ($p < 0.001$ in each case), and also lower than the odorized water intake measured in CCA 5 groups during testing ($p < 0.001$ in each case).

As shown in **Figure 3B**, the proportion of time spent in the conditioning context during pre-conditioning was similar between groups. In sham-lesioned animals, the proportion of time spent in the conditioning context during testing was lower than before conditioning whatever the type of procedure. This decrease indicated that short ISI lead to a CCA and that animals had associated the context with the US, whether exposed or not to the odorized water. In contrast, the time spent in the conditioning context did not differ between pre-conditioning and testing sessions in EC-lesioned groups, indicating that the lesion affected CCA. The ANOVA confirmed these observations and showed significant effects of Lesion [$F_{(1, 36)} = 15.13$, $p < 0.001$] and Session [$F_{(1, 36)} = 20.00$, $p < 0.0001$] but no effect of Type of procedure [$F_{(1, 36)} = 1.15$, p n.s.], and a significant Session X Lesion interaction [$F_{(1, 36)} = 16.52$, $p < 0.0001$]. *Post-hoc* comparisons confirmed that the proportion

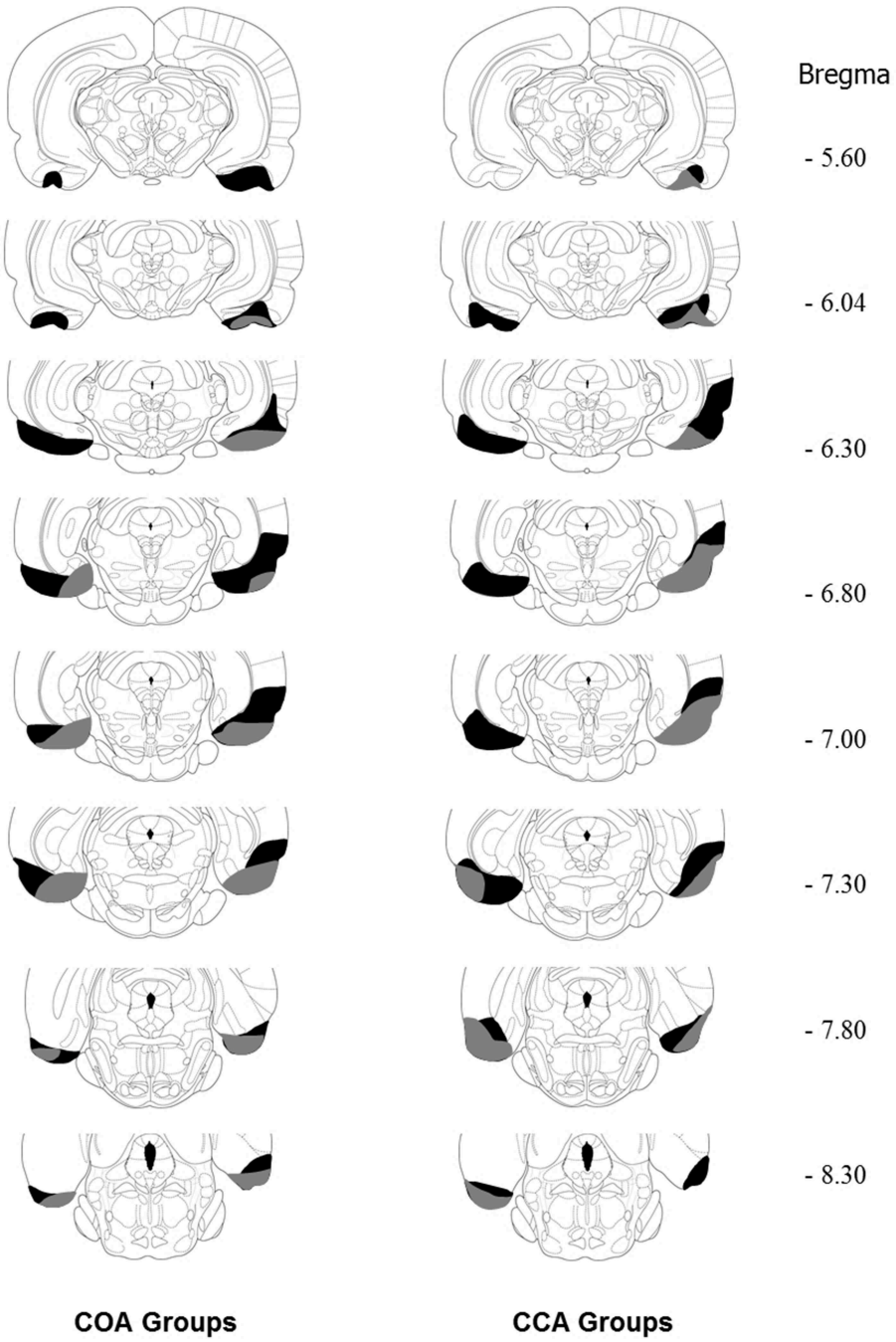


FIGURE 2 | Diagram of coronal sections (between −5.60 and −8.30 mm from bregma; according to the Paxinos and Watson stereotaxic atlas, 1998) showing the extent of the smallest (gray area) and the largest (black area) EC lesion in the COA and CCA groups. From *The Rat Brain in Stereotaxic Coordinates*, by Paxinos and Watson (1998). Copyright 2006 by Elsevier. Adapted with permission.

of time spent in the conditioning context during testing was lower than before conditioning in both sham-lesioned groups ($p < 0.05$ for COA group and $p < 0.001$ for CCA 5 group), but not in EC-lesioned groups. The proportion of time spent in the conditioning context during testing in sham-lesioned groups was also lower than the proportion of time spent in the conditioning context in EC-lesioned groups ($p < 0.001$ for sham COA 5 vs. EC COA 5 and $p < 0.001$ for sham CCA 5 vs. EC CCA 5).

Long ISI-induced Conditioned Odor and Context Aversions

The results obtained in the experimental groups conditioned with the long ISI are shown in **Figure 4**. **Figure 4A** represents the

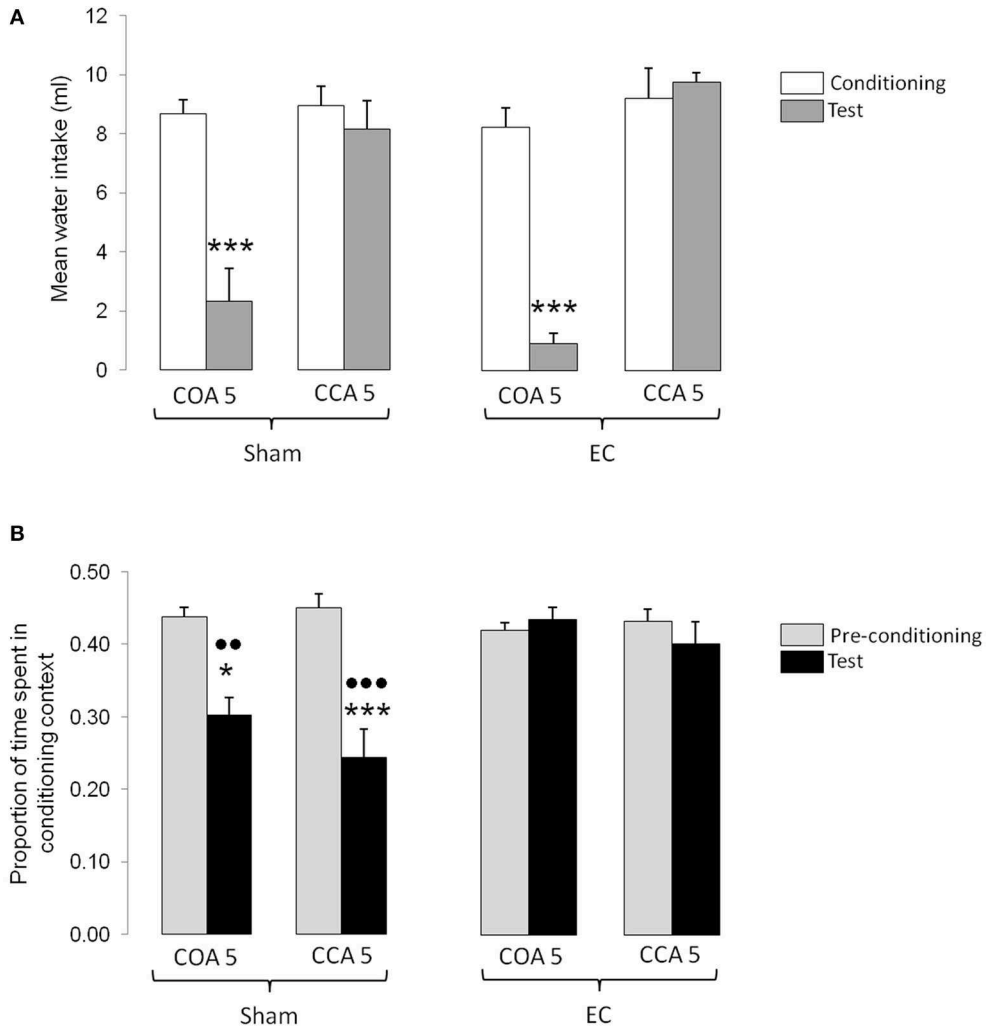


FIGURE 3 | Effect of a short ISI on COA and CCA in Sham- and EC-lesioned groups. (A) Represents the mean odorized water intakes (± S.E.M.) measured during the conditioning (white bars) and testing (gray bars) sessions in each experimental group. ***$p < 0.001$ as compared with the amount of odorized water intake during conditioning in the same group and with the amount of odorized water intake during testing in the corresponding CCA group. **(B)** represents the mean proportion of time (± S.E.M.) spent in the conditioning context for each experimental group. Bars represent the proportion of time spent in the conditioning context (mean time spent in the conditioning context/time of the session) calculated in the pre-conditioning (gray bars) and testing (black) sessions. *, ***$p < 0.05$ and 0.001 as compared with the proportion of time spent in the conditioning context during pre-conditioning in the same group;●●, ●●●$p < 0.01$ and 0.001 as compared with the proportion of time spent in the conditioning context during testing in the corresponding EC-lesioned-group. EC, lesion of the entorhinal cortex; Sham, sham-lesion of the entorhinal cortex; COA, conditioned odor aversion; CCA, conditioned context aversion.

mean water intakes (± S.E.M.) measured during conditioning and testing sessions whereas **Figure 4B** represents the mean proportion of time (± S.E.M.) spent in the conditioning context during the pre-conditioning and testing sessions. As shown in **Figure 4A**, the amount of odorized water intake during conditioning was similar in COA 120 groups indicating that the lesion did not affect the level of odorized water intake when the US followed its presentation. However, the amount of odorized water intake during conditioning was lower in CCA 120 groups suggesting an effect of the US when it was administered 60 min before the odorized water presentation. The higher amount of odorized water intake measured in the CCA 120 groups during testing (without US) confirmed this observation. As also shown in **Figure 4A**, the amount of odorized water intake between conditioning and testing was similar in Sham-lesioned COA 120 group. In contrast, the amount of odorized water decreased between conditioning and testing in EC-lesioned group thus indicating that animals associated the odor with the US with a long ISI. Statistical analyses confirmed these observations and revealed a significant effect of Lesion [$F_{(1, 33)} = 4.79$, $p < 0.05$], a significant interaction between Lesion and Type of procedure [$F_{(1, 33)} = 6.13$, $p < 0.05$], and a significant interaction between Lesion, Type of procedure and Session [$F_{(1, 33)} = 5.97$, $p < 0.05$]. *Post-hoc* comparisons confirmed that odorized water

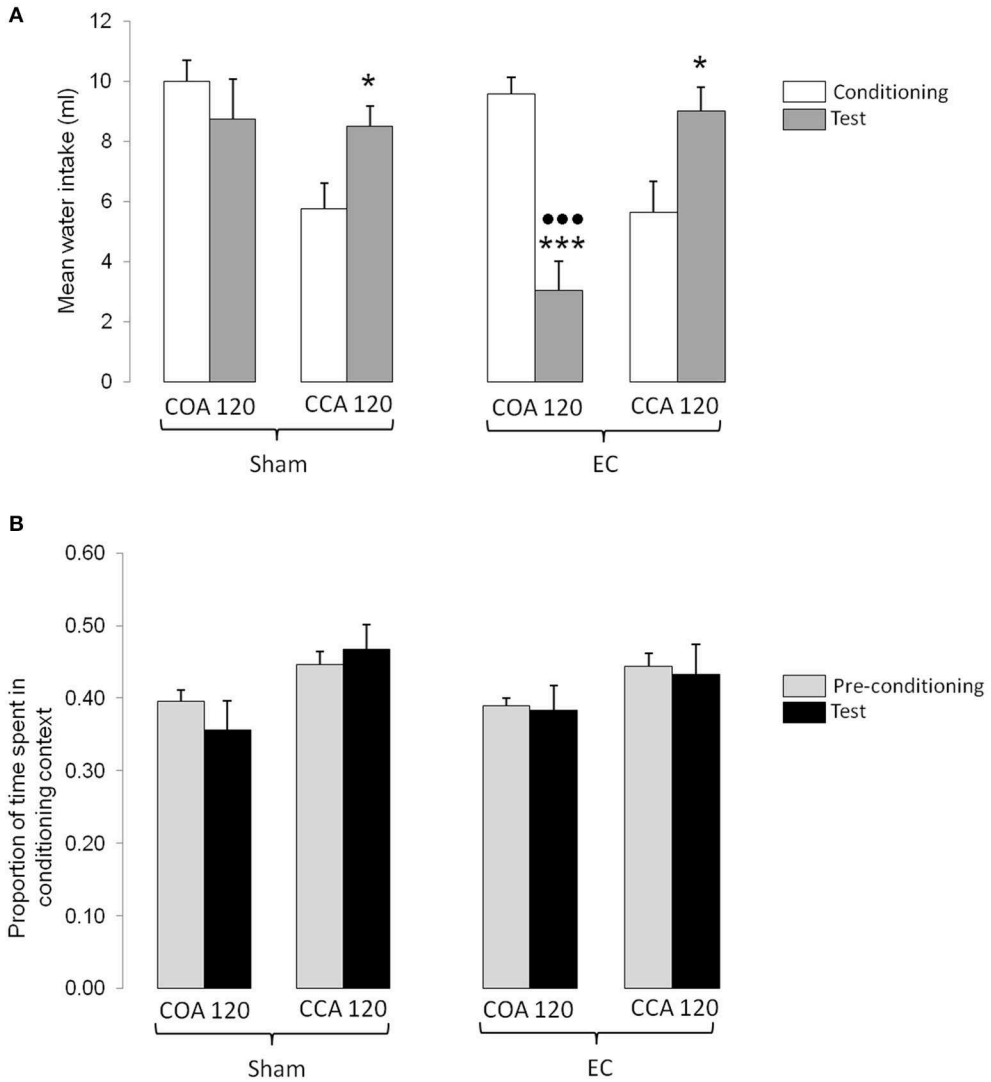


FIGURE 4 | Effect of a long ISI on COA and CCA in Sham- and EC-lesioned groups. (A) Represents the mean odorized water intakes (± S.E.M.) measured during the conditioning (white bars) and testing (gray bars) sessions in each experimental group. *, ***$p < 0.05$ and 0.001 as compared with the amount of odorized water intake during conditioning; ●●● $p < 0.001$ as compared with the amount of odorized water intake during testing in all the other groups. **(B)** represents the mean proportion of time (± S.E.M.) spent in the conditioning context for each experimental group. Bars represent the proportion of time spent in the conditioning context (mean time spent in the conditioning context/time of the session) calculated in the pre-conditioning (gray bars) and testing (black) sessions. EC, lesion of the entorhinal cortex; Control, sham-lesion of the entorhinal cortex; COA, conditioned odor aversion; CCA, conditioned context aversion.

intake was significantly lower during conditioning than during testing in CCA 120 groups ($p < 0.05$) and that the amount of odorized water intake was lower during testing than during conditioning in EC-lesioned COA 120 group ($p < 0.001$). *Post-hoc* comparisons also indicated that, in EC-lesioned COA 120 group, the amount of odorized water intake during testing was lower than in all the other groups ($p < 0.001$ for each comparison).

As shown in **Figure 4B**, the proportion of time spent in the conditioning context was similar before conditioning and testing in both COA and CCA 120 groups, irrespective of the type of lesion. This suggests that no CCA occurred when the US was administered more than 120 min after the beginning of the context exposure. **Figure 4B** also showed that the proportion of time spent in the conditioning context seemed higher in CCA than in COA groups, indicative of an initial and maintained higher preference for the conditioning context in CCA 120 groups, as compared to COA 120 groups. Statistical analysis confirmed this observation and revealed a significant effect of Type of procedure [$F_{(1, 33)} = 10.42$, $p < 0.01$], but not of the other factors nor of any interaction.

Discussion

The results of the present study show that EC lesions induced a deficit in CCA but did not disrupt COA learning; on the contrary,

EC-lesioned animals were able to associate the CS with the US even though the ISI was too long to enable sham-lesioned control animals to learn the task. Moreover, the establishment of COA with long ISI obtained in EC-lesioned rats was associated with altered CCA learning.

Sham-lesioned control animals in the CCA group did not display COA with the two intervals tested between LiCl injection and odorized water exposure. In addition, only sham-lesioned control animals that received the US 20 min after the beginning of context exposure (CCA 5) displayed CCA, thus confirming previous findings (Desmedt et al., 2003). These results show that a backward arrangement between CS and US (i.e., no competition between CS and context) leads to CCA but not to COA. Moreover, odorized water intake in the CCA 120 groups was significantly lower than in CCA 5 groups in both sham- and EC-lesioned animals. This result suggests that LiCl affected odorized water intake when injected 60 min before presentation (in CCA 120 groups) but not when it was administered 180 min later (in CCA 5 groups). This temporary interference between LiCl and odorized water intake might reflect a novelty-dependent reaction, the duration of which is limited to the period during which the animal experiences malaise (Domjan, 1977).

Interestingly, the amplitude of the CCA observed in the sham-lesioned COA 5 group was not reduced by simultaneous exposure to the CS. This result suggests that the context was not overshadowed by the CS, and confirms that CCA can occur concomitantly to COA when the CS is presented together with the context during COA learning (Hatfield et al., 1992). On the other hand, the failure to obtain CCA with long ISI might be due to a latent inhibition effect (Lubow and Moore, 1959): previous studies using a conditioned fear paradigm (e.g., Kiernan and Wesbrook, 1993; Killcross et al., 1998) showed that extensive exposure to the to-be-conditioned context resulted in a reduction in contextual fear. Thus, the long exposure (i.e., 140 min) to the conditioning context in the COA 120 and CCA 120 groups may have affected the context-US association by a latent inhibition effect.

Most importantly, the present results show that the EC lesion disrupted CCA. CCA requires learning relations between the different cues present in the learning context, and associating these cues with the US. Since EC-lesioned animals were able to associate the olfactory CS with the US, it is unlikely that the CCA deficit resulted from a failure of US processing. Rather, a large number of studies suggest that it might result from a deficit in context information processing. First, the EC is reciprocally connected to the hippocampus and BLA (e.g., Amaral and Witter, 1995; Ferry et al., 1997; Pitkänen et al., 2000) and it has been previously assumed to be involved in the representation of context (reviews in, e.g., Maren and Fanselow, 1997; Majchrzak et al., 2006; Ji and Maren, 2008; Rudy, 2009; Van Strien et al., 2009; but: Phillips and LeDoux, 1995; Good and Honey, 1997; Bannerman et al., 2001; Hales et al., 2014). The amygdala is a downstream target of the hippocampus for the association of context representation with US (e.g., Fanselow, 2010) and also influences storage of the hippocampus-dependent representation of the conditioning context (Huff and Rudy, 2004; Huff et al., 2005). Moreover, it was recently shown that the glutamatergic projection from BLA to EC (Pitkänen et al., 2000) is involved in the modulation of the acquisition of contextual fear conditioning (Sparta et al., 2014). This suggests that the EC lesion may have impaired CCA through disruption of contextual information processing by both hippocampus and amygdala.

The present results also confirmed that the EC lesion did not disrupt but rather enabled COA, with ISIs up to 120 min (Ferry et al., 1996, 1999, 2006, 2007; Ferry and Di Scala, 1997). Conditioned odor aversion learning (COA) requires association between olfactory CS memory trace and US (Bures and Buresova, 1990; Roldan and Bures, 1994), and we have previously suggested that the EC is involved in the control of olfactory CS memory trace duration through a functional interaction with the BLA (Ferry et al., 1996, 1999; Ferry and Di Scala, 1997). As odor CS and context can both associate with the US in an interdependent way (Rescorla and Wagner, 1972), it is reasonable to suggest that the establishment of COA with long ISI obtained in EC-lesioned animals may have resulted, at least in part, from inhibition of the context influence upon the odor-US association due to the deficit in context processing.

Histological analysis of the lesion extent showed that the aspirative technique damaged a large portion of the lateral EC and part of the medial EC; in the light of previous findings that selective lesion of the lateral but not the medial EC affected COA with long ISI (Ferry et al., 2006), the present effects on COA were likely due to the lesion of the lateral part of the EC. As for the CCA effect, the present results do not indicate which part of the EC was selectively involved. In addition, the aspirative technique induced lesions of axons of passage in the EC and the disruptive effect observed on CCA may have resulted from a deficit in the processing of information arising from or passing through the EC.

Using discrete brain structure inactivation techniques, future studies will probably help to clarify this point, although both parts of the EC seem to be involved in the same kind of mechanism, at least when it comes to spatial processing (Van Cauter et al., 2012).

Conclusion

Feeding behavior is part of a complex integrated adaptive system. The differentiation between safe and unsafe food items that conditions ingestive behavior depends, at least in part, on previous experience during which the cues characterizing either the food (i.e., odor, taste, texture, etc.) or the environment in which the food is present (contextual cues) acquired a hedonic valence after feeding, through CS-US associative learning. These kinds of association have been experimentally studied for years (Slotnick and Katz, 1974; Nigrosh et al., 1975; Slotnick, 1984) and experimental conditioned food aversion paradigms, such as conditioned taste or odor/taste-potentiated odor aversion learning, have provided fundamental insights into the mechanisms and CNS structures involved in food-reward/food-poisoning associations (see Miranda, 2012 for review). In the case of conditioned aversion learning, numerous studies have shown that context processing influences the strength of the conditioned aversion to a taste acquired in a

given context (e.g., Puente et al., 1988; Loy et al., 1993; Skinner et al., 1994; Nakajima et al., 1995; Boakes et al., 1997; Lopez and Cantora, 2003; Murphy and Skinner, 2005; Ishii et al., 2006). Using another type of conditioned food aversion paradigm, the present study clearly shows that the conditions in which COA is established concomitantly to context aversion depends on the time interval separating the presentation of the odor and context from the US. Importantly, the results show that the EC is a key structure in the processes underlying the associations between context, odor CS and US in COA learning. Eventually, our results suggest the EC could be more largely be involved in the acquisition of conditioned food aversion learning through a control upon the association (1) between the odor of a particular food and a gastric malaise (US) that followed its ingestion and (2) between the context in which this food has been encountered and the US.

References

Amaral, D. G., and Witter, M. P. (1995). "Hippocampal formation," in *The Rat Nervous System, 2nd Edn.,* ed G. Paxinos (San Diego, CA: Academic Press), 443–493.

Andrews, E. A., and Braveman, N. S. (1975). The combined effects of dosage level and interstimulus interval on the formation of one-trial poison-based aversions in rats. *Anim. Learn. Behav.* 3, 287–289. doi: 10.3758/BF03213446

Bannerman, D. M., Yee, B. K., Lemaire, M., Jarrard, L., Iversen, S. D., Rawlins, J. N. P., et al. (2001). Contextual fear conditioning is disrupted by lesions of the subcortical, but not entorhinal, connections to the hippocampus. *Exp. Brain Res.* 141, 304–311. doi: 10.1007/s002210100869

Biella, G., and de Curtis, M. (2000). Olfactory inputs activate the medial entorhinal cortex via the hippocampus. *J. Neurophysiol.* 83, 1924–1931.

Boakes, R. A., Westbrook, R. F., Elliott, M., and Swinbourne, A. L. (1997). Context dependencyof conditioned aversions to water and sweet tastes. *J. Exp. Psycho Anim. Behav. Process.* 23, 56–67. doi: 10.1037/0097-7403.23.1.56

Bouton, M. E., Jones, D. L., McPhillips, S. A., and Shwartzentruber, D. (1986). Potentiation and overshadowing in odor-aversion learning: role of method of odor presentation, the distal-proximal cue distinction, and the conditionability of odor. *Learn. Motiv.* 17, 115–138. doi: 10.1016/0023-9690(86)90006-8

Bures, J., Buresova, O. (1990). Reversible lesions allowr einterpretation of system level studies of brain mechanisms of behavior. *Concepts Neurosci.* 1, 69–89.

Burwell, R. D., and Amaral, D. G. (1998). Cortical afferents of the perirhinal, postrhinal, and entorhinal cortices in the rat. *J. Comp. Neurol.* 398, 179–205.

Capaldi, E. D., Campbell, D. H., Sheffer, J. D., and Bradford, J. P. (1987). Conditioned flavor preferences based on delayed caloric consequences. *J. Exp. Psychol. Anim. Behav. Process.* 13, 150–155. doi: 10.1037/0097-7403.13.2.150

Chapuis, J., Messaoudi, B., Ferreira, G., and Ravel, N. (2007). Importance of retronasal and orthonasal olfaction for odor aversion memory in rats. *Behav. Neurosci.* 121, 1383–1392. doi: 10.1037/0735-7044.121.6.1383

Desmedt, A., Hazvi, S., and Dudai, Y. (2003). Differential pattern of cAMP response element-binding protein activation in the rat brain after conditioned aversion as a function of the associative process engaged: taste versus context association. *J. Neurosci.* 23, 6102–6110.

Domjan, M. (1977). Selective suppression of drinking during a limited period following aversive drug treatment in rats. *J. Exp. Psychol. Anim. Behav. Process.* 3, 66–76. doi: 10.1037/0097-7403.3.1.66

Fanselow, M. S. (2010). Drom contextual fear to a dynamic view of memory systems. *Trends Cog. Sci.* 14, 7–15. doi: 10.1016/j.tics.2009.10.008

Fedorchak, P. M., and Bolles, R. C. (1987). Hunger enhances the expression of calorie- but not taste-mediated conditioned flavor preferences. *J. Exp. Psychol. Anim. Behav. Process.* 13, 73–79. doi: 10.1037/0097-7403.13.1.73

Ferry, B., and Di Scala, G. (1997). Bicuculline administration into basolateral amygdala facilitates trace conditioning of odor aversion in the rat. *Neurobiol. Learn. Mem.* 67, 80–83. doi: 10.1006/nlme.1996.3743

Ferry, B., Ferreira, G., Traissard, N., and Majchrzak, M. (2006). Selective involvement of the lateral entorhinal cortex in the control of the olfactory memory trace during conditioned odor aversion in the rat. *Behav. Neurosci.* 120, 1180–1186. doi: 10.1037/0735-7044.120.5.1180

Ferry, B., Gervasoni, D., and Vogt, C. (2014). *Stereotaxic Neurosurgery in Laboratory Rodent—Handbook on Best Practices.* New York, NY: Springer Verlag Intl. editions.

Ferry, B., Herbeaux, K., Cosquer, B., Traissard, N., Galani, R., and Cassel, J. C. (2007). Immunotoxic cholinergic lesions in the basal forebrain reverse the effects of entorhinal cortex lesions on conditioned odor aversion in the rat. *Neurobiol. Learn. Mem.* 88, 114–126. doi: 10.1016/j.nlm.2007.01.007

Ferry, B., Magistretti, P. J., and Pralong, E. (1997). Noradrenaline action on the glutamatergic transmission in the rat basolateral amygdala *in vitro*: α_2-adrenoreceptor-mediated inhibition and β-adrenoreceptor-mediated excitation. *Eur. J. Neurosci.* 9, 1356–1364. doi: 10.1111/j.1460-9568.1997.tb01490.x

Ferry, B., Oberling, P., Jarrard, L. E., and Di Scala, G. (1996). Facilitation of conditioned odor aversion learning by entorhinal cortex lesions in the rat. *Behav. Neurosci.* 110, 443–450. doi: 10.1037/0735-7044.110.3.443

Ferry, B., Wirth, S., and Di Scala, G. (1999). Functional interaction between entorhinal cortex and basolateral amygdala during trace conditioning of odor aversion in the rat. *Behav. Neurosci.* 113, 118–125. doi: 10.1037/0735-7044.113.1.118

Garcia, J., Ervin, F. R., and Koelling, R. A. (1966). Learning with prolonged delay of reinforcement. *Psychon. Sci.* 5, 121–122. doi: 10.3758/BF03328311

Gnatkovsky, V., Uva, L., and de Curtis, M. (2004). Topographic distribution of direct and hippocampus-mediated entorhinal cortex activity evoked by olfactory tract stimulation. *Eur. J. Neurosci.* 20, 1897–1905. doi: 10.1111/j.1460-9568.2004.03627.x

Good, M., and Honey, R. C. (1997). Dissociable effects of selective lesions to hippocampal subsystems on exploratory behavior, contextual learning, and spatial learning. *Behav. Neurosci.* 111, 487–493. doi: 10.1037/0735-7044.111.3.487

Haberly, L. B., and Price, J. L. (1978). Association and commissural fiber systems of the olfactory cortex of the rat: II. Systems originating in the olfactory peduncle. *J. Comp. Neurol.* 181, 781–808. doi: 10.1002/cne.901810407

Hales, J. B., Schlesiger, A. I., Leurgeb, J. K., Squire, L. R., Leutgeb, S., and Clark, R. E. (2014). Medial entorhinal cortex lesions only partially disrupt hippocampal place cells and hippocampus-dependent place memory. *Cell Reports* 9, 893–901. doi: 10.1016/j.celrep.2014.10.009

Hankins, W. G., Garcia, J., and Rusiniak, K. W. (1973). Dissociation of odor and taste in baitshyness. *Behav. Biol.* 8, 407–419. doi: 10.1016/S0091-6773(73)80081-1

Harris, J. A., Gorissen, M. C., Bailey, G. K., and Westbrook, R. F. (2000). Motivational state regulates the content of learned flavor preferences. *J. Exp. Psychol. Anim. Behav. Process.* 26, 15–30. doi: 10.1037/0097-7403.26.1.15

Hatfield, T., Graham, P. W., and Gallagher, M. (1992). Taste-potentiated odor aversion learning: role of the amygdaloid basolateral complex and central nucleus. *Behav Neurosci.* 106, 286–293. doi: 10.1037/0735-7044.106.2.286

Huff, N. C., and Rudy, J. W. (2004). The amygdala modulates hippocampus-dependent context memory formation and stores cue-shock associations. *Behav. Neurosci.* 118, 53–62. doi: 10.1037/0735-7044.118.1.53

Huff, N. C., Wright-Hardesty, K. J., Higgins, E. A., Matus-Amat, P., and Rudy, J. W. (2005). Context pre-exposure obscures amygdala modulation of contextual-fear conditioning. *Learn. Mem.* 12, 456–460. doi: 10.1101/lm.6705

Ishii, K., Iguchi, Y., and Sawa, K. (2006). Context dependency of aversion to familiar andnovel fluids. *Learn. Motiv.* 37, 113–130. doi: 10.1016/j.lmot.2005.04.002

Ji, J., and Maren, S. (2008). Lesions of the entorhinal cortex or fornix disrupt the context-dependence of fear extinction in rats. *Behav. Brain Res.* 194, 201–206. doi: 10.1016/j.bbr.2008.07.011

Kiernan, M. J., and Wesbrook, R. F. (1993). Effects of exposure to a to-be-showked environment upon the rat's freezing response: evidence for facilitation, latent

inhibition, and perceptual learning. *Q. J. Exp. Psychol. B* 46, 271–288. doi: 10.1080/14640749308401089

Killcross, A. S., Kiernan, M. J., Dwyer, D., and Westbrook, R. F. (1998). Loss of latent inhibition of contextual conditioning following non-reinforced context exposure in rats. *Q. J. Exp. Psychol. B*. 51, 75–90.

Krettek, J. E., and Price, J. L. (1977). The cortical projections of the mediodorsal nucleus and adjacent thalamic nuclei in the rat. *J. Comp. Neurol.* 171, 157–191. doi: 10.1002/cne.901710204

Lopez, M., and Cantora, R. (2003). Associative interference with taste aversions after con-textual discrimination learning. *Learn. Motiv.* 34, 372–388. doi: 10.1016/S0023-9690(03)00035-3

Lorden, J. F., Kenfield, M., and Braun, J. J. (1970). Response suppression to odors paired with toxicosis. *Learn. Motiv.* 1, 391–400. doi: 10.1016/0023-9690(70)90103-7

Loy, I., Alvarez, R., Rey, V., and Lopez, M. (1993). Context-US associations rather thanoccasion-setting in taste aversion learning. *Learn. Motiv.* 24, 55–72. doi: 10.1006/lmot.1993.1004

Lubow, R. E., and Moore, A. U. (1959). Latent inhibition: the effect of non reinforced pre-exposure to the conditioned stimulus. *J. Comp. Physiol. Psychol.* 52, 415–419. doi: 10.1037/h0046700

Majchrzak, M., Ferry, B., Marchand, A. R., Herbeaux, K., Seillier, A., and Barbelivien, A. (2006). Entorhinal cortex lesions disrupt fear conditioning to background context but spare fear conditioning to a tone in the rat. *Hippocampus* 16, 114–124. doi: 10.1002/hipo.20138

Maren, S., and Fanselow, M. S. (1997). Electrolytic lesions of the fimbria/fornix, dorsal hippocampus, or entorhinal cortex produce anterograde deficits in contextual fear conditioning in rats. *Neurobiol. Learn. Mem.* 67, 142–149. doi: 10.1006/nlme.1996.3752

Mehiel, R., and Bolles, R. C. (1984). Learned flavor preferences based on caloric outcome. *Anim. Learn. Behav.* 12, 421–427. doi: 10.3758/BF03199989

Miranda, M. I. (2012). Taste and odor recognition memory: the emotional flavor of life. *Rev. Neurosci.* 23, 481–499. doi: 10.1515/revneuro-2012-0064

Mouly, A. M., and Di Scala, G. (2006). Entorhinal cortex stimulation modulates amygdala and piriform cortex responses to olfactory bulb inputs in the rat. *Neuroscience* 137, 1131–1141. doi: 10.1016/j.neuroscience.2005.10.024

Murphy, M., and Skinner, D. M. (2005). Contextual control of fluid consumption: the effectsof context extinction. *Learn. Motiv.* 36, 297–311. doi: 10.1016/j.lmot.2004.11.002

Nakajima, S., Kobayashi, Y., and Imada, H., (1995). Contextual control of taste aver-sion in rats: the effects of context extinction. *Psychol. Record* 45, 309–318.

Nigrosh, B. J., Slotnick, B. M., and Nervin, J. A. (1975). Olfactory discrimination, reversal learning, and stimulus control in rats. *J. Physiol. Psychol.* 89, 285–294. doi: 10.1037/h0076821

Palmerino, C. C., Rusiniak, K. W., and Garcia, J. (1980). Flavor–Illness aversions: the peculiar roles of odor and taste in memory for poison. *Science* 208, 753–755. doi: 10.1126/science.7367891

Paxinos, G., and Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates, 4th Edn.* New York, NY: Academic Press.

Phillips, R. G., and LeDoux, J. E. (1995). Lesions of the fornix but not the entorhinal cortex or perirhinal cortex interfere with contextual fear conditioning. *J. Neurosci*. 15, 5308–5315.

Pitkänen, A., Pikkarainen, M., Nurminen, N., and Ylinen, A. (2000). Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. *Ann. N.Y. Acad. Sci.* 911, 369–391. doi: 10.1111/j.1749-6632.2000.tb06738.x

Puente, G. P., Cannon, D. S., Best, M. R., and Carrell, L. E. (1988). Occasion setting of fluid ingestion by contextual cues. *Learn. Motiv.* 19, 239–253. doi: 10.1016/0023-9690(88)90003-3

Rescorla, R. A., and Wagner, A. R. (1972). "A theory of Pavlovian conditioning; variations in the effectiveness of reinforcement and non reinforcement," in *Classical Conditioning II: Current Research and Theory*, eds A. H. Black andW. F. Prokasy (New York, NY: Appleton-Century- Crofts), 64–99.

Roldan, G., and Bures, J. (1994). Tetrodotoxin blockade of amygdala overlapping with poisoning impairs acquisition of conditioned taste aversion in rats. *Behav. Brain Res.* 65, 213–219. doi: 10.1016/0166-4328(94)90107-4

Rudy, J. W. (2009). Context representations, context functions, and the parahippocampal-hippocampal system. *Learn. Mem.* 16, 573–585. doi: 10.1101/lm.1494409

Skinner, D. M., Martin, G. M., Pridgar, A., and van der Kooy, D. (1994). Conditional control of fluid consumption in an occasion setting paradigm is independent of Pavlovian associations. *Learn. Motiv.* 25, 368–400. doi: 10.1006/lmot.1994.1019

Slotnick, B. M. (1984). Olfactory stimulus control in the rat. *Chem. Senses* 9, 157–165. doi: 10.1093/chemse/9.2.157

Slotnick, B. M., and Katz, H. M. (1974). Olfactory learning set formation in rats. *Science* 185, 796–798. doi: 10.1126/science.185.4153.796

Slotnick, B. M., Westbrook, F., and Darling, F. M. C. (1997). What the rat's nose tells the rat's mouth: long delay aversion conditioning with aqueous odors and potentiation of taste by odors. *Anim. Learn. Behav.* 25, 357–369. doi: 10.3758/BF03199093

Sparta, D. R., Smithuis, J., Stamatakis, A. M., Jennings, J. H., Kantak, P. A., Ung, R. L., et al. (2014). Inhibition of projections from the basolateral amygdala to the entorhinal cortex disrupts the acquisition of contextual fear. *Front. Behav. Neurosci.* 8:129. doi: 10.3389/fnbeh.2014.00129

Taukulis, H. K. (1974). Odor aversions produced over long CS-US delays. *Behav. Biol.* 10, 505–510. doi: 10.1016/S0091-6773(74)92111-7

Van Cauter, T., Camon, J., Alvernhe, A., Elduayen, C., Sargolini, F., and Save, E. (2012). Distinct roles of medial and lateral enrorhinal cortex in spatial cognition. *Cereb. Cortex* 23, 451–459. doi: 10.1093/cercor/bhs033

Van Strien, N. M., Cappaert, N. L. M., and Witter, M. P. (2009). The anatomy of memory: an interactive overview of the parahippocampal-hippocampal network. *Nature* 10, 272–282. doi: 10.1038/nrn2614

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 02 October 2015
doi: 10.3389/fnins.2015.00342

The entorhinal cortex is involved in conditioned odor and context aversions

Barbara Ferry[1], Karine Herbeaux[2], Hervé Javelot[3] and Monique Majchrzak[2]*

[1] Centre of Research in Neuroscience, CNRS UMR 5292 - INSERM U1028 - UCBL1, Lyon, France, [2] Laboratoire de Neurosciences Cognitives et Adaptatives, UMR 7364, Faculté de Psychologie, CNRS, Université de Strasbourg, Neuropôle de Strasbourg, GDR 2905 du CNRS, Strasbourg, France, [3] Etablissement Public de Santé Alsace Nord Brumath – Service Pharmacie - CHU de Strasbourg, Hôpital Civil, Clinique de Psychiatrie – Service de Psychiatrie II, Brumath, France

In a natural environment, avoidance of a particular food source is mostly determined by a previous intake experience during which sensory stimuli such as food odor, become aversive through a simple associative conditioned learning. Conditioned odor aversion learning (COA) is a food conditioning paradigm that results from the association between a tasteless scented solution (conditioned stimulus, CS) and a gastric malaise (unconditioned stimulus, US) that followed its ingestion. In the present experimental conditions, acquisition of COA also led to acquisition of aversion toward the context in which the CS was presented (conditioned context aversion, CCA). Previous data have shown that the entorhinal cortex (EC) is involved in the memory processes underlying COA acquisition and context fear conditioning, but whether EC lesion modulates CCA acquisition has never be investigated. To this aim, male Long-Evans rats with bilateral EC lesion received CS-US pairings in a particular context with different interstimulus intervals (ISI). The results showed that the establishment of COA with long ISI obtained in EC-lesioned rats is associated with altered CCA learning. Since ISI has been suggested to be the determining factor in the odor- and context-US association, our results show that the EC is involved in the processes that control both associations relative to ISI duration.

Keywords: entorhinal cortex, odor aversion, context aversion, food conditioning, odors

OPEN ACCESS

Edited by:
Markus Fendt,
Otto-von-Guericke University
Magdeburg, Germany

Reviewed by:
Jimmy Stehberg,
Universidad Andres Bello, Chile
Yasushi Kiyokawa,
The University of Tokyo, Japan

****Correspondence:***
Barbara Ferry,
CRNL, CNRS UMR 5292 - INSERM U1028 - UCBL1, 50 Avenue Tony Garnier, 69366 Lyon Cedex 07, France
barbara.ferry@cnrs.fr

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *02 July 2015*
Accepted: *09 September 2015*
Published: *02 October 2015*

Citation:
Ferry B, Herbeaux K, Javelot H and Majchrzak M (2015) The entorhinal cortex is involved in conditioned odor and context aversions. Front. Neurosci. 9:342. doi: 10.3389/fnins.2015.00342

Introduction

Finding adequate food sources', including water, is one of the most fundamental aspects of animal life. Guided by various kinds of stimuli present in the environment, animals move through space toward a resource goal, avoiding unsafe environments and the risk of themselves becoming food. This behavior involves the animal finding and recognizing particular cues, whether contingent, in a specific environment, or coming directly from the food source, that indicate the nearness and direction of the goal. Although innate, a lot of encounters with cue stimuli result in learned approaches or avoidances. In this situation, the capacity to anticipate the future and differentiate between safe or unsafe food items depends, at least in part, on previous experience in which the sensory stimuli characterizing a particular food (odor and taste) become associated with the positive (energy input) or negative (gastric malaise, poisoning)

consequences of ingestion through Pavlovian associative learning (e.g., Mehiel and Bolles, 1984; Capaldi et al., 1987; Fedorchak and Bolles, 1987; Harris et al., 2000). These kinds of association have been largely studied (Slotnick and Katz, 1974; Nigrosh et al., 1975; Slotnick, 1984) and the use of conditioned food aversion paradigms in research has provided fundamental insights into the brain mechanisms and structures involved in food-reward/food-poisoning associations (see Miranda, 2012 for a review). One such paradigm, conditioned odor aversion (COA), consists in avoidance of an odorized-tasteless solution (conditioned stimulus, CS) the ingestion of which precedes toxicosis (unconditioned stimulus, US). COA is a robust and long-lasting learned association that can be obtained with a single CS-US pairing (Lorden et al., 1970; Hankins et al., 1973; Taukulis, 1974), and with CS-US delays (interstimulus interval, ISI) ranging from minutes to hours depending on whether the CS is mixed (proximal presentation, inducing orthonasal, and retronasal stimulations: Slotnick et al., 1997; Chapuis et al., 2007) or presented close to the solution (distal presentation, inducing orthonasal stimulation: Garcia et al., 1966; Andrews and Braveman, 1975; Palmerino et al., 1980; Bouton et al., 1986; Ferry et al., 1996, 2006).

Many studies of the mechanisms and systems involved in the memory processes underlying COA learning have focused exclusively on conditioned responses toward the CS. In particular, some showed that the entorhinal cortex (EC) plays an important role in task acquisition. The EC is a parahippocampal structure that receives important olfactory projections (Krettek and Price, 1977; Haberly and Price, 1978; Burwell and Amaral, 1998) and is reciprocally connected to the hippocampus and amygdala (e.g., Amaral and Witter, 1995; Ferry et al., 1997; Pitkänen et al., 2000). In addition, electrophysiological data suggest that the processing of olfactory information in both these regions is modulated by the EC (e.g., Biella and de Curtis, 2000; Gnatkovsky et al., 2004; Mouly and Di Scala, 2006). Interestingly, we found that rats with EC lesions were able to learn to avoid an odor paired with toxicosis, using both distal and proximal CS presentation, even when the ISI was too long (120 min) for such learning to be observed in control animals (Ferry et al., 1996, 1999, 2006, 2007). Further experiments suggested that this facilitation of COA with long ISI may be the consequence of lesion-induced suppression of an inhibitory influence of the EC on brain areas involved in olfactory information processing, such as the basolateral amygdala (BLA; Ferry and Di Scala, 1997; Ferry et al., 1999; Mouly and Di Scala, 2006). Also and non-exclusively, persistence of the odor memory trace supporting COA with long ISI in EC-lesioned rats may involve altered processing of other cues present during conditioning. In concordance with this, several studies have shown that the EC is involved in the processing of information related to the context in which the CS-US association was established, mainly during fear conditioning (Maren and Fanselow, 1997; Ji and Maren, 2008; Majchrzak et al., 2006; but: Phillips and LeDoux, 1995; Good and Honey, 1997; Bannerman et al., 2001; Hales et al., 2014). However, although contextual cues contingent to a specific environment are indicators that also contribute to the behavioral response toward a food source, the relationship between contextual and odor cues during conditioned food aversion learning has been little studied. Context aversion has be-12mmen shown to occur concurrently to COA (Hatfield et al., 1992), but whether EC lesion modulates context aversion has never be investigated during COA.

The aim of the present experiment was to assess whether the establishment of COA with long ISI in EC-lesioned rats was associated with altered contextual information processing. To this end, acquisition of COA and conditioned context aversion (CCA) were tested in EC-lesioned animals using two different procedures. The first (COA), consisted in presenting an olfactory cue (odorized water, CS) in a particular context that was followed by LiCl-induced gastric malaise (US), with short, or long ISI. This forward arrangement between CS and context has previously been shown to result in odor and context aversion (Hatfield et al., 1992). The second (CCA), consisted in administering the US after the animals had been placed in a particular context, with the same short and long ISIs. In this procedure, the CS was presented after the US at the end of the session. This backward arrangement between the two stimuli resulted in acquisition of context aversion but not in COA.

Materials and Methods

Subjects

One hundred and two male Long-Evans rats (supplied from Janvier Labs, Le Genest-St-Isles France; weighing 250–275 g) were used. They were housed two per cage in transparent Makrolon cages (42 × 26 × 15 cm) under controlled temperature (22°C ± 2) and standard 12 h light/dark cycle (lights from 7:00 a.m. to 7:00 p.m.) in a colony room. The animals were provided with *ad libitum* access to food and water. After arrival, the animals were allowed to acclimate to the laboratory conditions for a period of 1 week before surgery.

All procedures involving animals and their care conformed to the institutional guidelines, which comply with international laws and policies (directive 2010/63/European Community) and have been approved by the ethics committee of the Université Claude Bernard Lyon 1 (CE2A-55). Permission references were 69–387517 for BF and 67–289 for MM. All other co-authors were under the responsibility of the former.

Surgery

All surgical procedures were conducted under optimal aseptic, analgesic, and ethical animal care conditions (see Ferry et al., 2014) by those authorized to do so. Rats were anesthetized by i.p. injection of a mix of ketamine (100 mg/kg)/xylasine (10 mg/kg). Following a prophylactic antibiotic treatment (penicillin 0.12 M.U./0.3 ml, i.m.) the rats were given bilateral lesions of the EC by aspiration ($n = 56$) as previously described (Ferry et al., 1999). Sham-lesioned animals were operated similarly but no aspiration was carried out ($n = 46$). Four animals died after surgery (three EC-lesioned and one sham-lesioned). All subjects recovered for 7–11 days after surgery with *ad libitum* access to food and water, and were singly housed until the end of the experiment.

Test Chambers

Habituation Chambers

Eight chambers (25 × 30 × 35 cm) located in a room adjacent to the vivarium were used for habituation to water consumption. They were made of clear perspex and had a wire mesh floor. The spout of a 25-ml glass tube (Richter tube, Strasbourg, France) could be introduced into the cage through a circular hole on the anterior wall of the chamber, located 2 cm above the floor. Intake was measured by reading to the nearest 0.5 ml, the level of liquid of the tube before and after each session.

Conditioning and Testing Chambers

Four place preference boxes located in a room adjacent to the vivarium were used. Each box was constituted by two large compartments of similar size (45 × 45 × 30 cm, compartments A and B) with distinctive visual and tactile features and a third smaller ship-wooden gray painted transit compartment (36 × 18 × 30 cm) that allowed animals to move between compartments A and B when the sliding door of their back wall was open. Compartment A had three wooden black walls and a floor made of tight and flexible wire mesh. Compartment B had three wooden walls with vertical black and white stripes and a floor made of large and rigid wire mesh. The front wall of both compartments was constituted by clear perspex. The spout of the Richter tube could be introduced into each compartment through a circular hole on the back wall of the chamber, located 2 cm above the floor.

Behavioral Procedure

All experimental sessions were carried out during the light portion of the cycle between 11:00 a.m. and 1:00 p.m. After post-surgical recovery, animals were handled (3 min/day) for 3 days and weighed daily to verify their adaptation to the deprivation schedule.

On the first day (Day 1), each animal was placed in the transit compartment of the place preference box with sliding doors open, and allowed to move freely in all compartments during a 15 min session. The time spent in each compartment was recorded. The compartment in which the animals spent more time was chosen as the conditioning context, and the other one was chosen as the neutral context.

Water bottles were removed from the home cage in the evening of Day 1 and a 23 h 45 min water deprivation schedule was initiated. During the water drinking habituation sessions (Day 2 to Day 6), rats had access to water once a day according to the following procedure: On Day 2 and 3 the animals had access to water in their home cage for 30 min on Day 2 and for 15 min on Day 3. From Day 4 to Day 6, animals had access to water for 15 min in the habituation chamber.

On Day 7, each animal was placed in the neutral context equipped with a Richter tube and had access to water for 15 min. Then, the Richter tube was removed and the animal received an i.p. injection of 0.9% NaCl (10 ml/kg). Animals spent an additional period of 60 min in the neutral context.

Conditioning session took place on Day 8 (**Figure 1**). Animals were divided in two experimental groups according to the conditioning procedure (adapted from Desmedt et al., 2003). Animals of COA groups were placed in the conditioning context where they had access for 15 min to odorized water in the Richter tube (CS; 0.01% isoamyl acetate solution). At the end of the 15 min, the Richter tube was removed and animals received an i.p. injection of a lithium chloride (LiCl) inducing gastric malaise (US; 0.15 M; 10 ml/kg) either 5 min (short ISI; COA 5 group) or 120 min (long ISI; COA 120 group) after the removal of the Richter tube. Animals spent the rest of the session in the conditioning context (total duration = 215 min).

Animals of the CCA groups were placed in the conditioning context and received an i.p. injection of LiCl at a timing similar to groups COA (either 20 or 140 min after the beginning of the session, referred successively as CCA 5 and CCA 120 groups for convenience). They spent the rest of the 215 min session in the same compartment and had access to the Richter tube filled with

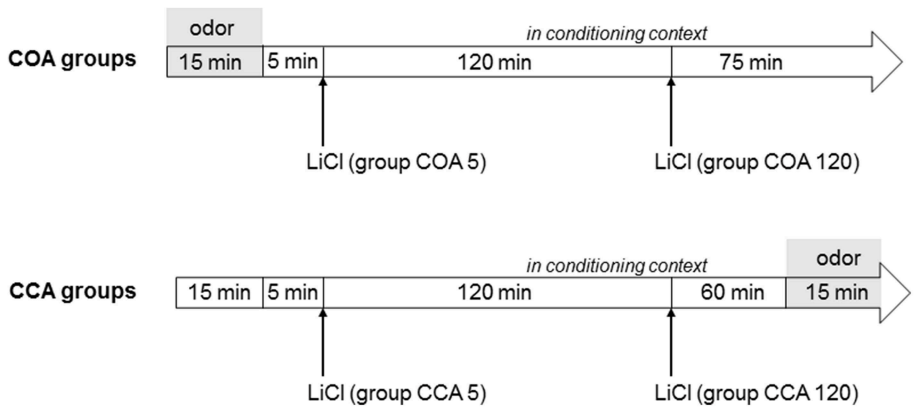


FIGURE 1 | Time schedule of the conditioning session for COA and CCA groups. In COA groups, the animals were subjected to presentation of the odor (symbolized by the gray area) in the conditioning context followed by the US with a short (5 min) or a long (120 min) interstimulus interval (COA 5 and COA 120 groups, respectively). The animals spent the rest of the session in the conditioning context. In CCA groups, the animals were placed in the conditioning context for 15 min and then received the US with a short (5 min) or long (120 min) interstimulus interval (CCA 5 and CCA 120 groups, respectively). The animals spent the rest of the session in the conditioning context in which the odor was presented during the last 15 min of the session (symbolized by the gray area). The total duration of the session was 215 min.

0.01% isoamyl acetate only during the last 15 min of the session. Intake of odorized water was measured by reading the level of liquid on the tube before and after the session in both COA and CCA groups.

EC-lesioned (EC) and sham-lesioned (Sham) rats were randomly assigned to one of these four conditioning procedures. Experimental groups were constituted as follows: COA 5 (EC: $n = 13$; Sham: $n = 11$), COA 120 (EC: $n = 14$; Sham: $n = 10$), CCA 5 (EC: $n = 13$; Sham: $n = 13$), and CCA 120 min (EC: $n = 13$; Sham: $n = 11$).

Conditioned aversions were assessed on Day 9 in the place preference box. To do this, animals were confined in the neutral context with access to the Richter tube filled with 0.01% isoamyl acetate for 15 min. At the end of the session, they returned to their home cages and the amount of liquid consumed during testing was measured. One hour after, animals were placed in the transit compartment with sliding doors open giving access to the two compartments A and B. Time spent in each compartment was measured during 30 min.

Histology

Ten days after completion of behavioral testing, each rat was given an overdose of sodium pentobarbital (100 mg/kg) and was transcardially perfused with 60 ml of saline (4°C) followed by phosphate-buffered 4% paraformaldehyde (pH 7.4; 4°C). The brain was then extracted, post-fixed for 4 h in the same fixative (4°C) and transferred into a 0.1 M phosphate-buffered 20% sucrose solution for about 36–40 h (4°C). All brains were frozen using isopentane (−40°C). Coronal sections, 30 μm, were cut on a freezing microtome (−23°C), and collected onto gelatine-coated slides. These sections were dried at room temperature and stained with cresyl violet. A microscopic inspection was then performed to determine the location and the extent of the lesions.

Data Analysis

Odor aversion was assessed by comparing the volume of odorized solution intake during the conditioning and testing sessions. Context aversion was assessed by comparing the proportion of time spent in the conditioning context before (pre-conditioning session) and after (testing session) conditioning. For each ISI, the data corresponding to the volumes (for COA) and the ratios (time spent in conditioning context/time of the session) were analyzed with a Three-Way repeated measures ANOVA with Lesion (EC vs. sham), Type of procedure (COA vs. CCA) as between subject factors and Session (conditioning vs. testing for COA assessment, pre-conditioning vs. testing for CCA assessment) as within subject factor. *Post-hoc* Newman-Keuls multiple range test (NK) was used to determine the source of detected significances in the ANOVAs. A probability level of <0.05 was accepted as statistically significant throughout.

Results

Histology

Seventeen EC-lesioned animals (with unilateral damage in the EC or extensive lesions of surrounding structures such as the perirhinal cortex, subiculum, or dentate gyrus) and four sham-lesioned animals with damage to the lateral part of the EC (resulting from the insertion of the curved needle) were excluded from the analysis. Final groups were constituted as follows: COA 5 (EC: $n = 9$; Sham: $n = 9$), COA 120 (EC: $n = 10$; Sham: $n = 9$), CCA 5 (EC: $n = 9$; Sham: $n = 13$), and CCA 120 min (EC: $n = 8$; Sham: $n = 10$).

Lesions were drawn from transversal sections stained with cresyl violet. Typical (i.e., smallest and largest) lesion extents observed in COA and CCA groups are illustrated in **Figure 2**. Histological analysis revealed that all the lesions included the lateral EC at the levels −5.60 to −8.30 from Bregma and a part of the medial EC at Bregma −7.80. The largest lesions were estimated to damage a part of the perirhinal cortex at the levels −6.30 to −7.80 mm.

Behavior

Short ISI-induced Conditioned Odor and Context Aversions

The results obtained in the experimental groups conditioned with the short ISI are shown in **Figure 3**. **Figure 3A** represents the mean odorized water intakes (± S.E.M.) measured during the conditioning and testing sessions whereas **Figure 3B** represents the mean proportion of time (± S.E.M.) spent in the conditioning context during the pre-conditioning and testing sessions. As shown in **Figure 3A**, the amount of odorized water intake during conditioning was similar between groups indicating that neither the lesion nor the early intoxication in CCA 5 groups (i.e., 180 min before) had an impact on intake. During testing the amount of odorized water intake was lower in animals exposed to the CS before intoxication, whatever the type of lesion. This indicated that short ISI lead to a COA when the odor is encountered before but not after intoxication. Statistical analyses confirmed these observations and revealed no significant effect of Lesion [$F_{(1, 36)} = 0.14$, p n.s.], a significant effect of Type of procedure [$F_{(1, 36)} = 55.37$, $p < 0.0001$], a significant effect of Session [$F_{(1, 36)} = 39.39$, $p < 0.0001$], and a significant interaction between Session and Type of procedure [$F_{(1, 36)} = 36.54$, $p < 0.0001$]. *Post-hoc* comparisons confirmed that, in both sham- and EC-lesioned COA 5 groups, the odorized water intake during testing was significantly lower than during conditioning ($p < 0.001$ in each case), and also lower than the odorized water intake measured in CCA 5 groups during testing ($p < 0.001$ in each case).

As shown in **Figure 3B**, the proportion of time spent in the conditioning context during pre-conditioning was similar between groups. In sham-lesioned animals, the proportion of time spent in the conditioning context during testing was lower than before conditioning whatever the type of procedure. This decrease indicated that short ISI lead to a CCA and that animals had associated the context with the US, whether exposed or not to the odorized water. In contrast, the time spent in the conditioning context did not differ between pre-conditioning and testing sessions in EC-lesioned groups, indicating that the lesion affected CCA. The ANOVA confirmed these observations and showed significant effects of Lesion [$F_{(1, 36)} = 15.13$, $p < 0.001$] and Session [$F_{(1, 36)} = 20.00$, $p < 0.0001$] but no effect of Type of procedure [$F_{(1, 36)} = 1.15$, p n.s.], and a significant Session X Lesion interaction [$F_{(1, 36)} = 16.52$, $p < 0.0001$]. *Post-hoc* comparisons confirmed that the proportion

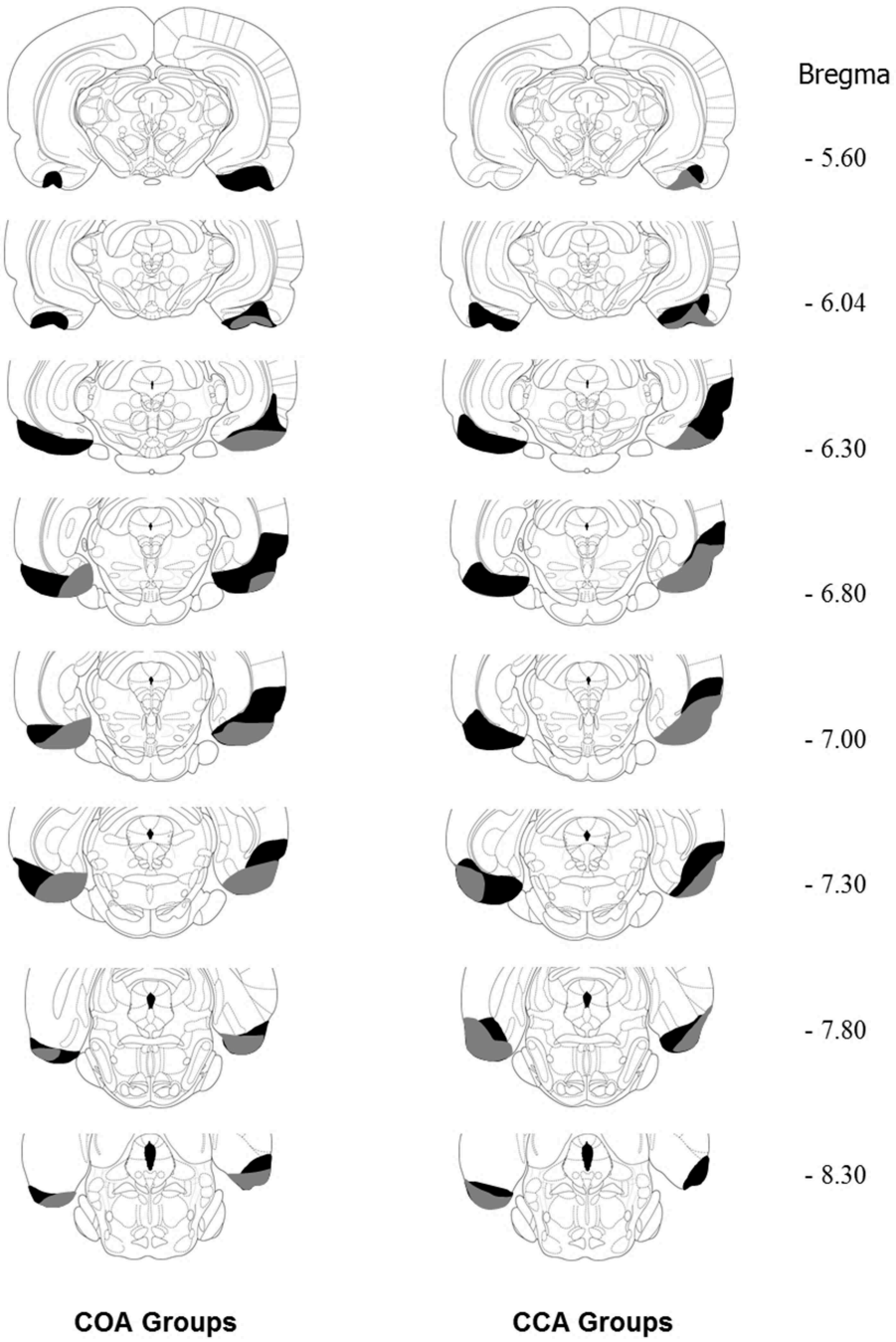


FIGURE 2 | Diagram of coronal sections (between −5.60 and −8.30 mm from bregma; according to the Paxinos and Watson stereotaxic atlas, 1998) showing the extent of the smallest (gray area) and the largest (black area) EC lesion in the COA and CCA groups. From *The Rat Brain in Stereotaxic Coordinates*, by Paxinos and Watson (1998). Copyright 2006 by Elsevier. Adapted with permission.

of time spent in the conditioning context during testing was lower than before conditioning in both sham-lesioned groups ($p < 0.05$ for COA group and $p < 0.001$ for CCA 5 group), but not in EC-lesioned groups. The proportion of time spent in the conditioning context during testing in sham-lesioned groups was also lower than the proportion of time spent in the conditioning context in EC-lesioned groups ($p < 0.001$ for sham COA 5 vs. EC COA 5 and $p < 0.001$ for sham CCA 5 vs. EC CCA 5).

Long ISI-induced Conditioned Odor and Context Aversions

The results obtained in the experimental groups conditioned with the long ISI are shown in **Figure 4**. **Figure 4A** represents the

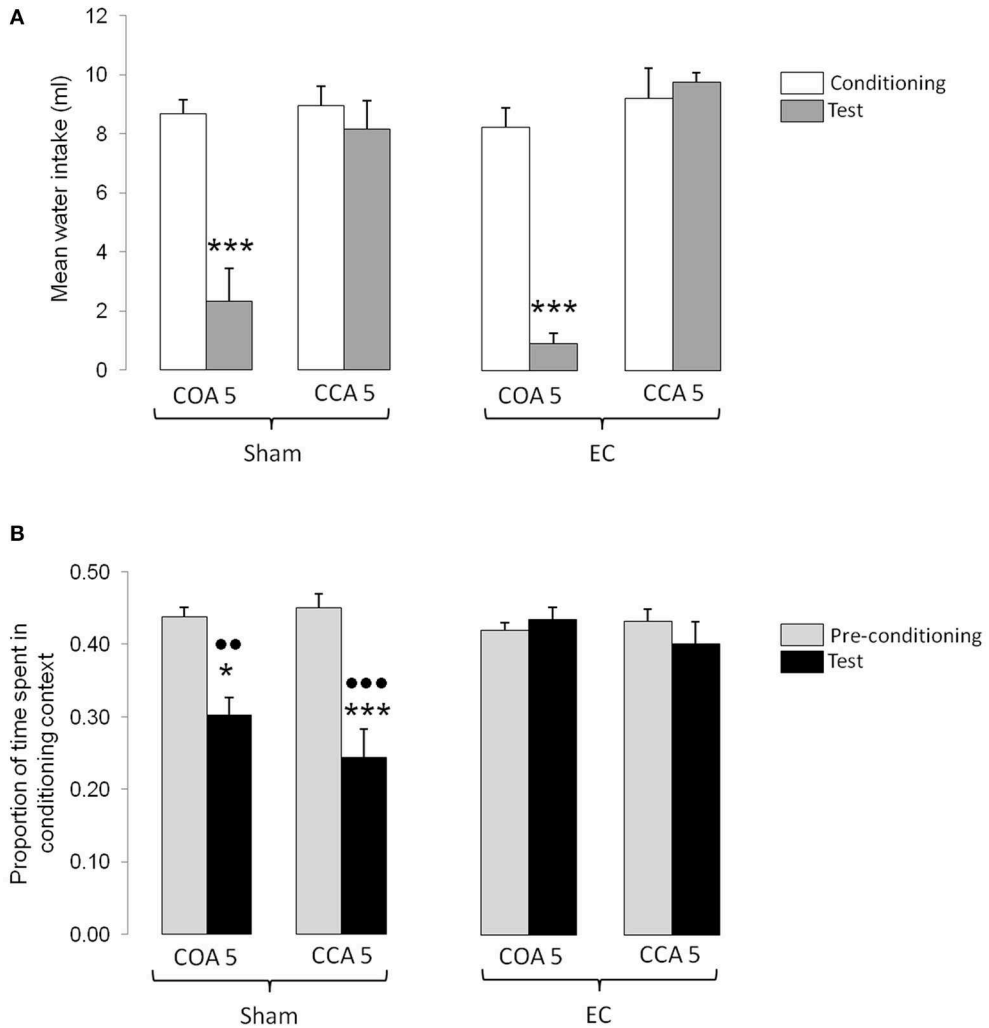


FIGURE 3 | Effect of a short ISI on COA and CCA in Sham- and EC-lesioned groups. (A) Represents the mean odorized water intakes (± S.E.M.) measured during the conditioning (white bars) and testing (gray bars) sessions in each experimental group. ***$p < 0.001$ as compared with the amount of odorized water intake during conditioning in the same group and with the amount of odorized water intake during testing in the corresponding CCA group. **(B)** represents the mean proportion of time (± S.E.M.) spent in the conditioning context for each experimental group. Bars represent the proportion of time spent in the conditioning context (mean time spent in the conditioning context/time of the session) calculated in the pre-conditioning (gray bars) and testing (black) sessions. *, ***$p < 0.05$ and 0.001 as compared with the proportion of time spent in the conditioning context during pre-conditioning in the same group;●●, ●●●$p < 0.01$ and 0.001 as compared with the proportion of time spent in the conditioning context during testing in the corresponding EC-lesioned-group. EC, lesion of the entorhinal cortex; Sham, sham-lesion of the entorhinal cortex; COA, conditioned odor aversion; CCA, conditioned context aversion.

mean water intakes (± S.E.M.) measured during conditioning and testing sessions whereas **Figure 4B** represents the mean proportion of time (± S.E.M.) spent in the conditioning context during the pre-conditioning and testing sessions. As shown in **Figure 4A**, the amount of odorized water intake during conditioning was similar in COA 120 groups indicating that the lesion did not affect the level of odorized water intake when the US followed its presentation. However, the amount of odorized water intake during conditioning was lower in CCA 120 groups suggesting an effect of the US when it was administered 60 min before the odorized water presentation. The higher amount of odorized water intake measured in the CCA 120 groups during testing (without US) confirmed this observation. As also shown in **Figure 4A**, the amount of odorized water intake between conditioning and testing was similar in Sham-lesioned COA 120 group. In contrast, the amount of odorized water decreased between conditioning and testing in EC-lesioned group thus indicating that animals associated the odor with the US with a long ISI. Statistical analyses confirmed these observations and revealed a significant effect of Lesion [$F_{(1, 33)} = 4.79$, $p < 0.05$], a significant interaction between Lesion and Type of procedure [$F_{(1, 33)} = 6.13$, $p < 0.05$], and a significant interaction between Lesion, Type of procedure and Session [$F_{(1, 33)} = 5.97$, $p < 0.05$]. *Post-hoc* comparisons confirmed that odorized water

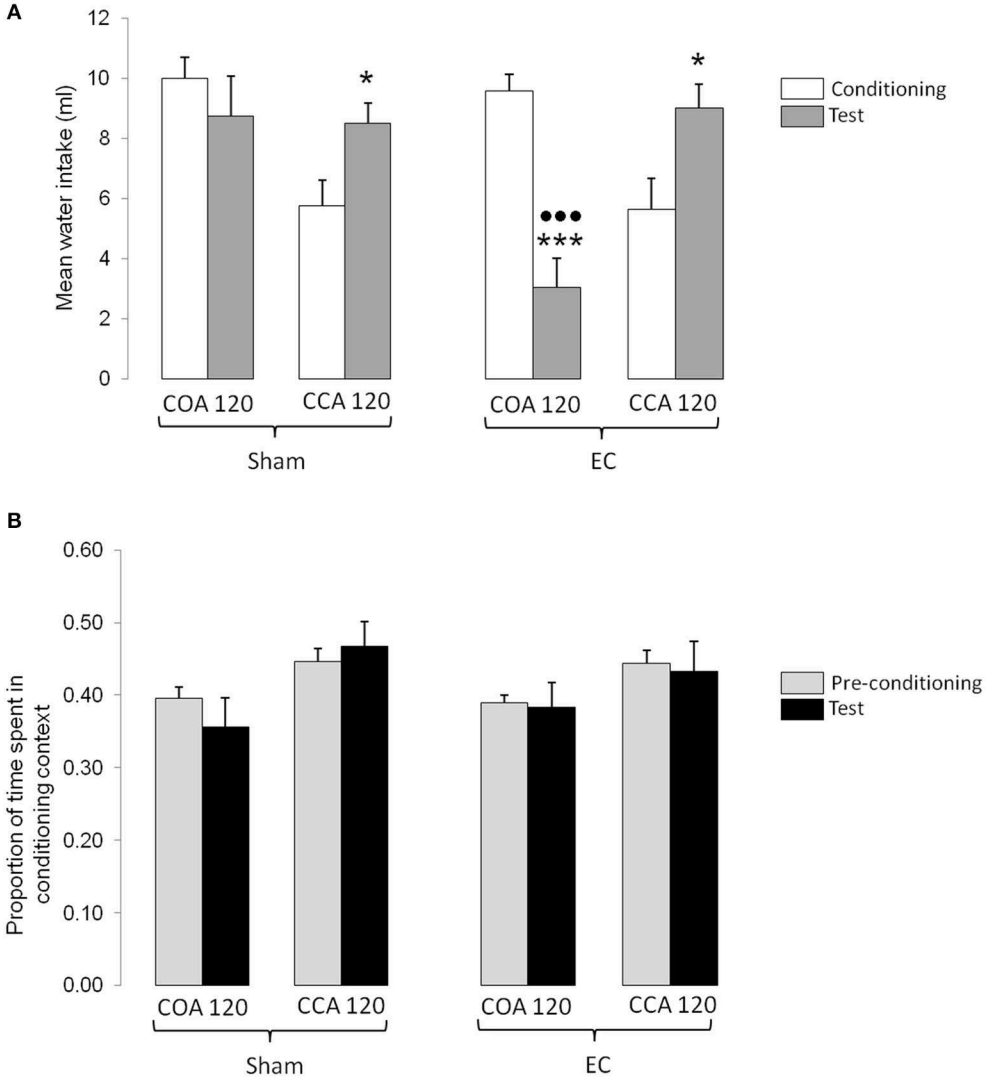


FIGURE 4 | Effect of a long ISI on COA and CCA in Sham- and EC-lesioned groups. (A) Represents the mean odorized water intakes (± S.E.M.) measured during the conditioning (white bars) and testing (gray bars) sessions in each experimental group. *, ***$p < 0.05$ and 0.001 as compared with the amount of odorized water intake during conditioning; ●●● $p < 0.001$ as compared with the amount of odorized water intake during testing in all the other groups. **(B)** represents the mean proportion of time (± S.E.M.) spent in the conditioning context for each experimental group. Bars represent the proportion of time spent in the conditioning context (mean time spent in the conditioning context/time of the session) calculated in the pre-conditioning (gray bars) and testing (black) sessions. EC, lesion of the entorhinal cortex; Control, sham-lesion of the entorhinal cortex; COA, conditioned odor aversion; CCA, conditioned context aversion.

intake was significantly lower during conditioning than during testing in CCA 120 groups ($p < 0.05$) and that the amount of odorized water intake was lower during testing than during conditioning in EC-lesioned COA 120 group ($p < 0.001$). *Post-hoc* comparisons also indicated that, in EC-lesioned COA 120 group, the amount of odorized water intake during testing was lower than in all the other groups ($p < 0.001$ for each comparison).

As shown in **Figure 4B**, the proportion of time spent in the conditioning context was similar before conditioning and testing in both COA and CCA 120 groups, irrespective of the type of lesion. This suggests that no CCA occurred when the US was administered more than 120 min after the beginning of the context exposure. **Figure 4B** also showed that the proportion of time spent in the conditioning context seemed higher in CCA than in COA groups, indicative of an initial and maintained higher preference for the conditioning context in CCA 120 groups, as compared to COA 120 groups. Statistical analysis confirmed this observation and revealed a significant effect of Type of procedure [$F_{(1, 33)} = 10.42$, $p < 0.01$], but not of the other factors nor of any interaction.

Discussion

The results of the present study show that EC lesions induced a deficit in CCA but did not disrupt COA learning; on the contrary,

EC-lesioned animals were able to associate the CS with the US even though the ISI was too long to enable sham-lesioned control animals to learn the task. Moreover, the establishment of COA with long ISI obtained in EC-lesioned rats was associated with altered CCA learning.

Sham-lesioned control animals in the CCA group did not display COA with the two intervals tested between LiCl injection and odorized water exposure. In addition, only sham-lesioned control animals that received the US 20 min after the beginning of context exposure (CCA 5) displayed CCA, thus confirming previous findings (Desmedt et al., 2003). These results show that a backward arrangement between CS and US (i.e., no competition between CS and context) leads to CCA but not to COA. Moreover, odorized water intake in the CCA 120 groups was significantly lower than in CCA 5 groups in both sham- and EC-lesioned animals. This result suggests that LiCl affected odorized water intake when injected 60 min before presentation (in CCA 120 groups) but not when it was administered 180 min later (in CCA 5 groups). This temporary interference between LiCl and odorized water intake might reflect a novelty-dependent reaction, the duration of which is limited to the period during which the animal experiences malaise (Domjan, 1977).

Interestingly, the amplitude of the CCA observed in the sham-lesioned COA 5 group was not reduced by simultaneous exposure to the CS. This result suggests that the context was not overshadowed by the CS, and confirms that CCA can occur concomitantly to COA when the CS is presented together with the context during COA learning (Hatfield et al., 1992). On the other hand, the failure to obtain CCA with long ISI might be due to a latent inhibition effect (Lubow and Moore, 1959): previous studies using a conditioned fear paradigm (e.g., Kiernan and Wesbrook, 1993; Killcross et al., 1998) showed that extensive exposure to the to-be-conditioned context resulted in a reduction in contextual fear. Thus, the long exposure (i.e., 140 min) to the conditioning context in the COA 120 and CCA 120 groups may have affected the context-US association by a latent inhibition effect.

Most importantly, the present results show that the EC lesion disrupted CCA. CCA requires learning relations between the different cues present in the learning context, and associating these cues with the US. Since EC-lesioned animals were able to associate the olfactory CS with the US, it is unlikely that the CCA deficit resulted from a failure of US processing. Rather, a large number of studies suggest that it might result from a deficit in context information processing. First, the EC is reciprocally connected to the hippocampus and BLA (e.g., Amaral and Witter, 1995; Ferry et al., 1997; Pitkänen et al., 2000) and it has been previously assumed to be involved in the representation of context (reviews in, e.g., Maren and Fanselow, 1997; Majchrzak et al., 2006; Ji and Maren, 2008; Rudy, 2009; Van Strien et al., 2009; but: Phillips and LeDoux, 1995; Good and Honey, 1997; Bannerman et al., 2001; Hales et al., 2014). The amygdala is a downstream target of the hippocampus for the association of context representation with US (e.g., Fanselow, 2010) and also influences storage of the hippocampus-dependent representation of the conditioning context (Huff and Rudy, 2004; Huff et al., 2005). Moreover, it was recently shown that the glutamatergic projection from BLA to EC (Pitkänen et al., 2000) is involved in the modulation of the acquisition of contextual fear conditioning (Sparta et al., 2014). This suggests that the EC lesion may have impaired CCA through disruption of contextual information processing by both hippocampus and amygdala.

The present results also confirmed that the EC lesion did not disrupt but rather enabled COA, with ISIs up to 120 min (Ferry et al., 1996, 1999, 2006, 2007; Ferry and Di Scala, 1997). Conditioned odor aversion learning (COA) requires association between olfactory CS memory trace and US (Bures and Buresova, 1990; Roldan and Bures, 1994), and we have previously suggested that the EC is involved in the control of olfactory CS memory trace duration through a functional interaction with the BLA (Ferry et al., 1996, 1999; Ferry and Di Scala, 1997). As odor CS and context can both associate with the US in an interdependent way (Rescorla and Wagner, 1972), it is reasonable to suggest that the establishment of COA with long ISI obtained in EC-lesioned animals may have resulted, at least in part, from inhibition of the context influence upon the odor-US association due to the deficit in context processing.

Histological analysis of the lesion extent showed that the aspirative technique damaged a large portion of the lateral EC and part of the medial EC; in the light of previous findings that selective lesion of the lateral but not the medial EC affected COA with long ISI (Ferry et al., 2006), the present effects on COA were likely due to the lesion of the lateral part of the EC. As for the CCA effect, the present results do not indicate which part of the EC was selectively involved. In addition, the aspirative technique induced lesions of axons of passage in the EC and the disruptive effect observed on CCA may have resulted from a deficit in the processing of information arising from or passing through the EC.

Using discrete brain structure inactivation techniques, future studies will probably help to clarify this point, although both parts of the EC seem to be involved in the same kind of mechanism, at least when it comes to spatial processing (Van Cauter et al., 2012).

Conclusion

Feeding behavior is part of a complex integrated adaptive system. The differentiation between safe and unsafe food items that conditions ingestive behavior depends, at least in part, on previous experience during which the cues characterizing either the food (i.e., odor, taste, texture, etc.) or the environment in which the food is present (contextual cues) acquired a hedonic valence after feeding, through CS-US associative learning. These kinds of association have been experimentally studied for years (Slotnick and Katz, 1974; Nigrosh et al., 1975; Slotnick, 1984) and experimental conditioned food aversion paradigms, such as conditioned taste or odor/taste-potentiated odor aversion learning, have provided fundamental insights into the mechanisms and CNS structures involved in food-reward/food-poisoning associations (see Miranda, 2012 for review). In the case of conditioned aversion learning, numerous studies have shown that context processing influences the strength of the conditioned aversion to a taste acquired in a

given context (e.g., Puente et al., 1988; Loy et al., 1993; Skinner et al., 1994; Nakajima et al., 1995; Boakes et al., 1997; Lopez and Cantora, 2003; Murphy and Skinner, 2005; Ishii et al., 2006). Using another type of conditioned food aversion paradigm, the present study clearly shows that the conditions in which COA is established concomitantly to context aversion depends on the time interval separating the presentation of the odor and context from the US. Importantly, the results show that the EC is a key structure in the processes underlying the associations between context, odor CS and US in COA learning. Eventually, our results suggest the EC could be more largely be involved in the acquisition of conditioned food aversion learning through a control upon the association (1) between the odor of a particular food and a gastric malaise (US) that followed its ingestion and (2) between the context in which this food has been encountered and the US.

References

Amaral, D. G., and Witter, M. P. (1995). "Hippocampal formation," in *The Rat Nervous System, 2nd Edn.,* ed G. Paxinos (San Diego, CA: Academic Press), 443–493.

Andrews, E. A., and Braveman, N. S. (1975). The combined effects of dosage level and interstimulus interval on the formation of one-trial poison-based aversions in rats. *Anim. Learn. Behav.* 3, 287–289. doi: 10.3758/BF03213446

Bannerman, D. M., Yee, B. K., Lemaire, M., Jarrard, L., Iversen, S. D., Rawlins, J. N. P., et al. (2001). Contextual fear conditioning is disrupted by lesions of the subcortical, but not entorhinal, connections to the hippocampus. *Exp. Brain Res.* 141, 304–311. doi: 10.1007/s002210100869

Biella, G., and de Curtis, M. (2000). Olfactory inputs activate the medial entorhinal cortex via the hippocampus. *J. Neurophysiol.* 83, 1924–1931.

Boakes, R. A., Westbrook, R. F., Elliott, M., and Swinbourne, A. L. (1997). Context dependencyof conditioned aversions to water and sweet tastes. *J. Exp. Psycho Anim. Behav. Process.* 23, 56–67. doi: 10.1037/0097-7403.23.1.56

Bouton, M. E., Jones, D. L., McPhillips, S. A., and Shwartzentruber, D. (1986). Potentiation and overshadowing in odor-aversion learning: role of method of odor presentation, the distal-proximal cue distinction, and the conditionability of odor. *Learn. Motiv.* 17, 115–138. doi: 10.1016/0023-9690(86)90006-8

Bures, J., Buresova, O. (1990). Reversible lesions allowr einterpretation of system level studies of brain mechanisms of behavior. *Concepts Neurosci.* 1, 69–89.

Burwell, R. D., and Amaral, D. G. (1998). Cortical afferents of the perirhinal, postrhinal, and entorhinal cortices in the rat. *J. Comp. Neurol.* 398, 179–205.

Capaldi, E. D., Campbell, D. H., Sheffer, J. D., and Bradford, J. P. (1987). Conditioned flavor preferences based on delayed caloric consequences. *J. Exp. Psychol. Anim. Behav. Process.* 13, 150–155. doi: 10.1037/0097-7403.13.2.150

Chapuis, J., Messaoudi, B., Ferreira, G., and Ravel, N. (2007). Importance of retronasal and orthonasal olfaction for odor aversion memory in rats. *Behav. Neurosci.* 121, 1383–1392. doi: 10.1037/0735-7044.121.6.1383

Desmedt, A., Hazvi, S., and Dudai, Y. (2003). Differential pattern of cAMP response element-binding protein activation in the rat brain after conditioned aversion as a function of the associative process engaged: taste versus context association. *J. Neurosci.* 23, 6102–6110.

Domjan, M. (1977). Selective suppression of drinking during a limited period following aversive drug treatment in rats. *J. Exp. Psychol. Anim. Behav. Process.* 3, 66–76. doi: 10.1037/0097-7403.3.1.66

Fanselow, M. S. (2010). Drom contextual fear to a dynamic view of memory systems. *Trends Cog. Sci.* 14, 7–15. doi: 10.1016/j.tics.2009.10.008

Fedorchak, P. M., and Bolles, R. C. (1987). Hunger enhances the expression of calorie- but not taste-mediated conditioned flavor preferences. *J. Exp. Psychol. Anim. Behav. Process.* 13, 73–79. doi: 10.1037/0097-7403.13.1.73

Ferry, B., and Di Scala, G. (1997). Bicuculline administration into basolateral amygdala facilitates trace conditioning of odor aversion in the rat. *Neurobiol. Learn. Mem.* 67, 80–83. doi: 10.1006/nlme.1996.3743

Ferry, B., Ferreira, G., Traissard, N., and Majchrzak, M. (2006). Selective involvement of the lateral entorhinal cortex in the control of the olfactory memory trace during conditioned odor aversion in the rat. *Behav. Neurosci.* 120, 1180–1186. doi: 10.1037/0735-7044.120.5.1180

Ferry, B., Gervasoni, D., and Vogt, C. (2014). *Stereotaxic Neurosurgery in Laboratory Rodent—Handbook on Best Practices.* New York, NY: Springer Verlag Intl. editions.

Ferry, B., Herbeaux, K., Cosquer, B., Traissard, N., Galani, R., and Cassel, J. C. (2007). Immunotoxic cholinergic lesions in the basal forebrain reverse the effects of entorhinal cortex lesions on conditioned odor aversion in the rat. *Neurobiol. Learn. Mem.* 88, 114–126. doi: 10.1016/j.nlm.2007.01.007

Ferry, B., Magistretti, P. J., and Pralong, E. (1997). Noradrenaline action on the glutamatergic transmission in the rat basolateral amygdala *in vitro*: α_2-adrenoreceptor-mediated inhibition and β-adrenoreceptor-mediated excitation. *Eur. J. Neurosci.* 9, 1356–1364. doi: 10.1111/j.1460-9568.1997.tb01490.x

Ferry, B., Oberling, P., Jarrard, L. E., and Di Scala, G. (1996). Facilitation of conditioned odor aversion learning by entorhinal cortex lesions in the rat. *Behav. Neurosci.* 110, 443–450. doi: 10.1037/0735-7044.110.3.443

Ferry, B., Wirth, S., and Di Scala, G. (1999). Functional interaction between entorhinal cortex and basolateral amygdala during trace conditioning of odor aversion in the rat. *Behav. Neurosci.* 113, 118–125. doi: 10.1037/0735-7044.113.1.118

Garcia, J., Ervin, F. R., and Koelling, R. A. (1966). Learning with prolonged delay of reinforcement. *Psychon. Sci.* 5, 121–122. doi: 10.3758/BF03328311

Gnatkovsky, V., Uva, L., and de Curtis, M. (2004). Topographic distribution of direct and hippocampus-mediated entorhinal cortex activity evoked by olfactory tract stimulation. *Eur. J. Neurosci.* 20, 1897–1905. doi: 10.1111/j.1460-9568.2004.03627.x

Good, M., and Honey, R. C. (1997). Dissociable effects of selective lesions to hippocampal subsystems on exploratory behavior, contextual learning, and spatial learning. *Behav. Neurosci.* 111, 487–493. doi: 10.1037/0735-7044.111.3.487

Haberly, L. B., and Price, J. L. (1978). Association and commissural fiber systems of the olfactory cortex of the rat: II. Systems originating in the olfactory peduncle. *J. Comp. Neurol.* 181, 781–808. doi: 10.1002/cne.901810407

Hales, J. B., Schlesiger, A. I., Leurgeb, J. K., Squire, L. R., Leutgeb, S., and Clark, R. E. (2014). Medial entorhinal cortex lesions only partially disrupt hippocampal place cells and hippocampus-dependent place memory. *Cell Reports* 9, 893–901. doi: 10.1016/j.celrep.2014.10.009

Hankins, W. G., Garcia, J., and Rusiniak, K. W. (1973). Dissociation of odor and taste in baitshyness. *Behav. Biol.* 8, 407–419. doi: 10.1016/S0091-6773(73)80081-1

Harris, J. A., Gorissen, M. C., Bailey, G. K., and Westbrook, R. F. (2000). Motivational state regulates the content of learned flavor preferences. *J. Exp. Psychol. Anim. Behav. Process.* 26, 15–30. doi: 10.1037/0097-7403.26.1.15

Hatfield, T., Graham, P. W., and Gallagher, M. (1992). Taste-potentiated odor aversion learning: role of the amygdaloid basolateral complex and central nucleus. *Behav Neurosci.* 106, 286–293. doi: 10.1037/0735-7044.106.2.286

Huff, N. C., and Rudy, J. W. (2004). The amygdala modulates hippocampus-dependent context memory formation and stores cue-shock associations. *Behav. Neurosci.* 118, 53–62. doi: 10.1037/0735-7044.118.1.53

Huff, N. C., Wright-Hardesty, K. J., Higgins, E. A., Matus-Amat, P., and Rudy, J. W. (2005). Context pre-exposure obscures amygdala modulation of contextual-fear conditioning. *Learn. Mem.* 12, 456–460. doi: 10.1101/lm.6705

Ishii, K., Iguchi, Y., and Sawa, K. (2006). Context dependency of aversion to familiar andnovel fluids. *Learn. Motiv.* 37, 113–130. doi: 10.1016/j.lmot.2005.04.002

Ji, J., and Maren, S. (2008). Lesions of the entorhinal cortex or fornix disrupt the context-dependence of fear extinction in rats. *Behav. Brain Res.* 194, 201–206. doi: 10.1016/j.bbr.2008.07.011

Kiernan, M. J., and Wesbrook, R. F. (1993). Effects of exposure to a to-be-showked environment upon the rat's freezing response: evidence for facilitation, latent

inhibition, and perceptual learning. *Q. J. Exp. Psychol. B* 46, 271–288. doi: 10.1080/14640749308401089

Killcross, A. S., Kiernan, M. J., Dwyer, D., and Westbrook, R. F. (1998). Loss of latent inhibition of contextual conditioning following non-reinforced context exposure in rats. *Q. J. Exp. Psychol. B*. 51, 75–90.

Krettek, J. E., and Price, J. L. (1977). The cortical projections of the mediodorsal nucleus and adjacent thalamic nuclei in the rat. *J. Comp. Neurol.* 171, 157–191. doi: 10.1002/cne.901710204

Lopez, M., and Cantora, R. (2003). Associative interference with taste aversions after con-textual discrimination learning. *Learn. Motiv.* 34, 372–388. doi: 10.1016/S0023-9690(03)00035-3

Lorden, J. F., Kenfield, M., and Braun, J. J. (1970). Response suppression to odors paired with toxicosis. *Learn. Motiv.* 1, 391–400. doi: 10.1016/0023-9690(70)90103-7

Loy, I., Alvarez, R., Rey, V., and Lopez, M. (1993). Context-US associations rather thanoccasion-setting in taste aversion learning. *Learn. Motiv.* 24, 55–72. doi: 10.1006/lmot.1993.1004

Lubow, R. E., and Moore, A. U. (1959). Latent inhibition: the effect of non reinforced pre-exposure to the conditioned stimulus. *J. Comp. Physiol. Psychol.* 52, 415–419. doi: 10.1037/h0046700

Majchrzak, M., Ferry, B., Marchand, A. R., Herbeaux, K., Seillier, A., and Barbelivien, A. (2006). Entorhinal cortex lesions disrupt fear conditioning to background context but spare fear conditioning to a tone in the rat. *Hippocampus* 16, 114–124. doi: 10.1002/hipo.20138

Maren, S., and Fanselow, M. S. (1997). Electrolytic lesions of the fimbria/fornix, dorsal hippocampus, or entorhinal cortex produce anterograde deficits in contextual fear conditioning in rats. *Neurobiol. Learn. Mem.* 67, 142–149. doi: 10.1006/nlme.1996.3752

Mehiel, R., and Bolles, R. C. (1984). Learned flavor preferences based on caloric outcome. *Anim. Learn. Behav.* 12, 421–427. doi: 10.3758/BF03199989

Miranda, M. I. (2012). Taste and odor recognition memory: the emotional flavor of life. *Rev. Neurosci.* 23, 481–499. doi: 10.1515/revneuro-2012-0064

Mouly, A. M., and Di Scala, G. (2006). Entorhinal cortex stimulation modulates amygdala and piriform cortex responses to olfactory bulb inputs in the rat. *Neuroscience* 137, 1131–1141. doi: 10.1016/j.neuroscience.2005.10.024

Murphy, M., and Skinner, D. M. (2005). Contextual control of fluid consumption: the effectsof context extinction. *Learn. Motiv.* 36, 297–311. doi: 10.1016/j.lmot.2004.11.002

Nakajima, S., Kobayashi, Y., and Imada, H., (1995). Contextual control of taste aver-sion in rats: the effects of context extinction. *Psychol. Record* 45, 309–318.

Nigrosh, B. J., Slotnick, B. M., and Nervin, J. A. (1975). Olfactory discrimination, reversal learning, and stimulus control in rats. *J. Physiol. Psychol.* 89, 285–294. doi: 10.1037/h0076821

Palmerino, C. C., Rusiniak, K. W., and Garcia, J. (1980). Flavor–Illness aversions: the peculiar roles of odor and taste in memory for poison. *Science* 208, 753–755. doi: 10.1126/science.7367891

Paxinos, G., and Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates, 4th Edn.* New York, NY: Academic Press.

Phillips, R. G., and LeDoux, J. E. (1995). Lesions of the fornix but not the entorhinal cortex or perirhinal cortex interfere with contextual fear conditioning. *J. Neurosci*. 15, 5308–5315.

Pitkänen, A., Pikkarainen, M., Nurminen, N., and Ylinen, A. (2000). Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. *Ann. N.Y. Acad. Sci.* 911, 369–391. doi: 10.1111/j.1749-6632.2000.tb06738.x

Puente, G. P., Cannon, D. S., Best, M. R., and Carrell, L. E. (1988). Occasion setting of fluid ingestion by contextual cues. *Learn. Motiv.* 19, 239–253. doi: 10.1016/0023-9690(88)90003-3

Rescorla, R. A., and Wagner, A. R. (1972). "A theory of Pavlovian conditioning; variations in the effectiveness of reinforcement and non reinforcement," in *Classical Conditioning II: Current Research and Theory*, eds A. H. Black andW. F. Prokasy (New York, NY: Appleton-Century- Crofts), 64–99.

Roldan, G., and Bures, J. (1994). Tetrodotoxin blockade of amygdala overlapping with poisoning impairs acquisition of conditioned taste aversion in rats. *Behav. Brain Res.* 65, 213–219. doi: 10.1016/0166-4328(94)90107-4

Rudy, J. W. (2009). Context representations, context functions, and the parahippocampal-hippocampal system. *Learn. Mem.* 16, 573–585. doi: 10.1101/lm.1494409

Skinner, D. M., Martin, G. M., Pridgar, A., and van der Kooy, D. (1994). Conditional control of fluid consumption in an occasion setting paradigm is independent of Pavlovian associations. *Learn. Motiv.* 25, 368–400. doi: 10.1006/lmot.1994.1019

Slotnick, B. M. (1984). Olfactory stimulus control in the rat. *Chem. Senses* 9, 157–165. doi: 10.1093/chemse/9.2.157

Slotnick, B. M., and Katz, H. M. (1974). Olfactory learning set formation in rats. *Science* 185, 796–798. doi: 10.1126/science.185.4153.796

Slotnick, B. M., Westbrook, F., and Darling, F. M. C. (1997). What the rat's nose tells the rat's mouth: long delay aversion conditioning with aqueous odors and potentiation of taste by odors. *Anim. Learn. Behav.* 25, 357–369. doi: 10.3758/BF03199093

Sparta, D. R., Smithuis, J., Stamatakis, A. M., Jennings, J. H., Kantak, P. A., Ung, R. L., et al. (2014). Inhibition of projections from the basolateral amygdala to the entorhinal cortex disrupts the acquisition of contextual fear. *Front. Behav. Neurosci.* 8:129. doi: 10.3389/fnbeh.2014.00129

Taukulis, H. K. (1974). Odor aversions produced over long CS-US delays. *Behav. Biol.* 10, 505–510. doi: 10.1016/S0091-6773(74)92111-7

Van Cauter, T., Camon, J., Alvernhe, A., Elduayen, C., Sargolini, F., and Save, E. (2012). Distinct roles of medial and lateral enrorhinal cortex in spatial cognition. *Cereb. Cortex* 23, 451–459. doi: 10.1093/cercor/bhs033

Van Strien, N. M., Cappaert, N. L. M., and Witter, M. P. (2009). The anatomy of memory: an interactive overview of the parahippocampal-hippocampal network. *Nature* 10, 272–282. doi: 10.1038/nrn2614

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 14 July 2015
doi: 10.3389/fnins.2015.00244

Tuning properties and dynamic range of type 1 vomeronasal receptors

Sachiko Haga-Yamanaka[1†], *Limei Ma*[1] *and C. Ron Yu*[1,2*]

[1] *Stowers Institute for Medical Research, Kansas City, MO, USA,* [2] *Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS, USA*

The mouse vomeronasal organ (VNO) expresses chemosensory receptors that detect intra-species as well as inter-species cues. The vomeronasal neurons are thought to be highly selective in their responses. The tuning properties of individual receptors remain difficult to characterize due to the lack of a robust heterologous expression system. Here, we take a transgenic approach to ectopically express two type 1 vomeronasal receptors in the mouse VNO and characterize their responses to steroid compounds. We find that V1rj2 and V1rj3 are sensitive to two sulfated estrogens (SEs) and can be activated by a broad variety of sulfated and glucuronidated steroids at high concentrations. Individual neurons exhibit narrow range of concentration-dependent activation. Collectively, a neuronal population expressing the same receptor covers a wide dynamic range in their responses to SEs. These properties recapitulate the response profiles of endogenous neurons to SEs.

Keywords: vomeronasal receptor, calcium imaging, sulfated steroids, transgenic mice, glucuronidated steroid, GCaMP2

OPEN ACCESS

Edited by:
Markus Fendt,
Otto-von-Guericke University
Magdeburg, Germany

Reviewed by:
Hiro Matsunami,
Duke University, USA
Carla Mucignat,
University of Padova, Italy
Roberto Tirindelli,
University of Parma, Italy

****Correspondence:***
C. Ron Yu,
Stowers Institute for Medical Research, 1000 E. 50th Street, Kansas City, MO 64110, USA
cry@stowers.org

†Present Address:
Sachiko Haga-Yamanaka, Department of Cell Biology and Neuroscience, University of California, Riverside, CA, USA

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *08 May 2015*
Accepted: *26 June 2015*
Published: *14 July 2015*

Citation:
Haga-Yamanaka S, Ma L and Yu CR (2015) Tuning properties and dynamic range of type 1 vomeronasal receptors. Front. Neurosci. 9:244. doi: 10.3389/fnins.2015.00244

Introduction

Terrestrial animals emit pheromones for intra-species chemo-communication. Within the same species, pheromones convey information about sexual, social, and reproductive status of individuals. They trigger a restricted repertoire of innate and stereotyped behaviors, including mating rituals, territorial aggression, and neuroendocrine responses (Wyatt, 2003). The vomeronasal organ (VNO) plays an important role for detecting pheromones in most terrestrial vertebrate species (Halpern and Martinez-Marcos, 2003; Swaney and Keverne, 2009). The mouse VNO expresses about 400 seven-transmembrane G protein-coupled receptors including the type 1 vomeronasal receptors (V1Rs), type 2 vomeronasal receptors (V2Rs), and formyl peptide receptors (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; Young and Trask, 2007; Liberles et al., 2009; Riviere et al., 2009; Young et al., 2010). Each vomeronasal sensory neuron (VSN) expresses either a single member of V1R or FPR, or a specific pair of V2R genes (Ibarra-Soria et al., 2014; Liberles, 2014). These receptors are dedicated to detect a variety of chemosensory cues and transmit the signal to the brain.

In natural environment, pheromone cues are present in bodily secretion and excretion, such as tears, feces, and urine (Ihara et al., 2013). The chemical nature of the pheromones ranges from volatile and non-volatile small molecular weight compounds to peptides and proteins (Novotny et al., 1985, 1986; Jemiolo et al., 1989; Leinders-Zufall et al., 2004; Kimoto et al., 2005; Chamero et al., 2007; Nodari et al., 2008; Riviere et al., 2009; Haga et al., 2010; Roberts et al., 2010, 2012; Ferrero et al., 2013). A unique pumping mechanism is required to bring the pheromone cues into the vomeronasal cavity where they are in contact with the dendrites of VSNs

(Meredith et al., 1980; Meredith, 1994). In addition to their chemical identities, pheromone concentrations also convey specific information (He et al., 2010; Ihara et al., 2013). The vomeronasal system is able to detect cues at various concentrations and convey concentration-invariant signals to the brain (Arnson and Holy, 2013).

VSNs are able to detect pheromone cues with high sensitivity and selectivity. In some studies, VSNs can respond specifically to ligands at sub-nanomolar concentrations (Leinders-Zufall et al., 2000, 2004, 2014; Kimoto et al., 2005; He et al., 2010; Isogai et al., 2011; Haga-Yamanaka et al., 2014). It is not understood how the VSN achieve a balance between receptor sensitivity and a wide dynamic range of detection. There are two plausible mechanisms that can be considered. In one scenario, neurons expressing the same receptor may exhibit different sensitivities to accommodate a wide range of ligand concentrations. Alternatively, neurons expressing different receptors with distinct sensitivities to the ligand broadens the detectable range of pheromones. Current available data do not distinguish these two hypotheses.

Thus, in order to understand the mechanisms of pheromone tuning, it is important to characterize the specific interactions between pheromones and their cognate receptors. However, after nearly 20 years of cloning of the first V1Rs, we have limited understanding of the receptor characteristics and functions. The main obstacle is the lack of a robust heterologous expression system that enables functional analysis of VR responses. In an earlier study, we overcome this challenge by generating transgenic mice that ectopically express V1Rs in VSNs (Haga-Yamanaka et al., 2014). The ectopic expression of vomeronasal receptors in their native environment enables us to identify V1rj2 and V1rj3 receptors as the cognate receptors for sulfated estrogens (SEs). In the current study, we further characterize the response profiles of V1rj2 and V1rj3 receptors to a panel of sulfated and glucuronidated steroids.

Materials and Methods

Mice

All mice were maintained in Lab Animal Service Facility of Stowers Institute at 12:12 light/dark cycle and provided with food and water *ad libitum*. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Stowers Institute and in compliance with NIH Guide for Care and Use of Animals. OMP-IRES-tTA (OIVT), Gγ8-tTA, tetO-GCaMP2, tetO-V1rj2-IRES-tdTomato, and tetO-V1rj3-IRES-tdTomato mice were described previously (Yu et al., 2004; Nguyen et al., 2007; He et al., 2008; Haga-Yamanaka et al., 2014). Mice containing OIVT, Gγ8-tTA, and tetO-GCaMP2 alleles were used to assess the wild type responses. Compound heterozygotic mice that contained tetO-V1rj2-IRES-tdTomato and tetO-V1rj3-IRES-tdTomato were generated to assess the V1rj2 and V1rj3 responses, respectively. VNO slices were prepared from 2 to 6 months old male and female mice.

Chemicals

Sulfated and glucuronidated steroids were purchased from Steraloids (Newport, RI, USA), and the catalog IDs were used to label the compounds. We dissolved the steroids in dimethyl sulfoxide (DMSO) to make 20 mM stock solutions, which were further diluted in Ringer's solution (in mM: 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 $NaHCO_3$, 10 HEPES, and 10 glucose) to the testing concentrations.

Calcium Imaging of VNO Slices

Details of imaging setup and procedures were described previously (Ma et al., 2011). Briefly, VNO slices were maintained in carboxygenated (95% O_2, 5% CO_2) mouse artificial cerebrospinal fluid (mACSF; in mM: 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, and 10 glucose) at room temperature. Carboxygenated mACSF was also used to superfuse VNO slices at a speed of 1 mL/min. The flow was kept unidirectional by placing the inlet and outlet at the apical and basal sides of VNO epithelium, respectively. Steroids were delivered through a HPLC injection valve mounted on the stage. To minimize mechanical artifacts, a continuous flow (∼0.3 mL/min) of Ringer's solution was maintained during the experiment. Solutions were switched by using the injection valve without disrupting the flow.

Time-lapse imaging acquisition of GCaMP2 signals from a VNO slice was performed on AxioScope FS2 (Carl Zeiss) microscope with a 20X/0.5NA water-dipping lens as described before (He et al., 2008; Ma et al., 2011; Yu, 2013; Haga-Yamanaka et al., 2014). Intervals between stimuli were at least 3 min. When a robust response to a given chemical was observed, we set the inter-stimulus interval to 5–10 min (varied with response level) to ensure adequate recovery. Data of individual genotypes or applications were collected from two to three VNO slices. Image processing and data analysis, including region of interest (ROI) detection and automated signal analyses, were performed using ImageJ and programs written in MATLAB. In order to compute F, a baseline fitting step was performed to model photo-bleaching effect and a peak detection step was performed to model the signal peak. ΔF was computed as the difference between the signal peak and the baseline. The computation was then manually validated to exclude possible errors. A threshold of 30% ΔF/F was imposed to identify VSNs as responding to a stimulus. Response amplitudes were normalized in each individual cell. For ensemble responses, the dose-response curve was fitted with a sigmoidal equation and EC50 were calculated using Prism (GraphPad).

Results

VSN Response to Sulfated Estrogens

Two of the SEs, 1, 3, 5(10)-estratrien-3, 17β-diol disulfate (E1050), and 1, 3, 5(10)-estratrien-3, 17β-diol 17-sulfate (E1103), strongly activate VSNs and can serve as estrus signals to induce mounting behaviors in male mice (Haga-Yamanaka et al., 2014). To understand the dose-dependent activation of VSNs, we performed Ca^{2+} imaging experiments using VNO slices from GCaMP2 mice. We observed VSN responses to E1050 and E1103 at 10^{-10} M in ∼2–4% of cells on a single slice (**Figures 1A,B**). The number of responding VSNs increased in a dose-dependent manner and reached a plateau of approximately 30 cells per

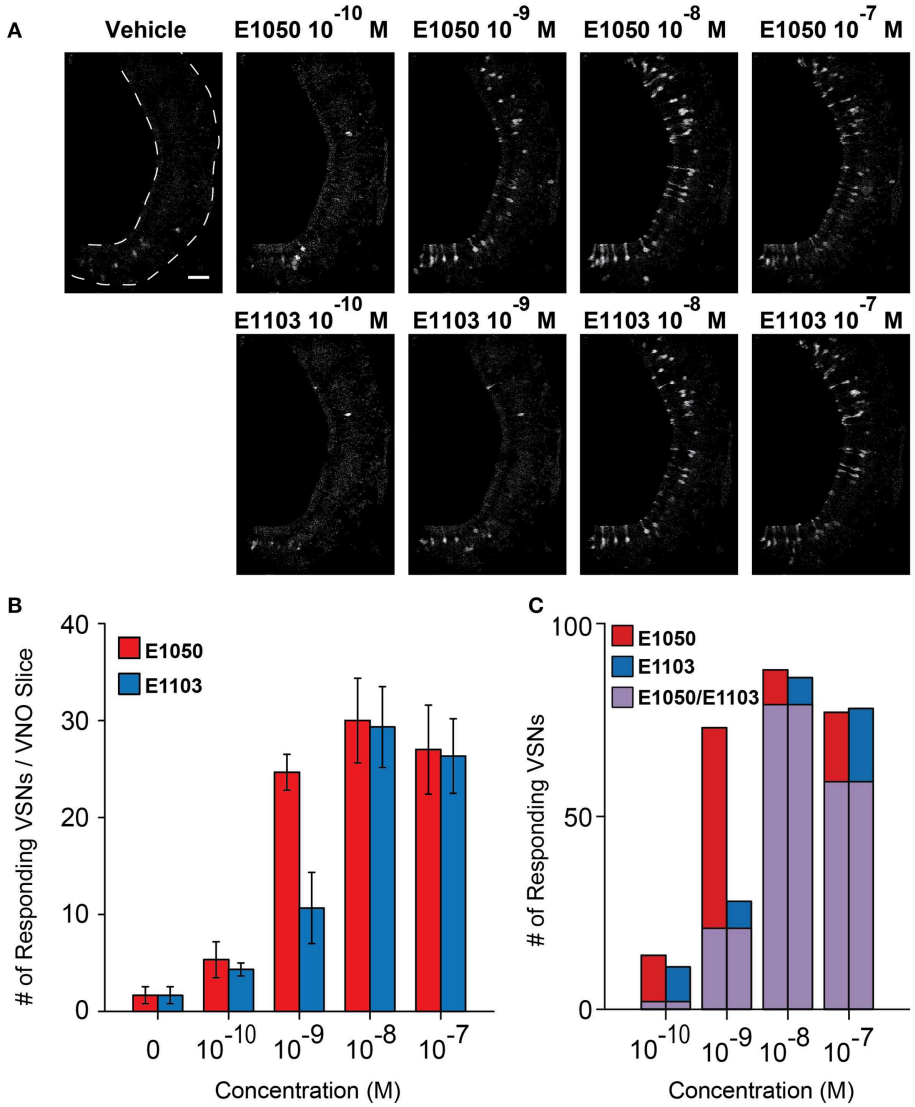


FIGURE 1 | Activation of VSN by E1050 and E1103. (A) Representative imaging experiments showing the VSN responses to E1050 (top) and E1103 (bottom). Scale bar, 50 μm. **(B)** Bar graph showing the number of VSNs activated by E1050 (red) and E1103 per slice (blue; $n = 3$ slices). Error bars, S.E.M. **(C)** Bar graph showing the number of E1050- and/or E1103-responding VSNs in 3 slices. Red, blue, and purple indicate VSNs activated by E1050, E1103, and both E1050 and E1103, respectively.

slice, which represented approximately 15% of the cell population (**Figures 1A,B**).

A comprehensive survey of the VSN response profiles showed that individual neurons exhibited diverse dose-response properties and sensitivities to E1050. Some cells showed the classic sigmoidal dose-response curves with increasing amplitude as a function of E1050 concentration (**Figures 2A,C,D**). The signal plateaued at higher concentrations, which indicated a saturation of the response. Neurons exhibiting these classic dose-response curves, however, only represented a fraction of the total VSNs. We found a large fraction (∼60%) of neurons displayed bell-shaped curves (**Figures 2B–D**). The peak response was reached at an intermediate concentration. Further increase of ligand concentration led to reduced response. A few cells displayed dose-response properties that did not fit either sigmoidal or bell-shaped curves (**Figures 2C,D**).

We also observed VSNs displayed different sensitivities to SE activation (**Figure 2D**). We used the first response concentration (FRC) as a measurement of sensitivity. Overall, the FRCs varied at least four orders of magnitude from 10^{-10} to 10^{-7} M. We observed some cells started to respond at 10^{-7} M, which was the highest concentration tested for these two SEs. We marked those high-threshold cells as with FRC at 10^{-7} M. Regardless of the shape of their dose-response curves, individual neurons had relatively narrow dynamic ranges.

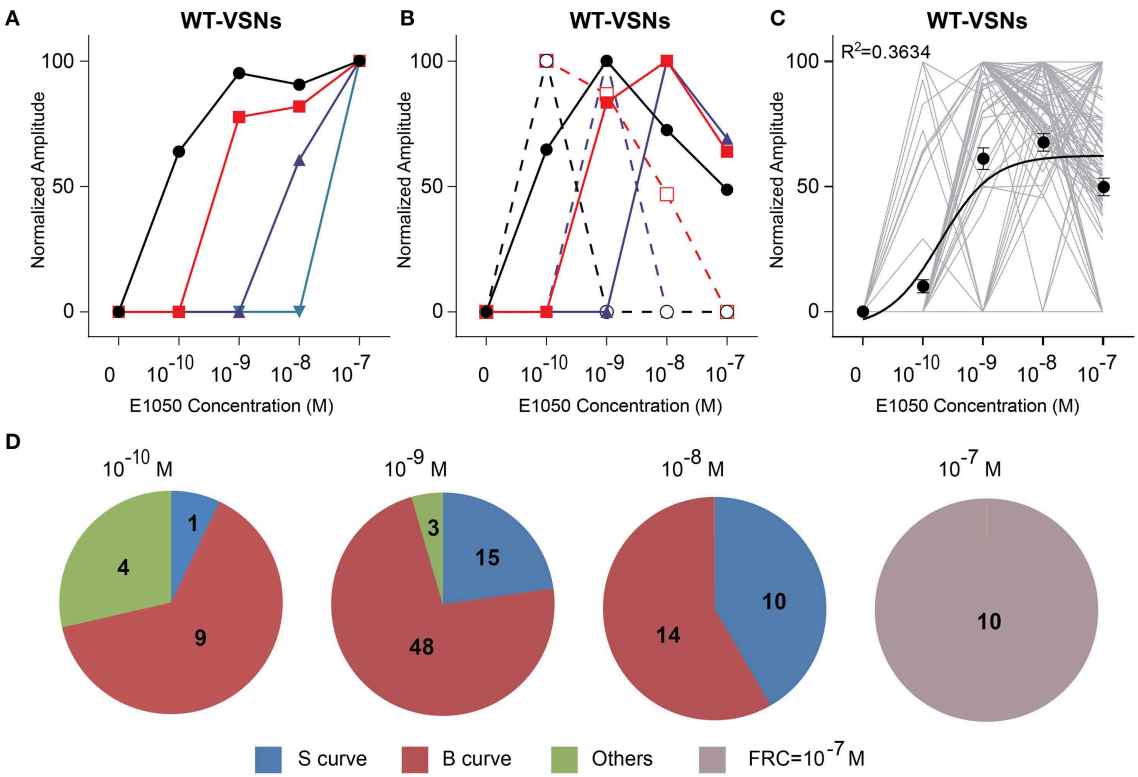


FIGURE 2 | Activation of VSN by E1050. (A,B) Examples of sigmoidal **(A)** and bell-shaped **(B)** dose-response curves to E1050. **(C)** Dose-response curves of individual cells (gray) and a sigmoidal curve (black) fitted to the average amplitude from all cells. Error bar, S.E.M. **(D)** Pie charts showing the number of cells exhibiting sigmoidal (S, blue), bell-shaped (B, red), and other (O, green) types of dose responses. Cells with FRC of 10^{-7} M are shown in gray. Concentration above each pie chart represents the first response concentration of the cells.

Approximately 90% of neurons showed the maximal response at 10x FRC.

Although individual neurons had different sensitivities and narrow dynamic ranges, VSN population can respond to a wide range of pheromone stimulation collectively (**Figure 2C**). The average response to E1050 had an EC50 of 1.921×10^{-10} M with a dynamic range of 1000 fold change in concentration.

The responses to E1103, a singly-sulfated estrogen compound, elicited VSN responses at as low as 10^{-10} M (**Figures 1A,B**). At this concentration, ~85% of the neurons activated by E1103 and E1050 were distinct (**Figure 1C**). Compared to E1050, the number of E1103 responding VSNs showed a slower increase with rising concentration and did not plateau until 10^{-8} M (**Figure 1B**). At concentrations higher than 10^{-9} M, the majority of E1103 responding VSNs overlapped with E1050 responding cells (**Figure 1C**). At individual cell level, the sensitivity ranged across three orders of magnitude. Consistent with the number of responding VSNs, we found that a large fraction of the cells showed peak response to E1103 at 10^{-8} M. Both sigmoidal and bell-shaped dose-response curves were observed (**Figures 3A,B,D**). On average, responses to E1103 had an EC50 of 1.348×10^{-9} M (**Figure 3C**).

The bell-shaped dose-response curve also has been observed in urine-responding VSN neurons (He et al., 2010). Because the VSNs showed little adaptation to repeated or prolonged stimuli (Holy et al., 2000; He et al., 2010), it was unlikely that the diminution of response at high ligand concentration was caused by cell fatigue or receptor desensitization. To further exclude these possibilities, we examined the response of individual cells to different ligands. As shown in **Figures 3E,F**, the same cell exhibited reduced response to high concentration (10^{-7} M) of E1103, but subsequent application of E1050 nonetheless elicited strong responses. The same cells, therefore, exhibited distinct dose-dependent activations to different ligands that was not related to stimulation sequence.

Activation of V1rj2 by Sulfated Estrogens

In a previous study, we showed that the V1rj clade receptors respond to SEs by using transgenic mice lines in which V1rj2 and V1rj3 were ectopically expressed (Haga-Yamanaka et al., 2014). These mice allowed us to further examine the activation of individual V1rj receptors activated by SEs.

V1rj2-expressing VSNs showed dose-dependent activation by both SEs (**Figures 4**, **5**). E1050 activated V1rj2-expressing VSNs at 10^{-10} M and the numbers of responding cells plateaued at 10^{-8} M (**Figure 4B**). These VSNs showed both the classic dose-response curves and bell-shaped curves (**Figures 4A,C,E**). In addition, the majority of V1rj2-expressing VSNs started

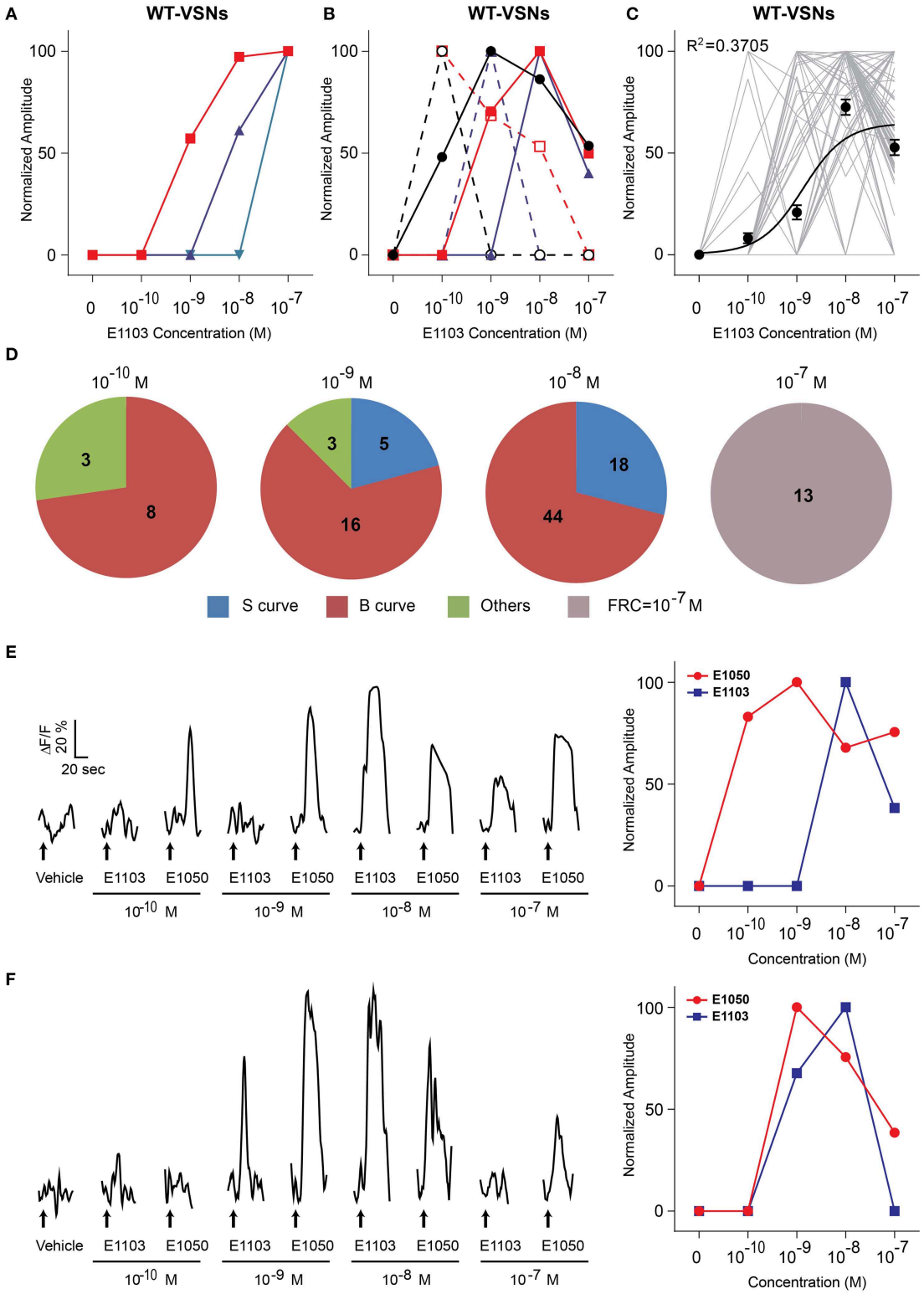


FIGURE 3 | Activation of VSN by E1103. (A,B) Examples of sigmoidal **(A)** and bell-shaped **(B)** dose-response curves to E1103. **(C)** Dose-response curves of individual cells (gray) and a sigmoidal curve (black) fitted to the average amplitude from all cells. Error bar, S.E.M. **(D)** Pie charts showing the number of cells exhibiting sigmoidal (S, blue), bell-shaped (B, red) and other (O, green) types of dose responses. Cells with FRC of 10^{-7} M are shown in gray. **(E,F)** Raw traces (left) and dose-response curves (right) of two representative cells simulated by E1050 and E1103 in a sequence with increasing concentrations. Arrows indicate the onset of stimulus delivery.

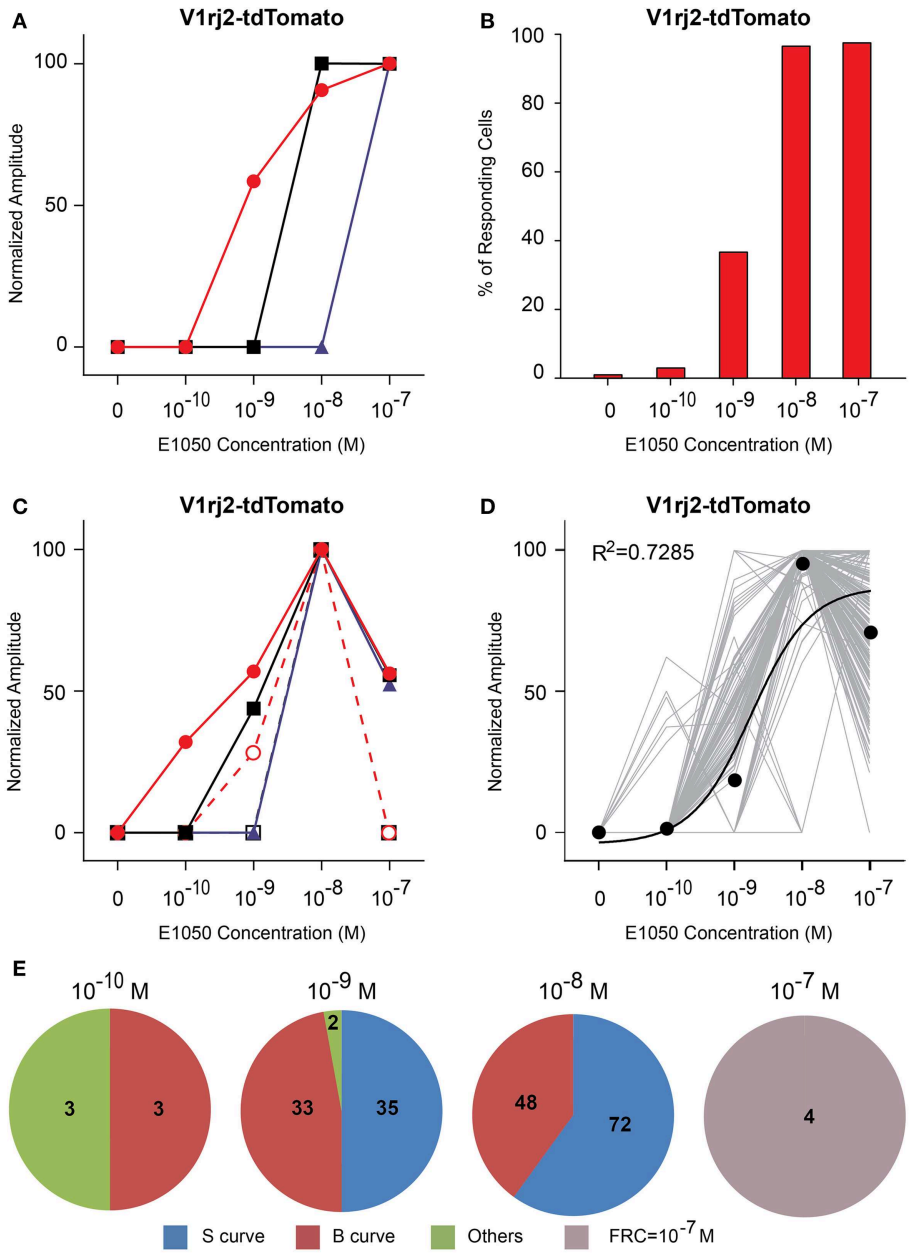


FIGURE 4 | Activation of V1rj2 by E1050. (A) Examples of sigmoidal dose-response curves to E1050 in V1rj2 cells. **(B)** Bar graph showing the percentage of E1050 activated V1rj2 cells ($n = 200$). **(C)** Examples of bell-shaped dose- response curves to E1050 in V1rj2 cells. **(D)** Dose-response curves of individual cells (gray) and a sigmoidal curve (black) fitted to the average amplitude from all cells. Error bar, S.E.M. **(E)** Pie charts showing the number of cells exhibiting sigmoidal (S, blue), bell-shaped (B, red), and other (O, green) types of dose responses. Cells with FRC of 10^{-7} M are shown in gray.

responding to E1050 at 10^{-9} M and reached the maximal response amplitude at 10^{-8} M (**Figure 4D**). On average, the V1rj2 neurons covered a dynamic range of 1000 folds (**Figure 4D**).

Compared to E1050, fewer V1rj2-expressing VSNs were activated by E1103 at 10^{-10} M (**Figure 5B**). Interestingly, the majority of VSNs exhibited classic dose-response curves (**Figure 5E**). Most of V1rj2-expressing VSNs started to respond at 10^{-8} M and peaked at 10^{-7} M (**Figures 5B,D**).

Activation of V1rj3 by Sulfated Estrogens

Compared to V1rj2 cells, VSNs expressing the V1rj3 receptor showed distinct response characteristics to E1050 (**Figure 6**). The majority of the cells only responded at or above 10^{-8} M (**Figure 6B**) and less than 30% of the cells

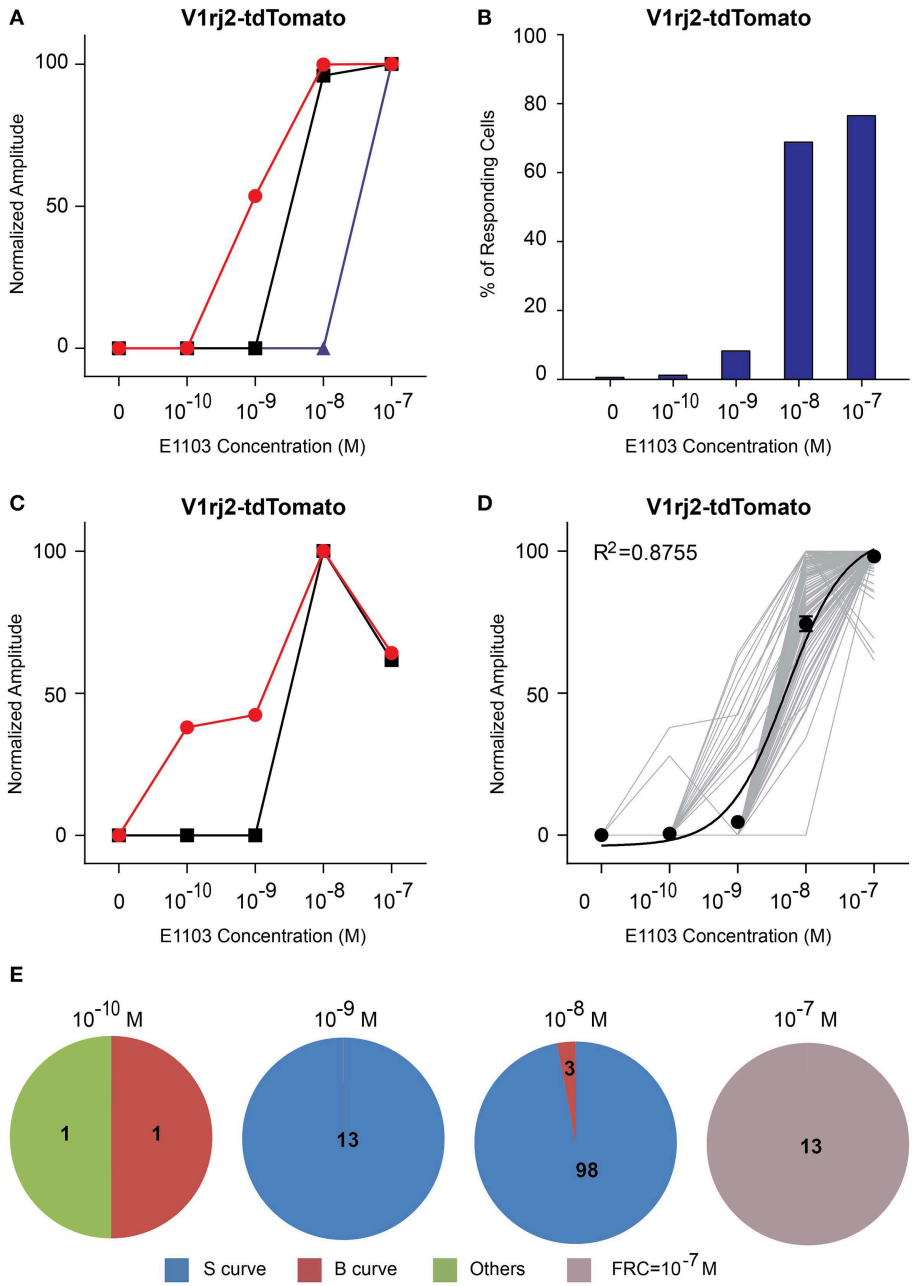


FIGURE 5 | Activation of V1rj2 by E1103. (A) Examples of sigmoidal dose-response curves to E1103 in V1rj2 cells. **(B)** Bar graph showing the percentage of E1103 activated V1rj2 cells ($n = 129$). **(C)** Examples of bell-shaped dose-dependent responses of V1rj2 cells to E1103. **(D)** Dose-response curves of individual cells (gray) and a sigmoidal curve (black) fitted to the average amplitude from all cells. Error bar, S.E.M. **(E)** Pie charts showing the number of cells exhibiting sigmoidal (S, blue), bell-shaped (B, red), and other (O, green) types of dose responses. Cells with FRC of 10^{-7} M are shown in gray.

exhibited bell-shaped response curves (**Figure 6E**). A significant number of V1rj3-expressing cells were activated by E1050 at 10^{-10} M and showed maximal response at this concentration (**Figure 6D**). These populations of VSNs make this single receptor cover a wide dynamic range in their response to SEs (**Figure 6D**).

The V1rj3-expressing cells also responded to E1103 with high sensitivities (**Figure 7**). About 50% of the cells responded at 10^{-10} M (**Figure 7B**). The number of responding cells plateaued at 10^{-8} M. Approximately 45% of the cells exhibited bell-shaped dose-response curves, with the maximal responses elicited between 10^{-10} and 10^{-8} M (**Figures 7D,E**).

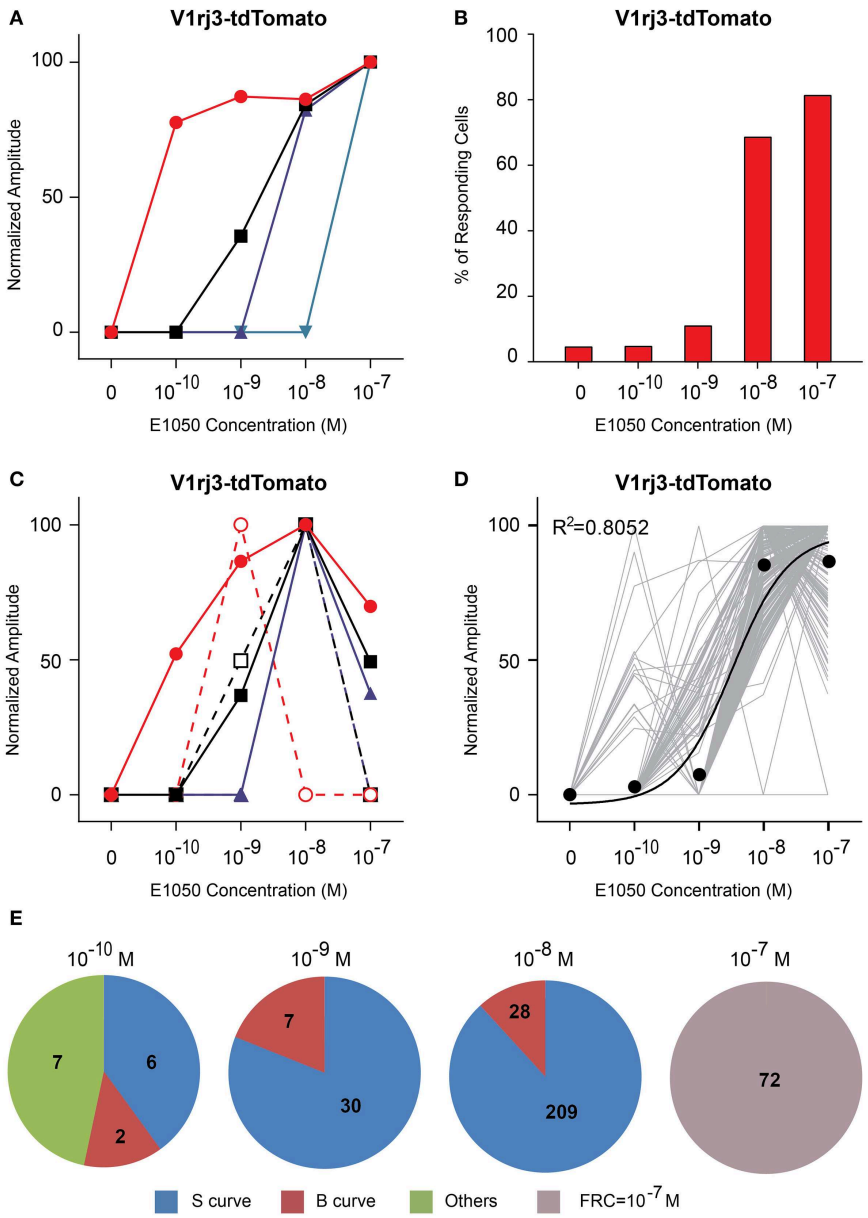


FIGURE 6 | Activation of V1rj3 by E1050. (A) Examples of sigmoidal dose-response curves to E1050 in V1rj3 cells. **(B)** Bar graph showing the percentage of E1050 activated V1rj3 cells ($n = 256$). **(C)** Examples of bell-shaped dose-dependent responses of V1rj3 cells to E1050. **(D)** Dose-response curves of individual cells (gray) and a sigmoidal curve (black) fitted to the average amplitude from all cells. Error bar, S.E.M. **(E)** Pie charts showing the number of cells exhibiting sigmoidal (S, blue), bell-shaped (B, red), and other (O, green) types of dose responses. Cells with FRC of 10^{-7} M are shown in gray.

These results suggested that the V1rj2 receptor was more sensitive to E1050, while the V1rj3 receptor was more sensitive to E1103. At the individual cell level, both V1rj2 and V1rj3 VSNs exhibited rather narrow dynamic range. The maximal response was typically observed at a concentration just 10x of FRC. Collectively, each receptor type was able to cover the concentration range of SE spanning three orders of magnitude.

Activation of V1rj2 Receptor by Glucuronidated Estrogens

Steroid hormones can be modified after initial synthesis and during circulation. Circulating estrogen molecules are modified by specific enzymes to become sulfated or glucuronidated estrogens (GEs), which are soluble and can be excreted in urine (Shackleton, 1986; Blair, 2010). We therefore examined whether GEs also activated the V1rj2 and V1rj3

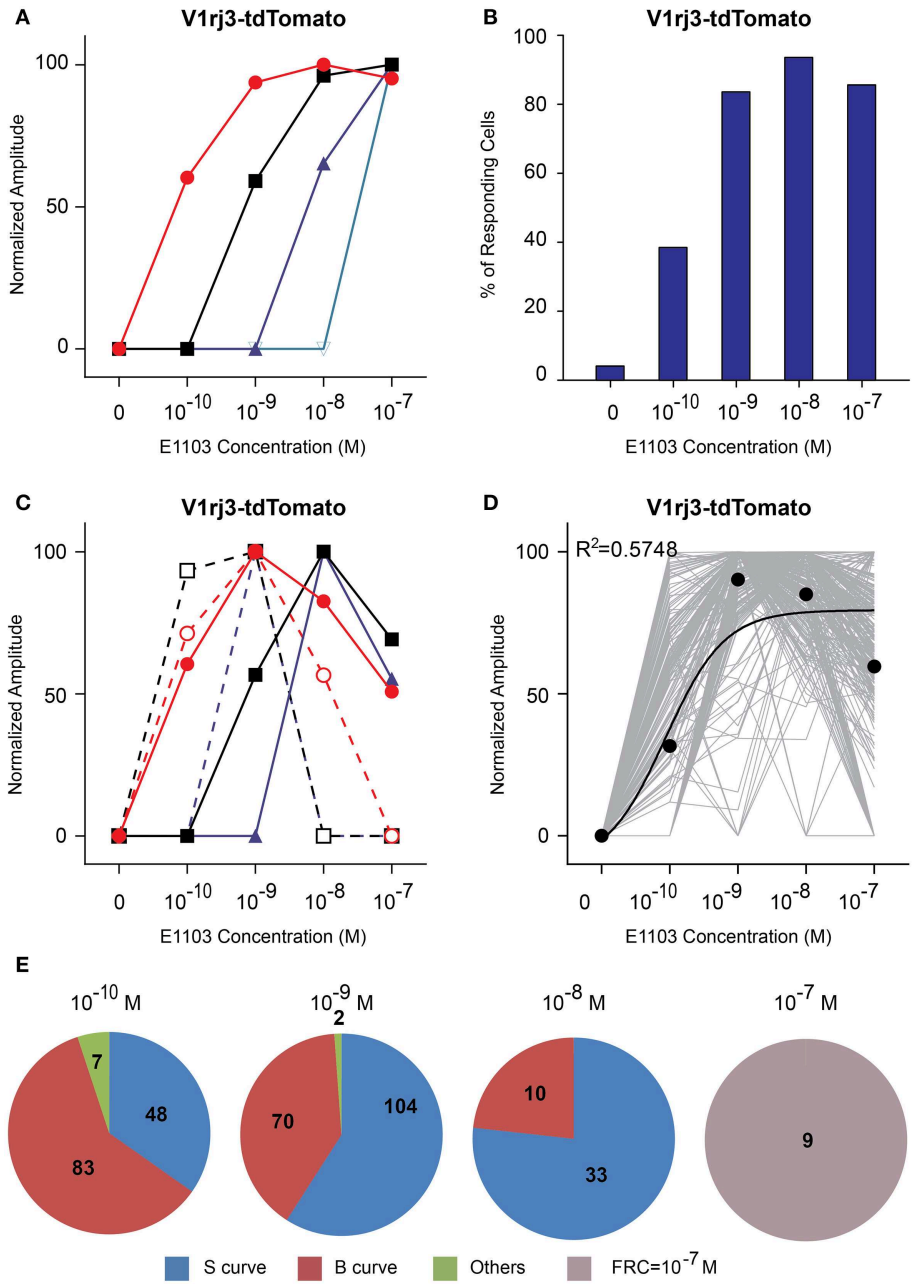


FIGURE 7 | Activation of V1rj3 by E1103. (A) Examples of sigmoidal dose-response curves to E1103 in V1rj3 cells. **(B)** Bar graph showing the percentage of E1103 activated V1rj3 cells ($n = 256$). **(C)** Examples of bell-shaped dose-dependent responses of V1rj3 cells to E1103. **(D)** Dose-response curves of individual cells (gray) and a sigmoidal curve (black) fitted to the average amplitude from all cells. Error bar, S.E.M. **(E)** Pie charts showing the number of cells exhibiting sigmoidal (S, blue), bell-shaped (B, red), and other (O, green) types of dose responses. Cells with FRC of 10^{-7} M are shown in gray.

receptors using mono-glucuronidated estrogen molecules 1, 3, 5(10)-estratrien- 3, 17β -diol 3-glucosiduronate (E1072) and 1, 3, 5(10)-estratrien-3, 17β- diol 17-glucosiduronate (E1073). The 3- and 17-hydroxyl groups are glucuronidated in E1072 and E1073, respectively.

At 10^{-7} M, E1072 stimulated responses in V1rj2 cells (**Figures 8A,B**). The response amplitude was comparable to that activated by E1050 and E1103 at the same concentration. On the other hand, E1073 did not induce any response in V1rj2 expressing VSNs at concentrations up to 10^{-5} M (**Figures 8A–C**). Dose-response analysis indicated that V1rj2 cells were highly sensitive to E1072. Neurons were activated at 10^{-10} M and reached the maximal response at 10^{-9} M (**Figure 8G**). These cells also showed narrow dynamic

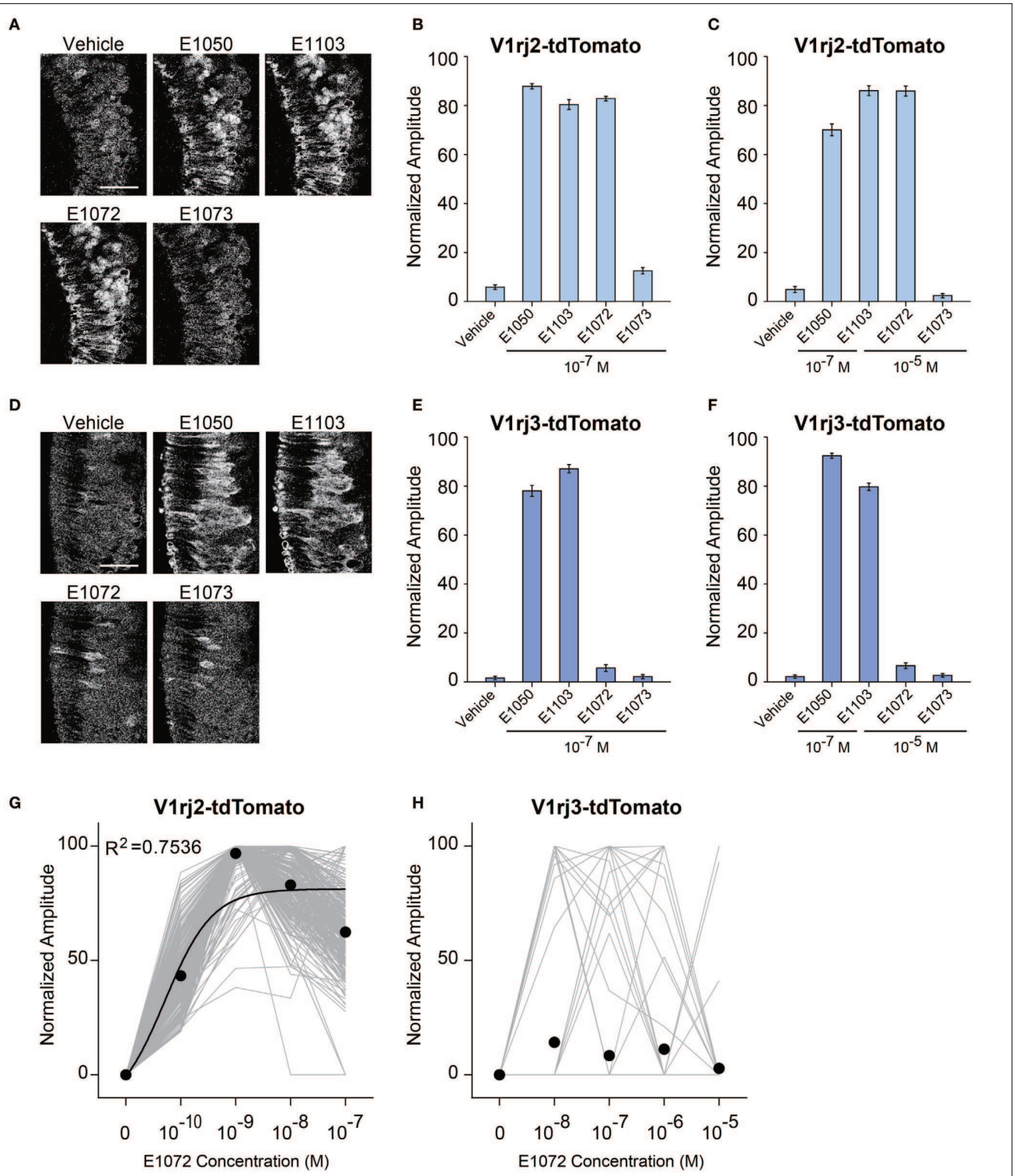


FIGURE 8 | Activation of V1rj2 and V1rj3 by Glucuronidated Estrogens. (A) Representative imaging experiments showing the responses of V1rj2 cells to vehicle control, E1050, E1103, E1072, and E1073 at 10^{-7} M. Scale bar, 50 μm. **(B)** Bar graph showing the normalized response to E1050, E1103, E1072, and E1073 in V1rj2 cells at indicated concentration ($n = 409$). **(C)** Bar graph showing the normalized response to E1050, E1103, E1072, and E1073 in V1rj2 cells at indicated concentrations ($n = 409$). **(D)** Representative imaging experiments showing the responses of V1rj3 cells to vehicle control, E1050, E1103, E1072, and E1073 at 10^{-7} M. **(E)** Bar graph showing the normalized response to E1050, E1103, E1072, and E1073 in V1rj3 cells at indicated concentration ($n = 204$). **(F)** Bar graph showing the normalized response to E1050, E1103, E1072, and E1073 in V1rj3 cells at indicated concentrations ($n = 318$). **(G,H)** Dose-response curve of V1rj2 **(G)** and V1rj3 **(H)** cells activated by E1072. Error bars, S.E.M.

ranges. Similar to the SEs, we observed bell-shaped dose-response curves that peaked at concentrations between 10^{-9} and 10^{-7} M. In contrast, V1rj3-expressing VSNs did not respond to E1072 or E1073 more than the vehicle controls (**Figures 8D–F,H**). These results demonstrated that E1072 was a ligand for V1rj2 and E1073 could not activate either receptors.

Responses of V1rj2 and V1rj3 to Other Sulfated Steroids

Previous studies have shown that individual VSNs are activated by different steroid compounds (Nodari et al., 2008; Meeks et al., 2010; Isogai et al., 2011). We also observed that V1rj2 expressing VSNs responded to a sulfated androgen, 5-androsten-3β, 17β-diol-disulfate (A7864), and other members of SEs, such as 17β-estradiol 3-sulfate (E1100) (Haga-Yamanaka et al., 2014). Here, we analyzed the response of V1rj2 and V1rj3 to these two compounds. Approximately 80% of V1rj2 expressing VSNs began to respond to both A7864 and E1100 at 10^{-7} M. The response amplitude plateaued at 10^{-7} M and increasing ligand concentration did not further augment the response amplitude (**Figure 9A**). A7864 and E1100 were able to activate V1rj3 at 10^{-5} M (**Figure 9B**).

To obtain a comprehensive understanding of ligand selectivity of these V1R receptors, we tested a panel of sulfated steroids at 10^{-5} M (**Figures 9C,D**). V1rj2 expressing VSNs were activated by multiple sulfated androgens including A2398, A2534, A7864, and A8530. They also responded to a couple of sulfated progesterones including P2135 and P3865, a corticosterone, Q5545, and all the SEs tested including E0588, E0893, and E1100 (**Figure 9C**). V1rj3 cells were more selective in their responses. They were activated by a couple of sulfated androgens, A2398 and A7864, sulfated progesterone, P2135, and one SE, E1100 (**Figure 9D**). V1rj3 cell did not respond to sulfated corticosterone stimulation. Thus, V1rj2 and V1rj3 receptors were activated by a diverse group of sulfated steroids and showed tuning preference to different compounds.

Structural Basis of V1rj2 and V1rj3 Activation

The two V1rj receptors share 81% amino acid sequence similarity but exhibit differential tuning and dose-response relationship to steroid derivatives. By comparing the molecular structures of the sulfated and glucuronidated steroids, it appears that a core estradiol-17β structure is essential for highly sensitive activation of the receptors (**Figure 10**). The 3- and 17β-hydroxyl groups appear to be important structural determinants. The steroid compounds maintain high potency when the 3-hydroxyl group is unmodified, sulfated or glucuronidated. However, compounds with a ketone group at this position, such as A6940, lose their ability to activate the receptors. The β configuration of the 17-hydroxyl group is important, too. E0893, which has α configuration, is two orders of magnitude less potent in activating the V1rj2 receptor than E0588 and E1100. This observation suggests that the configuration at the 17-β site plays an important role in activating the receptors. Glucuronidation at this site, as in E1073, blocks its activity, whereas the native or sulfated form retains activity.

We observe that the most potent compounds possess an unsaturated A ring, but permutations in this structure appear to be tolerated (**Figure 10C**). Compounds that possess unsaturated A ring, 18-methyl group or unsaturated bonds in the B ring activate the receptors as long as the 3 and 17β-hydroxyl groups are intact.

Discussion

By taking advantage of a transgenic system to ectopically express the V1Rs, we circumvent a major obstacle in characterizing vomeronasal receptors. Although the VRs can be expressed in heterologous systems, the receptors are retained in the endoplasmic reticulum and cannot be transported to the cell surface. Chaperone proteins, including receptor-transporting proteins (RTPs) and receptor expression enhancing proteins (REEPs), have been shown to facilitate transportation of some odorant receptors to the cell membrane (Saito et al., 2004; Mainland and Matsunami, 2012). None of these molecules can facilitate the surface expression of V1Rs. Although several molecules have been reported to be involved in VR surface expression, it is still challenging to examine individual VR function in heterologous expression systems (Ishii et al., 2003; Loconto et al., 2003; Dey and Matsunami, 2011). In our transgenic system, individual VRs can be ectopically expressed via a synthetic tetO promoter and transported to the cell surface utilizing the endogenous chaperons in VSNs. Thus, this system allows us to characterize VR response in its native environment.

Recapitulation of Native Responses

Previously we observed a diverse responses of VSNs to urinary pheromone stimulation (He et al., 2008, 2010). It was not clear whether the variations in responses arose from the mixture of pheromone cues in the urine or from diverse properties of the VSNs. In the transgenic lines, we also observe that the VSNs display a variety of dose-dependent activation by individual pheromone ligands. Sensitivities of individual cells to SEs range from sub-nanomolar to -micromolar concentrations. The overall dynamic range of the response of the two receptors are comparable to that of the endogenous receptors to SE ligands.

Individual V1rj2 or V1rj3 expressing neurons show both sigmoidal and bell-shaped dose-response curves, similar to those observed in wild type VSNs. Different dose-response relationships appear to be specific to individual cells. We notice that a smaller percentage of V1rj2 and V1rj3 cells exhibit bell-shaped dose-response curves than the wild type VSNs. The difference may reflect the level of expression of receptor proteins in the transgenic mice, which may affect the coupling between receptor and downstream signaling pathways. Alternatively, the dose-response properties may be intrinsic to individual cells. As the V1rj receptors are expressed in most of the VSNs in the transgenic line, the difference in cellular properties may affect how the ectopically expressed receptors respond to ligands.

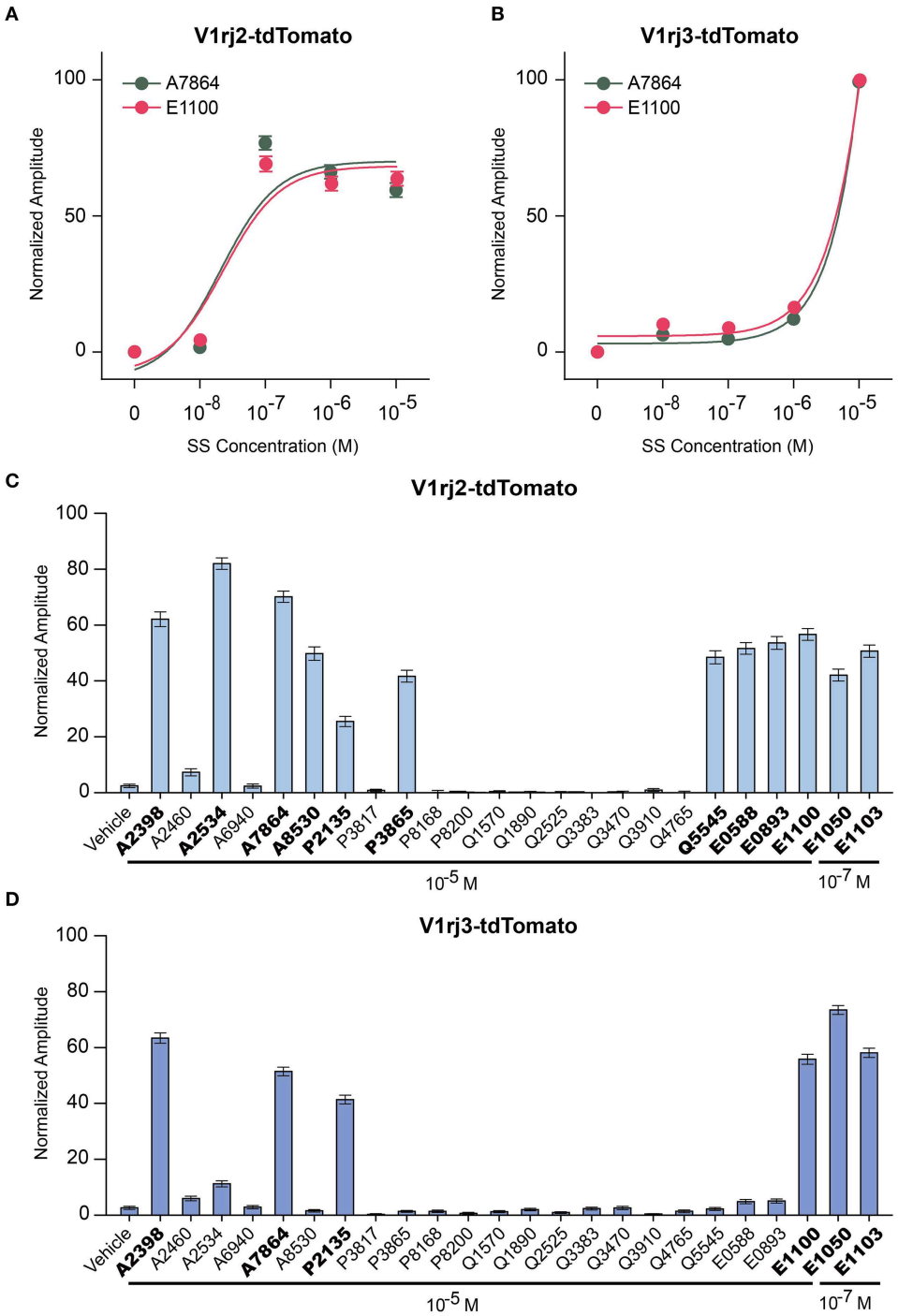


FIGURE 9 | Activation of V1rj2 and V1rj3 by Other Sulfated Steroids. **(A)** Dose-response curves of V1rj2 cells activated by A7864 ($n = 201$) and E1100 ($n = 199$). **(B)** Dose-response curves of V1rj3 cells activated by A7864 ($n = 249$) and E1100 ($n = 249$). **(C,D)** Bar graphs showing the normalized response of V1rj2 (**C**; $n = 183$) and V1rj3 (**D**; $n = 373$) cells a panel of sulfated steroid compounds. Error bars, S.E.M.

The cellular mechanism that generates the bell-shaped dose-response curves is unknown. Experimental evidence from multiple studies suggests that they are unlikely the result of receptor desensitization or cell fatigue. Calcium signal is usually correlated with spiking activity. When a cell is stimulated by a high concentration of ligand, it is possible that a large depolarization inactivates voltage-gated sodium and calcium channels such that the cell cannot sustain action potentials and Ca^{2+} entry through the Ca^{2+} channels. As voltage-gated channels can recover

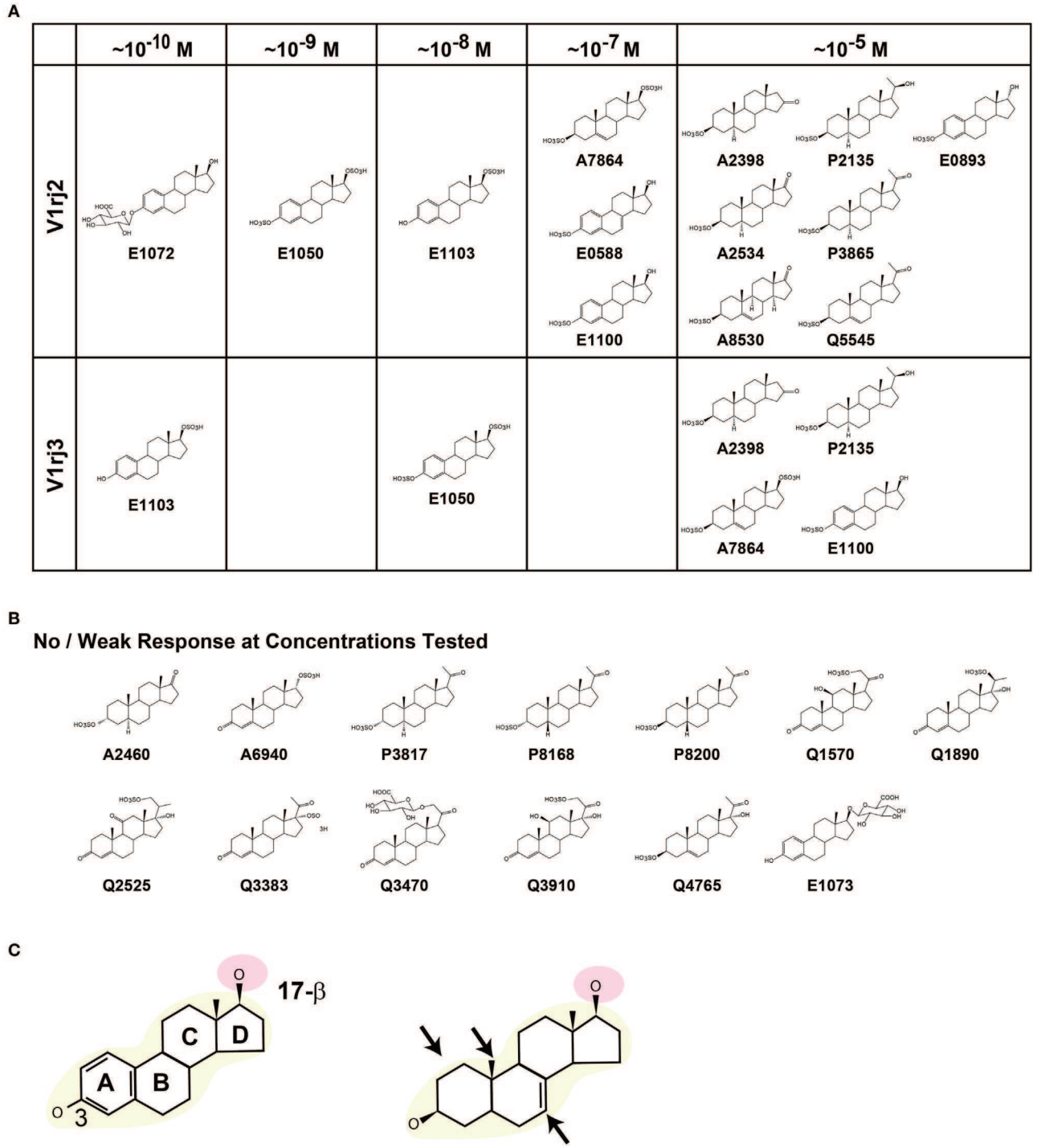


FIGURE 10 | Structural elements of steroid compounds that activate V1rj2 and V1rj3 receptors. (A) Steroid compounds that activate V1rj2 and V1rj3 receptors. The minimal concentrations of activation are indicated. **(B)** Molecular structures of sulfated or glucuronidated steroids that induce no or weak responses. **(C)** Schematic illustration of important structural elements required to activate the V1rj2 and V1rj3 receptors. The estradiol backbone (yellow) and 17-β hydroxyl group (pink) are highlighted. Structural permutations that are permissible are indicated by arrows in the right panel.

quickly from refractory period, this scenario could explain why the cells can respond to different ligands differently at high concentrations. Our result puts the focus on this phenomenon, and further analyses using electrophysiological recording could reveal the cellular mechanism.

Sensitivity and Dynamic Range of the VSNs

The ectopically expressed V1rj receptors are activated by SEs at concentrations as low as 10^{-10} M. It appears that more V1rj3 cells respond to the ligands at this low concentration than V1rj2 cells. The majority of the cells show response saturation within two orders of magnitude. An efficient intracellular signal amplification process is likely to couple the response. The VNO has unusual signal transduction mechanisms (Kim et al., 2012). Different signaling cascades could allow low concentration of ligands to elicit a neuronal response to achieve heightened sensitivity. An amplification process may also explain the relatively narrow dynamic range at individual cell level. A relatively moderate increase in ligand concentration may quickly saturate the responses. In some cells, overstimulation may cause desensitization, which can result in a decline of responses as shown in the bell-shaped dose-response curves to steroid compounds and to mouse urine (He et al., 2010).

VSNs expressing the same receptor exhibit different sensitivities. Since each cell also display sharp changes in dose-dependent activation, the sensitivity is unlikely to reflect differences in intrinsic amplification. It is more likely that sensitivity is determined by the amount of receptors expressed on the cell surface. Alternatively, the binding affinity of the VNO receptors might be affected by the membrane environment in which they are expressed. Our current study, however, does not have evidence to fully address this question.

Despite the narrow dynamic range of individual cells, the VSN population respond to a wide range of ligand concentrations. The average dose-response curve spans at least three orders of magnitude. This allows the VNO to accommodate a large quantitative variation of pheromones presented by animals. VSNs expressing the same vomeronasal receptor project stereotypically to multiple glomeruli in the accessory olfactory bulb (Belluscio et al., 1999; Rodriguez et al., 1999; Wagner et al., 2006; Haga et al., 2010; Ishii and Mombaerts, 2011). Each glomerulus likely receives input from VSNs expressing the same VR. This convergence pattern may allow each glomerulus to integrate signals transmitted by neurons with disparate sensitivity such that the glomerulus serves as a proxy of the VSN collectives. Alternatively, it is also possible that VSN axons segregate in their projection patterns according to ligand sensitivity such that each of the multiple glomeruli exhibits narrow concentration tuning. This scenario may provide a mechanism to readout concentration ratios for concentration-invariant perception of the pheromone cues. Detailed study of the glomerular responses in the AOB would distinguish these two models.

Our study also shows that neurons expressing the same receptor could be tuned to different steroid compounds at high concentrations. Therefore, in addition to chemical identities of ligands, concentration information about a ligand could also be transmitted by different receptors.

Tuning Specificity and Steric Configuration of the Molecules

V1rj2 and V1rj3 are sensitive to SE compounds. They are also activated to a lesser extent by other sulfated and glucuronidated steroids. The structural determinants of the activation appear to require the 3- and 17-hydroxyl groups and estradiol backbone. V1rj3 appears to be more sensitive to SEs, but are also tuned to fewer compounds than V1rj2. The specific interaction of these structural elements with the receptors may determine the specificity and sensitivity of V1rj2 and V1rj3 receptors. The structure basis that differentiates these two receptors remains unknown.

Acknowledgments

We thank A. Moran and members of the Lab Animal Services, and Elden Yu from Microscopy Center at the Stowers Institute for technical assistance. This work is supported by funding from the Stowers Institute and the US National Institutes of Health (National Institute on Deafness and Other Communication Disorders 008003) to CY. SH was supported by a postdoctoral fellowship from the Japan Society for the Promotion of Science.

References

Arnson, H. A., and Holy, T. E. (2013). Robust encoding of stimulus identity and concentration in the accessory olfactory system. *J. Neurosci.* 33, 13388–13397. doi: 10.1523/JNEUROSCI.0967-13.2013

Belluscio, L., Koentges, G., Axel, R., and Dulac, C. (1999). A map of pheromone receptor activation in the mammalian brain. *Cell* 97, 209–220. doi: 10.1016/S0092-8674(00)80731-X

Blair, I. A. (2010). Analysis of estrogens in serum and plasma from postmenopausal women: past present, and future. *Steroids* 75, 297–306. doi: 10.1016/j.steroids.2010.01.012

Chamero, P., Marton, T. F., Logan, D. W., Flanagan, K., Cruz, J. R., Saghatelian, A., et al. (2007). Identification of protein pheromones that promote aggressive behaviour. *Nature* 450, 899–902. doi: 10.1038/nature05997

Dey, S., and Matsunami, H. (2011). Calreticulin chaperones regulate functional expression of vomeronasal type 2 pheromone receptors. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16651–16656. doi: 10.1073/pnas.1018140108

Dulac, C., and Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals. *Cell* 83, 195–206. doi: 10.1016/0092-8674(95)90161-2

Ferrero, D. M., Moeller, L. M., Osakada, T., Horio, N., Li, Q., Roy, D. S., et al. (2013). A juvenile mouse pheromone inhibits sexual behaviour through the vomeronasal system. *Nature* 502, 368–371. doi: 10.1038/nature12579

Haga, S., Hattori, T., Sato, T., Sato, K., Matsuda, S., Kobayakawa, R., et al. (2010). The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. *Nature* 466, 118–122. doi: 10.1038/nature09142

Haga-Yamanaka, S., Ma, L., He, J., Qiu, Q., Lavis, L. D., Looger, L. L., et al. (2014). Integrated action of pheromone signals in promoting courtship behavior in male mice. *Elife* 3:e03025. doi: 10.7554/eLife.03025

Halpern, M., and Martinez-Marcos, A. (2003). Structure and function of the vomeronasal system: an update. *Prog. Neurobiol.* 70, 245–318. doi: 10.1016/S0301-0082(03)00103-5

He, J., Ma, L., Kim, S., Nakai, J., and Yu, C. R. (2008). Encoding gender and individual information in the mouse vomeronasal organ. *Science* 320, 535–538. doi: 10.1126/science.1154476

He, J., Ma, L., Kim, S., Schwartz, J., Santilli, M., Wood, C., et al. (2010). Distinct signals conveyed by pheromone concentrations to the mouse vomeronasal organ. *J. Neurosci.* 30, 7473–7483. doi: 10.1523/JNEUROSCI.0825-10.2010

Herrada, G., and Dulac, C. (1997). A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. *Cell* 90, 763–773. doi: 10.1016/S0092-8674(00)80536-X

Holy, T. E., Dulac, C., and Meister, M. (2000). Responses of vomeronasal neurons to natural stimuli. *Science* 289, 1569–1572. doi: 10.1126/science.289.5484.1569

Ibarra-Soria, X., Levitin, M. O., and Logan, D. W. (2014). The genomic basis of vomeronasal-mediated behaviour. *Mamm. Genome* 25, 75–86. doi: 10.1007/s00335-013-9463-1

Ihara, S., Yoshikawa, K., and Touhara, K. (2013). Chemosensory signals and their receptors in the olfactory neural system. *Neuroscience* 254, 45–60. doi: 10.1016/j.neuroscience.2013.08.063

Ishii, T., Hirota, J., and Mombaerts, P. (2003). Combinatorial coexpression of neural and immune multigene families in mouse vomeronasal sensory neurons. *Curr. Biol.* 13, 394–400. doi: 10.1016/S0960-9822(03)00092-7

Ishii, T., and Mombaerts, P. (2011). Coordinated coexpression of two vomeronasal receptor V2R genes per neuron in the mouse. *Mol. Cell. Neurosci.* 46, 397–408. doi: 10.1016/j.mcn.2010.11.002

Isogai, Y., Si, S., Pont-Lezica, L., Tan, T., Kapoor, V., Murthy, V. N., et al. (2011). Molecular organization of vomeronasal chemoreception. *Nature* 478, 241–245. doi: 10.1038/nature10437

Jemiolo, B., Andreolini, F., Xie, T. M., Wiesler, D., and Novotny, M. (1989). Puberty-affecting synthetic analogs of urinary chemosignals in the house mouse, *Mus domesticus*. *Physiol. Behav.* 46, 293–298. doi: 10.1016/0031-9384(89)90270-9

Kim, S., Ma, L., Jensen, K. L., Kim, M. M., Bond, C. T., Adelman, J. P., et al. (2012). Paradoxical contribution of SK3 and GIRK channels to the activation of mouse vomeronasal organ. *Nat. Neurosci.* 15, 1236–1244. doi: 10.1038/nn.3173

Kimoto, H., Haga, S., Sato, K., and Touhara, K. (2005). Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature* 437, 898–901. doi: 10.1038/nature04033

Leinders-Zufall, T., Brennan, P., Widmayer, P., Prashanth Chandramani, S., Maul-Pavicic, A., Jager, M., et al. (2004). MHC class I peptides as chemosensory signals in the vomeronasal organ. *Science* 306, 1033–1037. doi: 10.1126/science.1102818

Leinders-Zufall, T., Ishii, T., Chamero, P., Hendrix, P., Oboti, L., Schmid, A., et al. (2014). A family of nonclassical class I MHC genes contributes to ultrasensitive chemodetection by mouse vomeronasal sensory neurons. *J. Neurosci.* 34, 5121–5133. doi: 10.1523/JNEUROSCI.0186-14.2014

Leinders-Zufall, T., Lane, A. P., Puche, A. C., Ma, W., Novotny, M. V., Shipley, M. T., et al. (2000). Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature* 405, 792–796. doi: 10.1038/35015572

Liberles, S. D. (2014). Mammalian pheromones. *Annu. Rev. Physiol.* 76, 1–25. doi: 10.1146/annurev-physiol-021113-170334

Liberles, S. D., Horowitz, L. F., Kuang, D., Contos, J. J., Wilson, K. L., Siltberg-Liberles, J., et al. (2009). Formyl peptide receptors are candidate chemosensory receptors in the vomeronasal organ. *Proc. Natl. Acad. Sci. U.S.A.* 106, 9842–9847. doi: 10.1073/pnas.0904464106

Loconto, J., Papes, F., Chang, E., Stowers, L., Jones, E. P., Takada, T., et al. (2003). Functional expression of murine V2R pheromone receptors involves selective association with the M10 and M1 families of MHC class Ib molecules. *Cell* 112, 607–618. doi: 10.1016/S0092-8674(03)00153-3

Ma, L., Haga-Yamanaka, S., Yu, Q. E., Qiu, Q., Kim, S., and Yu, C. R. (2011). Imaging neuronal responses in slice preparations of vomeronasal organ expressing a genetically encoded calcium sensor. *J. Vis. Exp.* 58:e3404. doi: 10.3791/3404

Mainland, J., and Matsunami, H. (2012). RAMP like proteins: RTP and REEP family of proteins. *Adv. Exp. Med. Biol.* 744, 75–86. doi: 10.1007/978-1-4614-2364-5_7

Matsunami, H., and Buck, L. B. (1997). A multigene family encoding a diverse array of putative pheromone receptors in mammals. *Cell* 90, 775–784. doi: 10.1016/S0092-8674(00)80537-1

Meeks, J. P., Arnson, H. A., and Holy, T. E. (2010). Representation and transformation of sensory information in the mouse accessory olfactory system. *Nat. Neurosci.* 13, 723–730. doi: 10.1038/nn.2546

Meredith, M. (1994). Chronic recording of vomeronasal pump activation in awake behaving hamsters. *Physiol. Behav.* 56, 345–354. doi: 10.1016/0031-9384(94)90205-4

Meredith, M., Marques, D. M., O'connell, R. O., and Stern, F. L. (1980). Vomeronasal pump: significance for male hamster sexual behavior. *Science* 207, 1224–1226. doi: 10.1126/science.7355286

Nguyen, M. Q., Zhou, Z., Marks, C. A., Ryba, N. J., and Belluscio, L. (2007). Prominent roles for odorant receptor coding sequences in allelic exclusion. *Cell* 131, 1009–1017. doi: 10.1016/j.cell.2007.10.050

Nodari, F., Hsu, F. F., Fu, X., Holekamp, T. F., Kao, L. F., Turk, J., et al. (2008). Sulfated steroids as natural ligands of mouse pheromone-sensing neurons. *J. Neurosci.* 28, 6407–6418. doi: 10.1523/JNEUROSCI.1425-08.2008

Novotny, M., Harvey, S., Jemiolo, B., and Alberts, J. (1985). Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. U.S.A.* 82, 2059–2061. doi: 10.1073/pnas.82.7.2059

Novotny, M., Jemiolo, B., Harvey, S., Wiesler, D., and Marchlewska-Koj, A. (1986). Adrenal-mediated endogenous metabolites inhibit puberty in female mice. *Science* 231, 722–725. doi: 10.1126/science.3945805

Riviere, S., Challet, L., Fluegge, D., Spehr, M., and Rodriguez, I. (2009). Formyl peptide receptor-like proteins are a novel family of vomeronasal chemosensors. *Nature* 459, 574–577. doi: 10.1038/nature08029

Roberts, S. A., Davidson, A. J., McLean, L., Beynon, R. J., and Hurst, J. L. (2012). Pheromonal induction of spatial learning in mice. *Science* 338, 1462–1465. doi: 10.1126/science.1225638

Roberts, S. A., Simpson, D. M., Armstrong, S. D., Davidson, A. J., Robertson, D. H., McLean, L., et al. (2010). Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol.* 8:75. doi: 10.1186/1741-7007-8-75

Rodriguez, I., Feinstein, P., and Mombaerts, P. (1999). Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system. *Cell* 97, 199–208. doi: 10.1016/S0092-8674(00)80730-8

Ryba, N. J., and Tirindelli, R. (1997). A new multigene family of putative pheromone receptors. *Neuron* 19, 371–379. doi: 10.1016/S0896-6273(00)80946-0

Saito, H., Kubota, M., Roberts, R. W., Chi, Q., and Matsunami, H. (2004). RTP family members induce functional expression of mammalian odorant receptors. *Cell* 119, 679–691. doi: 10.1016/j.cell.2004.11.021

Shackleton, C. H. (1986). Profiling steroid hormones and urinary steroids. *J. Chromatogr.* 379, 91–156. doi: 10.1016/S0378-4347(00)80683-0

Swaney, W. T., and Keverne, E. B. (2009). The evolution of pheromonal communication. *Behav. Brain Res.* 200, 239–247. doi: 10.1016/j.bbr.2008.09.039

Wagner, S., Gresser, A. L., Torello, A. T., and Dulac, C. (2006). A multireceptor genetic approach uncovers an ordered integration of VNO sensory inputs in the accessory olfactory bulb. *Neuron* 50, 697–709. doi: 10.1016/j.neuron.2006.04.033

Wyatt, T. D. (2003). *Pheromones and Animal Behaviours*. Cambridge: Cambridge University Press.

Young, J. M., Massa, H. F., Hsu, L., and Trask, B. J. (2010). Extreme variability among mammalian V1R gene families. *Genome Res.* 20, 10–18. doi: 10.1101/gr.098913.109

Young, J. M., and Trask, B. J. (2007). V2R gene families degenerated in primates, dog and cow, but expanded in opossum. *Trends Genet.* 23, 212–215. doi: 10.1016/j.tig.2007.03.004

Yu, C. R. (2013). Calcium imaging of vomeronasal organ response using slice preparations from transgenic mice expressing G-CaMP2. *Methods Mol. Biol.* 1068, 211–220. doi: 10.1007/978-1-62703-619-1_15

Yu, C. R., Power, J., Barnea, G., O'donnell, S., Brown, H. E., Osborne, J., et al. (2004). Spontaneous neural activity is required for the establishment and maintenance of the olfactory sensory map. *Neuron* 42, 553–566. doi: 10.1016/S0896-6273(04)00224-7

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 18 March 2015
doi: 10.3389/fnins.2015.00073

Effect of social odor context on the emission of isolation-induced ultrasonic vocalizations in the BTBR T+tf/J mouse model for autism

Markus Wöhr *

Behavioral Neuroscience, Experimental and Physiological Psychology, Philipps-University of Marburg, Marburg, Germany

An important diagnostic criterion for social communication deficits in autism spectrum disorders (ASD) are difficulties in adjusting behavior to suit different social contexts. While the BTBR T+tf/J (BTBR) inbred strain of mice is one of the most commonly used mouse models for ASD, little is known about whether BTBR mice display deficits in detecting changes in social context and their ability to adjust to them. Here, it was tested therefore whether the emission of isolation-induced ultrasonic vocalizations (USV) in BTBR mouse pups is affected by the social odor context, in comparison to the standard control strain with high sociability, C57BL/6J (B6). It is known that the presence of odors from mothers and littermates leads to a calming of the isolated mouse pup, and hence to a reduction in isolation-induced USV emission. In accordance with their behavioral phenotypes with relevance to all diagnostic core symptoms of ASD, it was predicted that BTBR mouse pups would not display a calming response when tested under soiled bedding conditions with home cage bedding material containing maternal odors, and that similar isolation-induced USV emission rates would be seen in BTBR mice tested under clean and soiled bedding conditions. Unexpectedly, however, the present findings show that BTBR mouse pups display such a calming response and emit fewer isolation-induced USV when tested under soiled as compared to clean bedding conditions, similar to B6 mouse pups. Yet, in contrast to B6 mouse pups, which emitted isolation-induced USV with shorter call durations and lower levels of frequency modulation under soiled bedding conditions, social odor context had no effect on acoustic call features in BTBR mouse pups. This indicates that the BTBR mouse model for ASD does not display deficits in detecting changes in social context, but has a limited ability and/or reduced motivation to adjust to them.

Keywords: animal model, neurodevelopmental disorder, ultrasonic vocalization, ultrasonic communication, maternal odor

OPEN ACCESS

Edited by:
Markus Fendt, Otto-von-Guericke University Magdeburg, Germany

Reviewed by:
Francesca R. D'Amato, National Research Council, Italy
Ksenia Zuzanna Meyza, Nencki Institute of Experimental Biology, Poland

***Correspondence:**
Markus Wöhr, Behavioral Neuroscience, Experimental and Physiological Psychology, Philipps-University of Marburg, Gutenbergstr. 18, 35032 Marburg, Germany
markus.woehr@staff.uni-marburg.de

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *19 January 2015*
Accepted: *19 February 2015*
Published: *18 March 2015*

Citation:
Wöhr M (2015) Effect of social odor context on the emission of isolation-induced ultrasonic vocalizations in the BTBR T+tf/J mouse model for autism. Front. Neurosci. 9:73. doi: 10.3389/fnins.2015.00073

Introduction

The BTBR T+tf/J (BTBR) inbred strain of mice is one of the most commonly used mouse models for autism spectrum disorders (ASD). BTBR mice display behavioral phenotypes with relevance to all diagnostic core symptoms of ASD, namely persistent deficits in reciprocal social interaction and communication across multiple contexts, together with restricted, repetitive patterns

of behavior, activities, and interests (DSM-5, 2013), as compared to the social mouse strain C57BL/6J (B6; for review see: Blanchard et al., 2012; Meyza et al., 2013; Careaga et al., 2015).

Specifically, BTBR mice display reduced reciprocal social interaction behavior as juveniles (Yang et al., 2007b, 2009; McFarlane et al., 2008; Jones-Davis et al., 2013) and lack of sociability as adults (Bolivar et al., 2007; Moy et al., 2007; Yang et al., 2007a,b, 2009; McFarlane et al., 2008; Defensor et al., 2011; Jones-Davis et al., 2013) in standard laboratory settings, but also semi-natural environments (Pobbe et al., 2010), possibly due to reduced social motivation (Pearson et al., 2012; Martin et al., 2014). For assessing communication deficits, ultrasonic vocalizations (USV) are typically studied. As pups, BTBR mice display an unusual pattern of USV categories, including high levels of harmonics, two-syllable, and composite calls, but vocalize more than B6 mice when being isolated from mother and littermates (Scattoni et al., 2008). During adolescence, low emission rates of pro-social USV were observed in BTBR mice, consistent with their strongly reduced juvenile reciprocal social interaction behavior (Scattoni et al., 2013). Likewise, during reciprocal social interactions in adulthood also low emission rates of pro-social USV were obtained (Scattoni et al., 2011; Yang et al., 2013). Moreover, male BTBR mice do not emit USV to attract females and display reduced scent marking behavior in response to female urine cues, in stark contrast to male B6 mice (Wöhr et al., 2011b), while scent marking behavior in response to male urine cues was found to be unchanged (Roullet et al., 2011). Deficits in the social transmission of food preferences were also reported (McFarlane et al., 2008). Finally, BTBR mice show high levels of restricted, repetitive behavior, such as perseverative self-grooming and marble-burying (McFarlane et al., 2008; Yang et al., 2009; Pobbe et al., 2010; Pearson et al., 2011; Amodeo et al., 2012; Jones-Davis et al., 2013; Molenhuis et al., 2014) or altered exploratory behavior in the hole board task (Moy et al., 2008) and the repetitive novel object contact task (Pearson et al., 2011). They were also reported to display deficits in reversal learning in the Morris water maze (Moy et al., 2007; Yang et al., 2012a) and a set-shifting task (Molenhuis et al., 2014), yet conflicting results were obtained in T-maze reversal learning and related tasks (Moy et al., 2007; Amodeo et al., 2012; Guariglia and Chadman, 2013).

In addition, BTBR mice are characterized by alterations in brain development and morphology associated with ASD, including a lack of the corpus callosum (Wahlsten et al., 2003; Kusek et al., 2007; MacPherson et al., 2008; Jones-Davis et al., 2013), altered functional connectivity networks (Dodero et al., 2013; Ellegood et al., 2013, 2015; Miller et al., 2013; Gogolla et al., 2014; Sforazzini et al., 2015), as well as reduced hippocampal neurogenesis and changes in neurodevelopmental proteins (Stephenson et al., 2011). BTBR mice further display ASD-related alterations in neurotransmitter systems, including serotonin (Gould et al., 2011, 2014; Zhang et al., 2014), dopamine (Squillace et al., 2014), and acetylcholine (McTighe et al., 2013), as well as in neuromodulators, such as oxytocin (Silverman et al., 2010b) and endocannabinoids (Liu et al., 2009; Onaivi et al., 2011; Gould et al., 2014). Persistent immune dysregulation was also reported (Heo et al., 2011; Onore et al., 2013; Schwartzer et al., 2013; Zhang et al., 2013). Not surprisingly, the BTBR inbred strain of mice is therefore a mouse model for ASD that is commonly used to test new pharmacological compounds and strategies for their efficacy in reversing ASD-related behavioral phenotypes, such as negative allosteric modulation of the mGluR5 receptor (Silverman et al., 2012), long-term exposure to intranasal oxytocin (Bales et al., 2014), and others (Silverman et al., 2010a, 2013a,b; Burket et al., 2013, 2014; Amodeo et al., 2014a,b; Han et al., 2014; Karvat and Kimchi, 2014; Langley et al., 2015).

An important diagnostic criterion for social communication deficits in ASD are difficulties in adjusting behavior to suit different social contexts (DSM-5, 2013). However, little is known about whether the BTBR mouse model for ASD displays deficits in detecting changes in social context and their ability to adjust to them. Yet, the fact that the strain of the partner during reciprocal social interactions was reported to have minimal effects on the social behavioral repertoire displayed by BTBR mice is in stark contrast to the changes that were observed in B6 mice (Yang et al., 2012a) and suggests that BTBR mice have difficulties in adjusting their behavior to different social contexts. Here, it was tested therefore whether the emission of isolation-induced USV in BTBR mouse pups is affected by the social odor context, in comparison to the standard control strain with high sociability, B6. It is known that the presence of odors from mothers and littermates leads to a calming of the isolated mouse pup, and hence to a reduction in isolation-induced USV emission (Branchi et al., 1998; Marchlewska-Koj et al., 1999; Kapusta and Szentgyörgyi, 2004; Moles et al., 2004; Zanettini et al., 2010; for similar findings in voles and rats see: Oswalt and Meier, 1975; Conely and Bell, 1978; Kapusta et al., 1995; Szentgyörgyi et al., 2008; but see: Lemasson et al., 2005). Highlighting the relevance of this calming response for behavioral phenotyping of mouse models for ASD, it was further shown that μ-opioid deficient mice do not display a reduction in isolation-induced USV emission rates in the presence of odors from mothers and littermates (Moles et al., 2004), consistent with a variety of other social and communication deficits displayed by this ASD mouse model (Tian et al., 1997; Wöhr et al., 2011a; Cinque et al., 2012; Becker et al., 2014; Gigliucci et al., 2014; for review see: Oddi et al., 2013).

In accordance with their behavioral phenotypes with relevance to all diagnostic core symptoms of ASD, it was predicted that BTBR mouse pups would not display a calming response when tested under soiled bedding conditions with home cage bedding material containing maternal odors, and that similar isolation-induced USV emission rates would be seen in BTBR mice tested under clean and soiled bedding conditions, while lower isolation-induced USV emission rates would occur in B6 mice tested under soiled bedding conditions as compared to clean bedding conditions.

Materials and Methods

Animals and Housing

Subject mice were $N = 30$ BTBR T+tf/J (BTBR) and $N = 30$ C57BL/6J (B6) mice. Breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred at the National Institute of Mental Health in Bethesda, MD, USA. About 2 weeks after pairing for breeding, females were

individually housed and subsequently inspected daily for pregnancy and delivery. The day of birth was considered as postnatal day (PND) 0. All mice were housed in polycarbonate Makrolon cages (369 × 156 × 132 mm, 435 cm^2; 1145T; Tecniplast, Milan, Italy). Bedding, paper strips, a nestlet square, and a cardboard tube were provided in each cage. Standard rodent chow and water were available *ad libitum*. The colony room was maintained on a 12:12 light/dark cycle with lights on at 06:00 h, at 20°C temperature and 55% humidity. All procedures were conducted in strict compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the National Institute of Mental Health Animal Care and Use Committee.

Social Odor Context Manipulation

An experimental design with two independent factors was used in order to study the effects of social odor context on isolation-induced pup USV in a strain-dependent manner, namely strain (BTBR vs. B6) and social odor context (clean bedding vs. soiled bedding), with $N = 15$ per strain and social odor context. To this aim, mouse pups from four different litters per strain (litter size: BTBR: 7.50 ± 1.50; B6: 7.50 ± 1.26; typically with a male: female ratio of approximately 50:50 in both strains) were tested on PND8, using either clean bedding or soiled bedding from the home cage. Random group assignment was used, with approximately 50% of pups per sex from a given litter being tested under clean bedding or soiled bedding conditions, respectively. Pups were tested only once to avoid carry over effects. Home cages used to obtain soiled bedding material were not cleaned for at least 2 days prior testing in order to expose mouse pups to sufficiently distinct odor stimuli.

Isolation-Induced USV—Recording

Pups were isolated from their mother and littermates on PND8 for 5 min under room temperature (22–24°C; humidity: 3–55%). Pups were removed individually from the nest at random and gently placed into an isolation container made of glass (10 × 8 × 7 cm; open surface), containing either clean bedding or soiled bedding depending on experimental group. The isolation container was surrounded by a sound attenuating box (18 × 18 × 18 cm) made of Styrofoam (thickness of walls: 4 cm). USV emission was monitored by an UltraSoundGate Condenser Microphone CM16 (Avisoft Bioacoustics, Berlin, Germany) placed in the roof of the sound attenuating box, 10 cm above the floor. The microphone was connected via an UltraSoundGate 116 USB audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 250,000 Hz in 16 bit format by Avisoft RECORDER (version 2.97; Avisoft Bioacoustics). The microphone was sensitive to frequencies of 15–180 kHz with a flat frequency response (±6 dB) between 25 and 140 kHz. After the 5 min isolation period, body weight and body temperature were determined. Body weight was measured using a palmscale (PS6-250; My Weigh Europe, Hückelhoven, Germany). For body temperature determination a DiGiSense Thermistor Thermometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used. Body temperature was measured by gentle application of the thermal probe onto the stomach of the mouse pup for 20 s. Isolation occurred between 8:00 and 12:00 h during the light phase of the 12:12 h light/dark cycle. Prior to each test, behavioral equipment was cleaned using a 70% ethanol solution, followed by water, and dried with paper towels.

Isolation-Induced USV—Analysis

For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (version 4.50; Avisoft Bioacoustics) and a fast Fourier transform was conducted (512 FFT length, 100% frame, Hamming window, and 75% time window overlap), resulting in spectrograms with 488 Hz of frequency resolution and 0.512 ms of time resolution. Detection of isolation-induced USV was provided by an automatic threshold-based algorithm (amplitude threshold: −40 dB) and a hold-time mechanism (hold time: 10 ms). Since no USV were detected below 30 kHz, a high-pass filter of 30 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. The accuracy of USV detection by the software was verified manually by an experienced user. When necessary, missed USV were marked by hand to be included in the automatic parameter analysis. Total number of isolation-induced USV was calculated for the entire 5 min recording session. Based on previous studies on isolation-induced USV in mouse pups (Wöhr et al., 2008, 2011b; Kurz et al., 2010; Yang et al., 2012b), the following additional parameters were included: latency to start calling, total calling time, call duration, peak frequency, peak amplitude, and frequency modulation. Peak frequency and peak amplitude were derived from the average spectrum of the entire USV. Peak amplitude, i.e., loudness, was defined as the point with the highest energy within the spectrum. Peak frequency was defined as the frequency at the location of the peak amplitude within the spectrum. The extent of frequency modulation was defined as the difference between the lowest and the highest peak frequency within each USV. In addition, USV subtypes were determined by means of density blots (Wöhr, 2014), depicting call duration and frequency modulation. Finally, to assess the temporal organization of isolation-induced USV emission, sequential analyses were performed by correlating the durations of given isolation-induced USV with the durations of the previous ones (N-1), the ones two before (N-2), and the ones three before (N-3), as described before (Wöhr, 2014).

Statistical Analysis

For statistical comparisons, Two-Way ANOVAs with the between-subject factors strain (BTBR vs. B6) and social odor context (clean bedding vs. soiled bedding) were calculated, followed by unpaired *t*-tests when appropriate. Pearson's product moment statistics were used to run correlation analyses between the durations of given isolation-induced USV with the durations of the previous ones (N-1), the ones two before (N-2), and the ones three before (N-3) in mouse pups that emitted >3 isolation-induced USV. Paired *t*-tests were used to compare correlation coefficients against chance level. Sex had no effect on the emission of isolation-induced USV (all $p > 0.100$). A *p*-value of < 0.050 was considered statistically significant.

Results

Effects of Social Odor Context on Isolation-Induced USV in BTBR and B6 Mouse Pups

Social odor context significantly affected the emission of isolation-induced USV [main effect social odor context: $F_{(1, 56)} = 15.646$, $p < 0.001$; **Figure 1A**], with emission rates significantly differing between strains [main effect strain: $F_{(1, 56)} = 124.807$, $p < 0.001$; interaction social odor context × strain: $F_{(1, 56)} = 0.001$, $p = 0.983$; **Figure 1A**]. Specifically, both, BTBR and B6 mouse pups tested under home cage bedding conditions emitted significantly fewer isolation-induced USV than littermates tested under clean cage bedding conditions [$t_{(28)} = 2.939$, $p = 0.007$ and $t_{(28)} = 2.664$, $p = 0.013$; respectively]. In BTBR mouse pups, a significant reduction in isolation-induced USV was seen in the first 2 min of testing [min 1–5: $t_{(28)} = 6.557$, $p < 0.001$; $t_{(28)} = 2.255$, $p = 0.032$; $t_{(28)} = 0.605$, $p = 0.550$; $t_{(28)} = 0.270$, $p = 0.789$; $t_{(28)} = 0.482$, $p = 0.633$; respectively; **Figure 2A**], whereas in B6 mouse pups significant reductions were seen in the first 4 min of testing [min 1–5; $t_{(28)} = 2.835$, $p = 0.008$; $t_{(28)} = 2.703$, $p = 0.012$; $t_{(28)} = 2.638$, $p = 0.013$; $t_{(28)} = 2.332$, $p = 0.027$; $t_{(28)} = 1.470$, $p = 0.153$; respectively; **Figure 2B**]. As expected, BTBR mouse pups emitted significantly more isolation-induced USV than B6 mouse pups under clean bedding conditions [$t_{(28)} = 7.487$, $p < 0.001$], and in line with the results obtained under clean bedding conditions, BTBR mouse pups also emitted significantly more isolation-induced USV than B6 mouse pups under soiled bedding conditions [$t_{(28)} = 8.385$, $p < 0.001$].

Consistently, total calling time was significantly lower in mouse pups tested in soiled bedding than in littermates tested in clean bedding [main effect social odor context: $F_{(1, 56)} = 14.127$, $p < 0.001$; **Figure 1B**], with total calling times significantly differing between strains [main effect strain: $F_{(1, 56)} = 269.882$, $p < 0.001$; interaction social odor context × strain: $F_{(1, 56)} = 2.536$, $p = 0.117$; **Figure 1B**]. Importantly, however, the reduction was again seen in both, BTBR and B6 mouse pups [$t_{(28)} = 2.996$, $p = 0.006$ and $t_{(28)} = 2.408$, $p = 0.023$; respectively], despite total calling times being significantly higher in BTBR than in B6 mouse pups under clean and soiled bedding conditions [$t_{(28)} = 14.396$, $p < 0.001$ and $t_{(28)} = 9.511$, $p < 0.001$; respectively]. The reductions in isolation-induced USV emission rates and total calling times seen in mouse pups tested in a soiled odor context were not due to longer latencies to start calling [main effect social odor context: $F_{(1, 56)} = 1.085$, $p = 0.302$; not shown], yet latencies to start calling differed significantly between strains [main effect strain: $F_{(1, 56)} = 9.654$, $p = 0.003$; interaction social

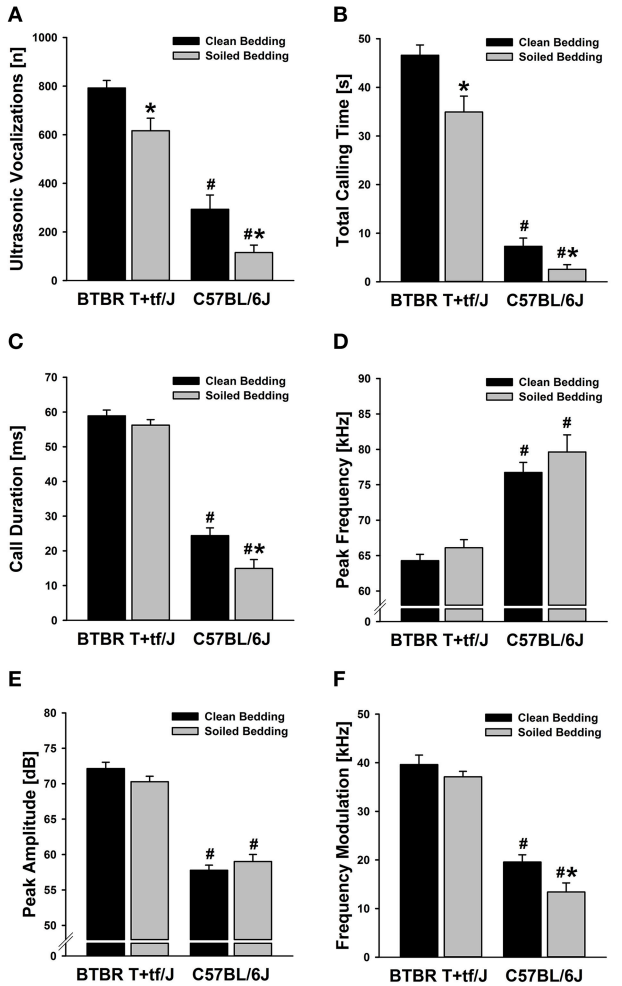


FIGURE 1 | Effects of social odor context on isolation-induced ultrasonic vocalizations (USV) emitted by BTBR T+tf/J (left) and C57BL/6J (right) mouse pups ($N = 15$ per strain and social odor context). (A) Total number of isolation-induced USV [n] and **(B)** total calling time in seconds [s] in BTBR T+tf/J and C57BL/6J mouse pups tested under clean (black) and soiled (gray) bedding conditions. **(C)** Call duration in milliseconds [ms], **(D)** peak frequency in kilohertz [kHz], **(E)** peak amplitude in decibel [dB], and **(F)** frequency modulation in kilohertz [kHz] of isolation-induced USV emitted by BTBR T+tf/J and C57BL/6J mouse pups tested under clean (black) and soiled (gray) bedding conditions. Data are presented as means ± standard errors of the mean. *$p < 0.050$ for soiled bedding vs. clean bedding; #$p < 0.050$ for BTBR T+tf/J vs. C57BL/6J mouse pups.

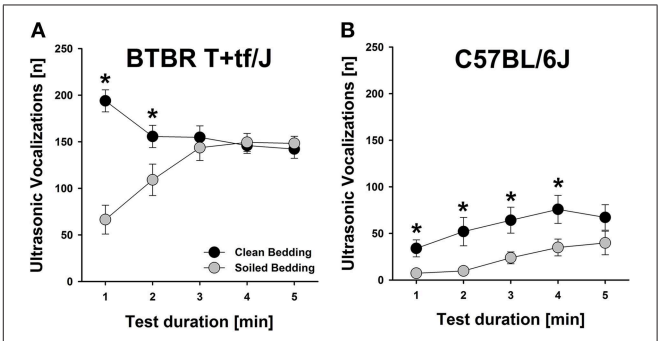


FIGURE 2 | Effects of social odor context on isolation-induced ultrasonic vocalizations (USV) emitted by BTBR T+tf/J (left) and C57BL/6J (right) mouse pups ($N = 15$ per strain and social odor context)—Time course. Number of isolation-induced USV [n] in **(A)** BTBR T+tf/J and **(B)** C57BL/6J mouse pups tested under clean (black) and soiled (gray) bedding conditions per minute [min]. Data are presented as means ± standard errors of the mean. *$p < 0.050$ for soiled bedding vs. clean bedding.

odor context × strain: $F_{(1, 56)} = 0.061$, $p = 0.805$; not shown]. Specifically, the latency to start calling was significantly reduced in BTBR mouse pups as compared to B6 mouse pups under both, clean and soiled bedding conditions [$t_{(28)} = 2.110$, $p = 0.044$ and $t_{(28)} = 2.281$, $p = 0.030$; respectively], consistent with an overall higher level of isolation-induced USV emission in BTBR mouse pups.

Importantly, two out of four acoustic call features determined were also significantly affected by social odor context, namely mean call duration [main effect social odor context: $F_{(1, 54)} = 8.937$, $p = 0.004$; **Figure 1C**] and frequency modulation [main effect social odor context: $F_{(1, 54)} = 6.927$, $p = 0.011$; **Figure 1F**], with both of them also significantly differing between strains [main effect strain: $F_{(1, 54)} = 349.882$, $p < 0.001$ and $F_{(1, 54)} = 175.969$, $p < 0.001$; respectively; interaction social odor context × strain: $F_{(1, 54)} = 2.817$, $p = 0.099$ and $F_{(1, 54)} = 1.221$, $p = 0.274$; respectively; **Figures 1C,F**]. Interestingly, however, when comparing BTBR mouse pups tested in the two social odor contexts, there were no significant differences in mean call duration [$t_{(28)} = 1.152$, $p = 0.259$] and frequency modulation [$t_{(28)} = 1.116$, $p = 0.274$]. In contrast to BTBR mouse pups, mean call duration was affected by social odor context in B6 mouse pups, which emitted significantly shorter isolation-induced USV when tested in soiled bedding than littermates tested in clean bedding [$t_{(26)} = 2.789$, $p = 0.010$]. Furthermore, frequency modulation was affected by social odor context, with B6 mouse pups tested in soiled bedding emitting significantly less frequency-modulated isolation-induced USV than littermates tested in clean bedding [$t_{(26)} = 2.556$, $p = 0.017$], again in contrast to BTBR mouse pups. Under both, clean and soiled bedding conditions, isolation-induced USV emitted by BTBR mouse pups were significantly longer [$t_{(28)} = 12.464$, $p < 0.001$ and $t_{(28)} = 9.511$, $p < 0.001$; respectively] and higher in frequency modulation [$t_{(28)} = 12.373$, $p < 0.001$ and $t_{(28)} = 10.964$, $p < 0.001$; respectively] when compared to isolation-induced USV emitted by B6 mouse pups.

Finally, however, the two other acoustic call features determined were not significantly affected by social odor context, namely peak frequency [main effect social odor context: $F_{(1, 54)} = 2.324$, $p = 0.133$; **Figure 1D**] and peak amplitude [main effect social odor context: $F_{(1, 54)} = 0.130$, $p = 0.719$; **Figure 1E**], yet significant differences between strains were detected for both measures [main effect strain: $F_{(1, 54)} = 070.351$, $p < 0.001$ and $F_{(1, 54)} = 228.079$, $p < 0.001$; respectively; interaction social odor context × strain: $F_{(1, 54)} = 0.123$, $p = 0.727$ and $F_{(1, 54)} = 3.329$, $p = 0.074$; respectively; **Figures 1D,E**]. Specifically, under both, clean and soiled bedding conditions, isolation-induced USV emitted by BTBR mouse pups were significantly lower in peak frequency [$t_{(28)} = 8.050$, $p < 0.001$ and $t_{(28)} = 5.172$, $p < 0.001$; respectively], but higher in peak amplitude [$t_{(28)} = 12.373$, $p < 0.001$ and $t_{(28)} = 9.101$, $p < 0.001$; respectively] when compared to isolation-induced USV emitted by B6 mouse pups.

Of note, body weight and temperature did not differ significantly between the two social odor contexts [main effect social odor context: $F_{(1, 56)} = 0.066$, $p = 0.797$ and $F_{(1, 56)} = 0.003$, $p = 0.954$; respectively; not shown], yet significant differences between strains were detected for both measures [main effect strain: $F_{(1,56)} = 40.815$, $p < 0.001$ and $F_{(1, 56)} = 6.110$, $p = 0.017$; respectively; interaction social odor context × strain: $F_{(1, 56)} = 0.007$, $p = 0.932$ and $F_{(1, 56)} = 0.001$, $p = 0.995$; respectively; not shown]. Specifically, as expected, body weight was significantly higher in BTBR than in B6 mouse pups under clean and soiled bedding conditions [$t_{(28)} = 4.053$, $p < 0.001$ and $t_{(28)} = 5.147$, $p < 0.001$; respectively]. Yet, when comparing strains tested either in clean or soiled bedding, no significant differences in body temperature were detected, but BTBR tended to have higher body temperatures than B6 [$t_{(28)} = 1.748$, $p = 0.091$ and $t_{(28)} = 1.748$, $p = 0.091$; respectively].

Detailed Spectrographic Analysis—Call Clustering and Temporal Organization in BTBR and B6 Mouse Pups

A more detailed analysis was performed to identify clusters of isolation-induced USV emitted by BTBR and B6 mouse pups under clean and soiled bedding conditions by means of density plots. For generating density plots, the two acoustic call features most strongly affected by social odor context were used, namely call duration and frequency modulation. In BTBR mouse pups tested under clean bedding conditions, four prominent call clusters were detected. One cluster was characterized by short call durations (<10 ms) and low levels of frequency modulation (<10 kHz). The other three clusters were characterized by long call durations (30–90 ms), with varying levels of frequency modulation, namely low (<20 kHz), moderate (30–40 kHz), and high (40–70 kHz; **Figure 3A**). Consistent with the lack of significant differences between clean and soiled bedding conditions in mean call duration and frequency modulation in BTBR mouse pups, the social odor context had only minor effects on call clustering, with the call clusters characterized by long call durations (30–90 ms) and low (<20 kHz) or moderate (30–40 kHz) levels of frequency modulation being more prominent (**Figure 3B**). While there were only minor social odor context effects on call clustering in BTBR mouse pups, call clustering markedly differed between BTBR and B6 mouse pups. Whereas in BTBR four prominent call clusters were detected, only two prominent call clusters were detected in B6 mouse pups. One cluster was characterized by short call durations (<40 ms) and low levels of frequency modulation (<30 kHz) and therefore broader than the corresponding call cluster in BTBR mouse pups. The second one was characterized by comparably long call durations (30–70 ms) and moderate levels of frequency modulation (40–50 kHz), and thus possibly corresponding to the call cluster in BTBR that was characterized by long call durations (30–90 ms) and moderate frequency modulation (30–40 kHz; **Figure 3C**). In B6 mouse pups, social odor context affected call clustering, with the second call cluster characterized by comparably long call durations (30–70 ms) and moderate levels of frequency modulation (40–50 kHz) being less prominent and coherent under soiled bedding conditions, in line with the overall reduced mean call duration and frequency modulation (**Figure 3D**).

An additional sequential analysis of the durations of subsequent isolation-induced USV finally indicated that the call

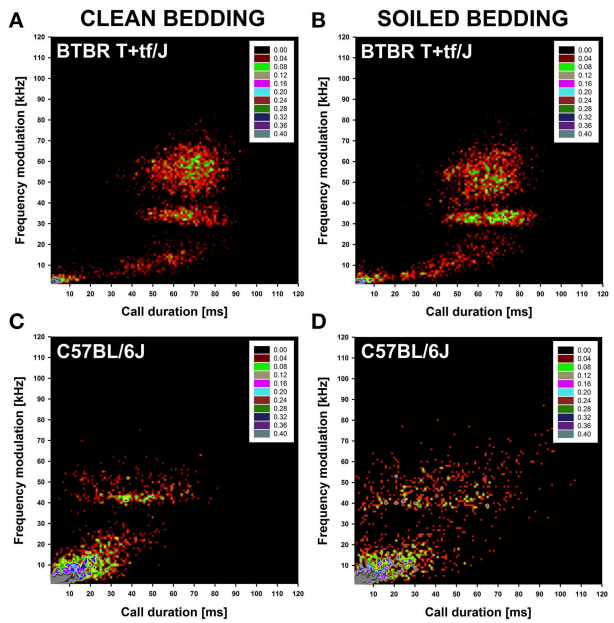


FIGURE 3 | Distribution of individual isolation-induced ultrasonic vocalizations (USV) in BTBR T+tf/J (upper panel) and C57BL/6J (lower panel) mouse pups (*N* = 15 per strain and social odor context). Density plots depicting the distribution of individual isolation-induced USV depending on call duration in milliseconds [ms] and frequency modulation in kilohertz [kHz] in BTBR T+tf/J mouse pups tested under **(A)** clean and **(B)** soiled bedding conditions and in C57BL/6J mouse pups tested under **(C)** clean and **(D)** soiled bedding conditions. Color coding reflects frequencies as percentages.

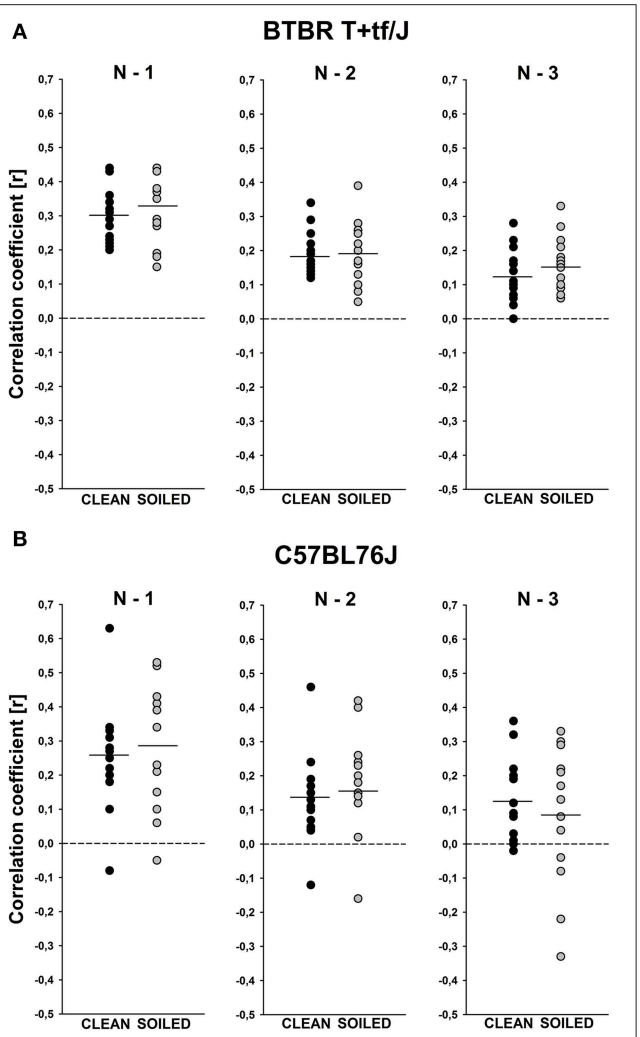


FIGURE 4 | Sequential analysis of the durations of subsequent isolation-induced ultrasonic vocalizations (USV) indicating non-random call emission patterns in BTBR T+tf/J (upper panel) and C57BL/6J (lower panel) mouse pups (*N* = 15 per strain and social odor context). Correlations between the call durations of given isolation-induced USV and the call durations of the previous ones (N–1), the call durations of the ones two before (N–2), or the call durations of the ones three before (N–3) for **(A)** BTBR T+tf/J and **(B)** C57BL/6J mouse pups tested under clean (black circles) and soiled (gray circles) bedding conditions. Each circle represents one mouse pup that emitted >3 isolation-induced USV. The solid line represents the mean per condition.

emission pattern is not random in BTBR mouse pups tested under both, clean and soiled, bedding conditions, since the durations of given isolation-induced USV could be predicted by the durations of the previous ones [N–1; clean bedding: $t_{(14)} = 15.248$, $p < 0.001$; soiled bedding: $t_{(14)} = 12.774$, $p < 0.001$], by the ones two before [N–2; clean bedding: $t_{(14)} = 10.497$, $p < 0.001$; soiled bedding: $t_{(14)} = 8.022$, $p < 0.001$], and by the ones three before [N–3; clean bedding: $t_{(14)} = 6.353$, $p < 0.001$; soiled bedding: $t_{(14)} = 7.313$, $p < 0.001$; **Figure 4A**]. Evidence for such a non-random call emission pattern was also obtained in B6 mouse pups, again, under both, clean and soiled, bedding conditions [N–1; clean bedding: $t_{(13)} = 6.255$, $p < 0.001$; soiled bedding: $t_{(12)} = 5.647$, $p < 0.001$; N–2; clean bedding: $t_{(13)} = 3.970$, $p = 0.002$; soiled bedding: $t_{(12)} = 3.187$, $p = 0.008$; N–3; clean bedding: $t_{(13)} = 3.992$, $p = 0.002$; soiled bedding: $t_{(12)} = 1.479$, $p = 0.165$; **Figure 4B**]. Correlation coefficients did not differ between BTBR and B6 or between clean and soiled bedding conditions (all $p > 0.100$).

Discussion

An important diagnostic criterion for social communication deficits in ASD are difficulties in adjusting behavior to suit different social contexts (DSM-5, 2013). In experimental studies assessing social context effects on social behavior, for instance, individuals with ASD display insensitivity to social reputation as assessed by the occurrence of charitable donations in the presence or absence of an observer (Izuma et al., 2011; Cage et al., 2013) or flattery behavior following rating of pictures depending on the drawer's presence (Chevallier et al., 2012b). Individuals with ASD further show resistance to social pressure in the Asch conformity experiment (Bowler and Worley, 1994; Yafai et al., 2014), more fixed strategies disregarding the partner's beliefs in a social hunting game (Yoshida et al., 2010) or trustworthiness in an economic trust game (Ewing et al., 2015), and lack of social gaze influences on motor action control (Schilbach et al., 2012). Also, in a study focusing on social communication, individuals with ASD were found to use attention-directing behavior less frequently

than controls and their behavior varied less across social contexts (Landry and Loveland, 1989). Together, these experimental findings echo anecdotal reports of parents emphasizing that individuals with ASD seem only mildly influenced by considerations of impression management (for review see: Chevallier et al., 2012a). Finally, social context appears to have opposite effects on the occurrence of repetitive patterns of behavior in healthy human subjects and individuals with ASD. While in healthy human subjects repetitive behavior is inhibited in social situations (Asendorpf, 1980), studies in individuals with ASD indicate that repetitive behavior is unchanged or even increased when exposed to a social context (Baron-Cohen, 1989; Carruthers, 1996).

Considering the diagnostic criteria for ASD and the experimental findings obtained in human ASD studies, surprisingly little is known about difficulties in adjusting behavior to suit different social contexts in mouse models for ASD (Wöhr and Scattoni, 2013). Even in the BTBR mouse model for ASD, which is one of the most commonly used mouse models (for review see: Blanchard et al., 2012; Meyza et al., 2013; Careaga et al., 2015), no study explicitly addressed this issue so far. The present findings show for the first time that BTBR mouse pups adjust their emission of isolation-induced USV to different social contexts. Specifically, they displayed a calming response and emitted fewer isolation-induced USV when tested under soiled bedding conditions with home cage bedding material containing maternal odors as compared to clean bedding conditions, similar to B6 mouse pups.

This is in contrast to what was expected, considering that BTBR mice display behavioral phenotypes with relevance to all diagnostic core symptoms of ASD (for review see: Blanchard et al., 2012; Meyza et al., 2013; Careaga et al., 2015). The present findings are further in contrast to a study by Yang et al. (2012a) who reported that the strain of the partner during reciprocal social interactions has minimal effects on the social behavioral repertoire displayed by BTBR mice, suggesting that adult BTBR mice have difficulties in adjusting their behavior to different social contexts.

The fact that emission of isolation-induced USV is affected by social context in BTBR mouse pups might be viewed as a challenge for the BTBR inbred strain of mice as a mouse model for ASD. However, it has to be emphasized that very little evidence is available up to now supporting the notion that the inhibition of isolation-induced USV caused by the presence of odors from mothers and littermates allows the reliable assessment of ASD-relevant behavioral alterations in mouse pups. Probably the strongest finding in support of such a notion was reported by Moles et al. (2004). They showed that μ-opioid deficient mice do not display a reduction in isolation-induced USV emission rates when tested under social odor conditions, whereas in wildtype controls a clear reduction was evident. The lack of a calming response in μ-opioid deficient mice is consistent with a variety of other social and communication deficits displayed by this ASD mouse model (Tian et al., 1997; Wöhr et al., 2011a; Cinque et al., 2012; Becker et al., 2014; Gigliucci et al., 2014; for review see: Oddi et al., 2013).

It has further to be emphasized that also little is known about the general mechanisms underlying the inhibition of isolation-induced USV caused by the presence of odors from mothers and littermates in mouse pups. In a pioneering study by Branchi et al. (1998), comparing three different social odor contexts, namely clean bedding material, bedding material from the home cage, and bedding material from a male cage, CD-1 mice tended to vocalize less in the latter two social odor contexts. In similar studies by Marchlewska-Koj et al. (1999) and Kapusta and Szentgyörgyi (2004), CBA mouse pups emitted shorter isolation-induced USV when tested under home cage bedding conditions as compared to clean bedding conditions, while isolation-induced USV emission rates and peak frequency were not affected. Finally, Lemasson et al. (2005) reported no effect of home cage odor on isolation-induced USV emission rates in B6 mice. In comparison to most available studies, the inhibition reported in the present study appears therefore to be comparatively strong, which is particularly surprising in case of the BTBR mouse model for ASD. One of the possible reasons for the comparatively strong odor effects is that isolation-induced USV were recorded for 5 min, whereas relatively short recording durations were used in most other studies (Branchi et al., 1998; Marchlewska-Koj et al., 1999; Kapusta and Szentgyörgyi, 2004; Lemasson et al., 2005). However, the detailed time course analysis speaks against this idea, as the inhibition of isolation-induced USV in mouse pups tested under soiled bedding conditions was most prominent in the first few minutes of testing, particularly in BTBR mouse pups.

While BTBR mouse pups unexpectedly displayed a calming response and emitted fewer isolation-induced USV when tested under soiled as compared to clean bedding conditions, social odor context had no effect on acoustic call features, such as call duration, peak frequency, peak amplitude, and frequency modulation, in BTBR mouse pups. This is in stark contrast to what was seen in B6 mouse pups, which emitted isolation-induced USV with shorter call durations and lower levels of frequency modulation under soiled bedding conditions as compared to clean bedding conditions.

At least three possible mechanisms for the reduced adjustment to different social contexts in BTBR mouse pups can be considered. Firstly, the fact that social odor context had no effect on acoustic call features in BTBR mouse pups could be due to deficits in detecting changes in the social context caused by olfactory impairments. However, BTBR mice displayed normal olfactory abilities, both in non-social test paradigms, such as the buried food task (Moy et al., 2007), as well as in social ones, like the preference for social novelty task (Moy et al., 2007; McFarlane et al., 2008). Consistently, olfactory habituation/dishabituation in response to a sequence of non-social and social odors was evident in BTBR mice, yet it was clearly less prominent than in B6 mice (Yang et al., 2012a). It was further reported that exploratory behavior displayed by BTBR mice in the hole board task can be altered by presenting soiled bedding (Moy et al., 2008). Moreover, in a recent study on female-induced USV and scent marking behavior in adult male mice, both, BTBR and B6 males, spent a similar amount of time in proximity to a salient

olfactory social cue, a spot of female urine, indicating that female urine evoked similar levels of interest in BTBR and B6 males (Wöhr et al., 2011b). Also in pups evidence for intact social olfactory abilities was provided. Specifically, in the homing test, in which mouse pups are exposed to clean bedding on one side and soiled bedding from the home cage on the other, it took BTBR mouse pups less time to reach the side containing the soiled bedding than B6 mouse pups, yet the finding is difficult to interpret due to an overall increased level of locomotor activity in BTBR mouse pups (Scattoni et al., 2008). Finally, the present results show that both, BTBR and B6 mouse pups, display a calming response and emit fewer isolation-induced USV when tested under soiled as compared to clean bedding conditions. Together, this supports the interpretation that BTBR mice are able to process social olfactory cues, both in infancy and adulthood, indicating that the observed deficit in behavioral adjustment to different social contexts in BTBR mouse pups is not due to olfactory impairments.

Secondly, a limited ability to adjust to different social contexts could be the reason for the fact that social odor context had no effect on acoustic call features in BTBR mouse pups. The unusual repertoire of USV categories seen in BTBR mouse pups, including high levels of harmonics, two-syllable, and composite calls (Scattoni et al., 2008), possibly speaks for a limited ability of BTBR mouse pups to modulate the acoustic call features of isolation-induced USV, yet the richness of USV subtypes (Scattoni et al., 2008) and call clusters in the present study speaks against it.

Thirdly, a reduced motivation to adjust to different social contexts could also be the reason and on the basis of the data available it is currently not possible to differentiate between the two possible mechanisms. In support of the latter mechanism it was shown that BTBR mice are characterized by a reduction in social motivation (Pearson et al., 2012; Martin et al., 2014). Specifically, Pearson et al. (2012) found no evidence for social conditioned place preference in BTBR but in B6 mice. Likewise, Martin et al. (2014) reported that BTBR mice had lower breaking points than B6 mice when lever pressing for a social reward, namely access to a conspecific. Yet, it has to be mentioned that breaking points for food reward were also lower in BTBR, questioning the specificity of the motivational deficit for the social domain. Finally, it has to be emphasized that a reduced motivation to adjust to different social context could also be due to altered levels of anxiety, with anxiety levels being possibly elevated in BTBR mice (Benno et al., 2009; Frye and Llaneza, 2010; Pobbe et al., 2011; Gould et al., 2014; Langley et al., 2015).

In line with the findings obtained by Scattoni et al. (2008) and Schwartzer et al. (2013), BTBR mouse pups emitted more isolation-induced USV than B6 mouse pups in the present study. It is currently unclear what is causing this strain difference in isolation-induced USV emission rates. It is tempting to speculate that the strain effect is due to the marked difference in body weight and/or size between BTBR and B6. Yet, Scattoni et al. (2008) showed that FVB/NJ mouse pups vocalized almost as little as B6 mouse pups, despite being close to BTBR mouse pups in body weight. What also appears possible is that the strain effect is caused by a difference in anxiety-related behavior. Isolation-induced USV have been repeatedly associated with anxiety in various behavioral studies (for review see: Schwarting and Wöhr, 2012). For instance, mice selectively bred for high anxiety-related behavior on the elevated plus maze emit more isolation-induced USV as pups than mice selectively bred for low anxiety levels (Krömer et al., 2005; Frank et al., 2009); a finding confirmed and extended by Kessler et al. (2011) who showed that this difference is not affected by cross-fostering and thus likely reflects a line-dependent change in innate anxiety. Also pharmacological studies support this view (for review see: Miczek et al., 1995). For instance, anxiolytic benzodiazepines and other positive modulators of GABA receptors inhibit isolation-induced USV in mouse pups (Benton and Nastiti, 1988; Nastiti et al., 1991; Cirulli et al., 1994; Fish et al., 2000; Takahashi et al., 2009). High levels of isolation-induced USV in BTBR mouse pups could hence reflect higher responses to stress or higher levels of anxiety-like traits. In fact, Schwartzer et al. (2013) found that the already high isolation-induced USV emission rates in BTBR mouse pups can be further enhanced by Poly I:C exposure during pregnancy, mimicking a viral infection and known to increase anxiety-related behavior in adulthood, including higher emission rates of fear-induced USV (Yee et al., 2012). Recently, Langley et al. (2015) further reported increased anxiety-related behavior in juvenile BTBR mice in the elevated plus maze. Moreover, Pobbe et al. (2011) described more defensiveness to animate threat stimuli, such as a predator, and an inconsistent response pattern in elevated plus maze and zero maze in adult BTBR mice. Most studies, however, did not report an anxiety-like phenotype in adult BTBR mice in standard tasks, including elevated plus-maze, zero maze, and light-dark box (Moy et al., 2007; McFarlane et al., 2008; Benno et al., 2009; Yang et al., 2009; Silverman et al., 2010b; Chadman, 2011; Molenhuis et al., 2014). Also, significantly higher plasma corticosterone levels and exaggerated responses to stress were repeatedly reported in juvenile and adult BTBR mice (Benno et al., 2009; Frye and Llaneza, 2010; Gould et al., 2014), yet no evidence for an abnormal stress response was detected by Silverman et al. (2010b) in adulthood. Thus, it is not clear whether high levels of isolation-induced USV in BTBR mouse pups reflect higher responses to stress or higher levels of anxiety-like traits and future studies on anxiety-related behavior in infant and juvenile BTBR mice appear indicated. Finally, it is also not clear whether strain differences in anxiety-like behavior and the production of isolation-induced USV are due to differences in maternal behavior. Yang et al. (2007b) reported typical maternal caregiving behavior in BTBR females and no major changes in the behavioral repertoire of BTBR offspring were seen following cross-fostering to B6 females. However, when BTBR embryos were transferred to B6 females, significant improvements in social and repetitive behavior were observed, yet anxiety-like behavior and isolation-induced USV were not assessed and it is unclear whether the observed changes are due to differences in the maternal immune environment or social factors (Zhang et al., 2013).

It is further in line with the findings obtained by Scattoni et al. (2008) that the isolation-induced USV emitted by BTBR mouse pups were longer in call duration, lower in peak frequency, but higher in peak amplitude than the ones emitted by B6 mouse pups. The higher level of frequency modulation of isolation-induced USV emitted by BTBR muse pups observed in the present study is probably reflecting the larger proportion of harmonics, two-syllable, and composite calls, as reported by Scattoni et al. (2008) before. The present findings show that these strain differences are robust and reliably detectable in two different social odor contexts, namely clean and soiled bedding conditions. Such strain differences might again be due to differences in body weight and/or size, but also related characteristics, including the length of their vocal cords.

The present study further identified clusters of isolation-induced USV emitted by BTBR and B6 mouse pups under clean and soiled bedding conditions by means of density plots. In BTBR mouse pups, four prominent call clusters were detected, virtually independent from social odor context, further highlighting their limited ability and/or reduced motivation to adjust to different social contexts. One cluster was characterized by short call durations and low levels of frequency modulation. The other three clusters were characterized by long call durations, with varying levels of frequency modulation, namely low, moderate, and high. Therefore, by means of the quantitative approach applied here, no clear evidence for the existence of 10 distinct USV subtypes as reported by Scattoni et al. (2008) was obtained. However, it has to be emphasized that the 10 USV subtypes differentiated by Scattoni et al. (2008) were identified by means of visual analyses of waveform patterns; a strategy that allows to take various different call features into account, while the quantitative approach applied here is based on two factors only, namely call duration and frequency modulation. Yet, despite the different approach and the difference in USV subtypes/clusters, remarkable consistencies were obtained. For instance, Scattoni et al. (2008) reported that USV subtypes characterized by a high level of frequency-modulation, such as harmonics, two-syllable, and frequency step calls, are longer in duration than less frequency-modulated USV subtypes. This is perfectly in line with the present findings obtained by means of density plots. Future studies are needed to test whether certain USV subtypes reported by Scattoni et al. (2008) are exclusively present in specific call clusters.

Call clustering markedly differed between BTBR and B6 mouse pups. While in BTBR four prominent call clusters were detected, only two prominent call clusters were detected in B6 mouse pups. One cluster was characterized by short call durations and low levels of frequency modulation, whereas the second one was characterized by comparably long call durations and moderate levels of frequency modulation. Interestingly, in B6 mouse pups, social odor context affected call clustering, with the second call cluster characterized by comparably long call durations and moderate levels of frequency modulation being less prominent and coherent, in line with the overall reduced mean call duration and frequency modulation. Shorter call durations in mouse pups tested in soiled bedding were reported before (Marchlewska-Koj et al., 1999; Kapusta and Szentgyörgyi, 2004).

Finally, an additional sequential analysis of the durations of subsequent isolation-induced USV indicated that the USV emission pattern is not random in BTBR mouse pups tested under both, clean and soiled bedding conditions, and that the temporal pattern does not differ significantly from the one obtained in B6 mouse pups. Specifically, in both, BTBR and B6 mouse pups, the durations of given isolation-induced USV could be predicted by the durations of the previous ones. Considering the unusual pattern of USV categories displayed by BTBR mouse pups (Scattoni et al., 2008), it might seem surprising that the temporal organization as assessed here appears to be unaltered in the BTBR mouse model for ASD, particularly because a distorted sequential organization was recently reported in a genetic mouse model for ASD, the *Shank1* deficient mouse (Wöhr, 2014). *Shank1* deficient mice display a variety of behavioral alterations with relevance to ASD (Hung et al., 2008; Silverman et al., 2011; Wöhr et al., 2011b; Sungur et al., 2014; for a USV emission pattern analysis in *Shank2* deficient mice see: Ey et al., 2013).

Conclusion

In accordance with their behavioral phenotypes with relevance to all diagnostic core symptoms of ASD, it was predicted that BTBR mouse pups would not display a calming response when tested under soiled bedding conditions with home cage bedding material containing maternal odors, and that similar isolation-induced USV emission rates would be seen in BTBR mice tested under clean and soiled bedding conditions. Unexpectedly, however, the present findings show that BTBR mouse pups display such a calming response and emit fewer isolation-induced USV when tested under soiled as compared to clean bedding conditions, similar to B6 mouse pups. Yet, in contrast to B6 mouse pups, which emitted isolation-induced USV with shorter call durations and lower levels of frequency modulation under soiled bedding conditions, social odor context had no effect on acoustic call features in BTBR mouse pups. This indicates that the BTBR mouse model for ASD does not display deficits in detecting changes in social context, but has a limited ability and/or reduced motivation to adjust to them.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (DFG WO 1732/1-1) and the National Institute of Mental Health Intramural Research Program. Data were collected by MW at the National Institute of Mental Health, USA, and analyzed by MW at the Philipps-University of Marburg, Germany. I wish to thank Dr. Jacqueline N. Crawley, National Institute of Mental Health, for giving me the opportunity to conduct this research.

References

Amodeo, D. A., Jones, J. H., Sweeney, J. A., and Ragozzino, M. E. (2012). Differences in BTBR T+ tf/J and C57BL/6J mice on probabilistic reversal learning and stereotyped behaviors. *Behav. Brain Res.* 227, 64–72. doi: 10.1016/j.bbr.2011.10.032

Amodeo, D. A., Jones, J. H., Sweeney, J. A., and Ragozzino, M. E. (2014a). Risperidone and the 5-HT2A receptor antagonist M100907 improve probabilistic reversal learning in BTBR T + tf/J mice. *Autism Res.* 7, 555–567. doi: 10.1002/aur.1395

Amodeo, D. A., Yi, J., Sweeney, J. A., and Ragozzino, M. E. (2014b). Oxotremorine treatment reduces repetitive behaviors in BTBR T+ tf/J mice. *Front. Synaptic Neurosci.* 6:17. doi: 10.3389/fnsyn.2014.00017

Asendorpf, J. (1980). Nichtreaktive Stressmessung: bewegungsstereotypien als Aktivierungsindikatoren. *Z. Exp. Angew. Psychol.* 27, 44–58.

Bales, K. L., Solomon, M., Jacob, S., Crawley, J. N., Silverman, J. L., Larke, R. H., et al. (2014). Long-term exposure to intranasal oxytocin in a mouse autism model. *Transl. Psychiatry* 4, e480. doi: 10.1038/tp.2014.117

Baron-Cohen, S. (1989). Do autistic children have obsessions and compulsions? *Br. J. Clin. Psychol.* 28, 193–200. doi: 10.1111/j.2044-8260.1989.tb01369.x

Becker, J. A., Clesse, D., Spiegelhalter, C., Schwab, Y., Le Merrer, J., and Kieffer, B. L. (2014). Autistic-like syndrome in mu opioid receptor null mice is relieved by facilitated mGluR4 activity. *Neuropsychopharmacology* 39, 2049–2060. doi: 10.1038/npp.2014.59

Benno, R., Smirnova, Y., Vera, S., Liggett, A., and Schanz, N. (2009). Exaggerated response to stress in the BTBR T+tf/J mouse: an unusual behavioral phenotype. *Behav. Brain Res.* 197, 462–465. doi: 10.1016/j.bbr.2008.09.041

Benton, D., and Nastiti, K. (1988). The influence of psychotropic drugs on the ultrasonic calling of mouse pups. *Psychopharmacology (Berl.)* 95, 99–102. doi: 10.1007/BF00212775

Blanchard, D. C., Defensor, E. B., Meyza, K. Z., Pobbe, R. L., Pearson, B. L., Bolivar, V. J., et al. (2012). BTBR T+tf/J mice: autism-relevant behaviors and reduced fractone-associated heparan sulfate. *Neurosci. Biobehav. Rev.* 36, 285–296. doi: 10.1016/j.neubiorev.2011.06.008

Bolivar, V. J., Walters, S. R., and Phoenix, J. L. (2007). Assessing autism-like behaviour in mice: variations in social interactions among inbred strains. *Behav. Brain Res.* 176, 21–26. doi: 10.1016/j.bbr.2006.09.007

Bowler, D., and Worley, K. (1994). Susceptibility to social-influence in adults with Asperger's syndrome—a research note. *J. Child Psychol. Psychiatry* 35, 689–697. doi: 10.1111/j.1469-7610.1994.tb01214.x

Branchi, I., Santucci, D., Vitale, A., and Alleva, E. (1998). Ultrasonic vocalizations by infant laboratory mice: a preliminary spectrographic characterization under different conditions. *Dev. Psychobiol.* 33, 249–256.

Burket, J. A., Benson, A. D., Tang, A. H., and Deutsch, S. I. (2013). D-Cycloserine improves sociability in the BTBR T+ Itpr3tf/J mouse model of autism spectrum disorders with altered Ras/Raf/ERK1/2 signaling. *Brain Res. Bull.* 96, 62–70. doi: 10.1016/j.brainresbull.2013.05.003

Burket, J. A., Benson, A. D., Tang, A. H., and Deutsch, S. I. (2014). Rapamycin improves sociability in the BTBR T(+)Itpr3(tf)/J mouse model of autism spectrum disorders. *Brain Res. Bull.* 100, 70–75. doi: 10.1016/j.brainresbull.2013.11.005

Cage, E., Pellicano, E., Shah, P., and Bird, G. (2013). Reputation management: evidence for ability but reduced propensity in autism. *Autism Res.* 6, 433–442. doi: 10.1002/aur.1313

Careaga, M., Schwartzer, J., and Ashwood, P. (2015). Inflammatory profiles in the BTBR mouse: how relevant are they to autism spectrum disorders? *Brain Behav. Immun.* 43, 11–16. doi: 10.1016/j.bbi.2014.06.006

Carruthers, P. (1996). "Autism as mind-blindness: an elaboration and partial defence," in *Theories of Theories of Mind*, eds P. Carruthers and P. K. Smith (Cambridge: Cambridge University Press), 257–273.

Chadman, K. K. (2011). Fluoxetine but not risperidone increases sociability in the BTBR mouse model of autism. *Pharmacol. Biochem. Behav.* 97, 586–594. doi: 10.1016/j.pbb.2010.09.012

Chevallier, C., Kohls, G., Troiani, V., Brodkin, E. S., and Schultz, R. T. (2012a). The social motivation theory of autism. *Trends Cogn. Sci.* 16, 231–239. doi: 10.1016/j.tics.2012.02.007

Chevallier, C., Molesworth, C., and Happ,é, F. (2012b). Diminished social motivation negatively impacts reputation management: autism spectrum disorders as a case in point. *PLoS ONE* 7:e31107. doi: 10.1371/journal.pone.0031107

Cinque, C., Pondiki, S., Oddi, D., Di Certo, M. G., Marinelli, S., Troisi, A., et al. (2012). Modeling socially anhedonic syndromes: genetic and pharmacological manipulation of opioid neurotransmission in mice. *Transl. Psychiatry* 2, e155. doi: 10.1038/tp.2012.83

Cirulli, F., Santucci, D., Laviola, G., Alleva, E., and Levine, S. (1994). Behavioral and hormonal responses to stress in the newborn mouse: effects of maternal deprivation and chlordiazepoxide. *Dev. Psychobiol.* 27, 301–316. doi: 10.1002/dev.420270505

Conely, L., and Bell, R. W. (1978). Neonatal ultrasounds elicited by odor cues. *Dev. Psychobiol.* 11, 193–197. doi: 10.1002/dev.420110302

Defensor, E. B., Pearson, B. L., Pobbe, R. L., Bolivar, V. J., Blanchard, D. C., and Blanchard, R. J. (2011). A novel social proximity test suggests patterns of social avoidance and gaze aversion-like behavior in BTBR T+ tf/J mice. *Behav. Brain Res.* 217, 302–308. doi: 10.1016/j.bbr.2010.10.033

Dodero, L., Damiano, M., Galbusera, A., Bifone, A., Tsaftsaris, S. A., Scattoni, M. L., et al. (2013). Neuroimaging evidence of major morpho-anatomical and functional abnormalities in the BTBR T+TF/J mouse model of autism. *PLoS ONE* 8:e76655. doi: 10.1371/journal.pone.0076655

DSM-5. (2013). *Diagnostic and Statistical Manual of Mental Disorders*, *5th Edn*. Arlington, VA: American Psychiatric Publishing.

Ellegood, J., Anagnostou, E., Babineau, B. A., Crawley, J. N., Lin, L., Genestine, M., et al. (2015). Clustering autism: using neuroanatomical differences in 26 mouse models to gain insight into the heterogeneity. *Mol. Psychiatry*. 20, 118–125. doi: 10.1038/mp.2014.98

Ellegood, J., Babineau, B. A., Henkelman, R. M., Lerch, J. P., and Crawley, J. N. (2013). Neuroanatomical analysis of the BTBR mouse model of autism using magnetic resonance imaging and diffusion tensor imaging. *Neuroimage* 70, 288–300. doi: 10.1016/j.neuroimage.2012.12.029

Ewing, L., Caulfield, F., Read, A., and Rhodes, G. (2015). Appearance-based trust behaviour is reduced in children with autism spectrum disorder. *Autism*. doi: 10.1177/1362361314559431. [Epub ahead of print].

Ey, E., Torquet, N., Le Sourd, A. M., Leblond, C. S., Boeckers, T. M., Faure, P., et al. (2013). The Autism ProSAP1/Shank2 mouse model displays quantitative and structural abnormalities in ultrasonic vocalisations. *Behav. Brain Res.* 256, 677–689. doi: 10.1016/j.bbr.2013.08.031

Fish, E. W., Sekinda, M., Ferrari, P. F., Dirks, A., and Miczek, K. A. (2000). Distress vocalizations in maternally separated mouse pups: modulation via 5-HT(1A), 5-HT(1B) and GABA(A) receptors. *Psychopharmacology (Berl.)* 149, 277–285. doi: 10.1007/s002130000370

Frank, E., Kessler, M. S., Filiou, M. D., Zhang, Y., Maccarrone, G., Reckow, S., et al. (2009). Stable isotope metabolic labeling with a novel N-enriched bacteria diet for improved proteomic analyses of mouse models for psychopathologies. *PLoS One* 4:e7821. doi: 10.1371/journal.pone.0007821

Frye, C. A., and Llaneza, D. C. (2010). Corticosteroid and neurosteroid dysregulation in an animal model of autism, BTBR mice. *Physiol. Behav.* 100, 264–267. doi: 10.1016/j.physbeh.2010.03.005

Gigliucci, V., Leonzino, M., Busnelli, M., Luchetti, A., Palladino, V. S., D'Amato, F. R., et al. (2014). Region specific up-regulation of oxytocin receptors in the opioid oprm1 (-/-) mouse model of autism. *Front. Pediatr.* 2:91. doi: 10.3389/fped.2014.00091

Gogolla, N., Takesian, A. E., Feng, G., Fagiolini, M., and Hensch, T. K. (2014). Sensory integration in mouse insular cortex reflects GABA circuit maturation. *Neuron* 83, 894–905. doi: 10.1016/j.neuron.2014.06.033

Gould, G. G., Burke, T. F., Osorio, M. D., Smolik, C. M., Zhang, W. Q., Onaivi, E. S., et al. (2014). Enhanced novelty-induced corticosterone spike and upregulated serotonin 5-HT1A and cannabinoid CB1 receptors in adolescent BTBR mice. *Psychoneuroendocrinology* 39, 158–169. doi: 10.1016/j.psyneuen.2013.09.003

Gould, G. G., Hensler, J. G., Burke, T. F., Benno, R. H., Onaivi, E. S., and Daws, L. C. (2011). Density and function of central serotonin (5-HT) transporters, 5-HT1A and 5-HT2A receptors, and effects of their targeting on BTBR T+tf/J mouse social behavior. *J. Neurochem.* 116, 291–303. doi: 10.1111/j.1471-4159.2010.07104.x

Guariglia, S. R., and Chadman, K. K. (2013). Water T-maze: a useful assay for determination of repetitive behaviors in mice. *J. Neurosci. Methods* 220, 24–29. doi: 10.1016/j.jneumeth.2013.08.019

Han, S., Tai, C., Jones, C. J., Scheuer, T., and Catterall, W. A. (2014). Enhancement of inhibitory neurotransmission by GABAA receptors having α2,3-subunits ameliorates behavioral deficits in a mouse model of autism. *Neuron* 81, 1282–1289. doi: 10.1016/j.neuron.2014.01.016

Heo, Y., Zhang, Y., Gao, D., Miller, V. M., and Lawrence, D. A. (2011). Aberrant immune responses in a mouse with behavioral disorders. *PLoS ONE* 6:e20912. doi: 10.1371/journal.pone.0020912

Hung, A. Y., Futai, K., Sala, C., Valtschanoff, J. G., Ryu, J., Woodworth, M. A., et al. (2008). Smaller dendritic spines, weaker synaptic transmission, but enhanced spatial learning in mice lacking Shank1. *J. Neurosci.* 28, 1697–1708. doi: 10.1523/JNEUROSCI.3032-07.2008

Izuma, K., Matsumoto, K., Camerer, C. F., and Adolphs, R. (2011). Insensitivity to social reputation in autism. *Proc. Natl. Acad. Sci. U.S.A.* 108, 17302–17307. doi: 10.1073/pnas.1107038108

Jones-Davis, D. M., Yang, M., Rider, E., Osbun, N. C., da Gente, G. J., Li, J., et al. (2013). Quantitative trait loci for interhemispheric commissure development and social behaviors in the BTBR T? tf/J mouse model of autism. *PLoS ONE* 8:e61829. doi: 10.1371/journal.pone.0061829

Kapusta, J., Marchlewska-Koj, A., and Sales, G. D. (1995). Home bedding modifies ultrasonic vocalization of infant bank voles. *J. Chem. Ecol.* 21, 577–582. doi: 10.1007/BF02033702

Kapusta, J., and Szentgyörgyi, H. (2004). Ultrasonic response of CBA pups to the odour of genetically different mice. *Behaviour* 141, 157–167. doi: 10.1163/156853904322890799

Karvat, G., and Kimchi, T. (2014). Acetylcholine elevation relieves cognitive rigidity and social deficiency in a mouse model of autism. *Neuropsychopharmacology* 39, 831–840. doi: 10.1038/npp.2013.274

Kessler, M. S., Bosch, O. J., Bunck, M., Landgraf, R., and Neumann, I. D. (2011). Maternal care differs in mice bred for high vs. low trait anxiety: impact of brain vasopressin and cross-fostering. *Soc. Neurosci.* 6, 156–168. doi: 10.1080/17470919.2010.495567

Krömer, S. A., Kessler, M. S., Milfay, D., Birg, I. N., Bunck, M., Czibere, L., et al. (2005). Identification of glyoxalase-I as a protein marker in a mouse model of extremes in trait anxiety. *J. Neurosci.* 25, 4375–4384. doi: 10.1523/JNEUROSCI.0115-05.2005

Kurz, A., Wöhr, M., Walter, M., Bonin, M., Auburger, G., Gispert, S., et al. (2010). Alpha-synuclein deficiency affects brain Foxp1 expression and ultrasonic vocalization. *Neuroscience* 166, 785–795. doi: 10.1016/j.neuroscience.2009.12.054

Kusek, G. K., Wahlsten, D., Herron, B. J., Bolivar, V. J., and Flaherty, L. (2007). Localization of two new X-linked quantitative trait loci controlling corpus callosum size in the mouse. *Genes Brain Behav.* 6, 359–363. doi: 10.1111/j.1601-183X.2006.00264.x

Landry, S. H., and Loveland, K. A. (1989). The effect of social context on the functional communication skills of autistic children. *J. Autism Dev. Disord.* 19, 283–299. doi: 10.1007/BF02211847

Langley, E. A., Krykbaeva, M., Blusztajn, J. K., and Mellott, T. J. (2015). High maternal choline consumption during pregnancy and nursing alleviates deficits in social interaction and improves anxiety-like behaviors in the BTBR T+Itpr3tf/J mouse model of autism. *Behav. Brain Res.* 278, 210–220. doi: 10.1016/j.bbr.2014.09.043

Lemasson, M., Delb,é, C., Gheusi, G., Vincent, J. D., and Lledo, P. M. (2005). Use of ultrasonic vocalizations to assess olfactory detection in mouse pups treated with 3-methylindole. *Behav. Processes* 68, 13–23. doi: 10.1016/j.beproc.2004.09.001

Liu, Q. R., Pan, C. H., Hishimoto, A., Li, C. Y., Xi, Z. X., Llorente-Berzal, A., et al. (2009). Species differences in cannabinoid receptor 2 (CNR2 gene): identification of novel human and rodent CB2 isoforms, differential tissue expression and regulation by cannabinoid receptor ligands. *Genes Brain Behav.* 8, 519–530. doi: 10.1111/j.1601-183X.2009.00498.x

MacPherson, P., McGaffigan, R., Wahlsten, D., and Nguyen, P. V. (2008). Impaired fear memory, altered object memory and modified hippocampal synaptic plasticity in split-brain mice. *Brain Res.* 1210, 179–188. doi: 10.1016/j.brainres.2008.03.008

Marchlewska-Koj, A., Kapusta, J., and Olejniczak, P. (1999). Ultrasonic response of CBA newborn mice to bedding odour. *Behaviour* 136, 269–278. doi: 10.1163/156853999501324

Martin, L., Sample, H., Gregg, M., and Wood, C. (2014). Validation of operant social motivation paradigms using BTBR T+tf/J and C57BL/6J inbred mouse strains. *Brain Behav.* 4, 754–764. doi: 10.1002/brb3.273

McFarlane, H. G., Kusek, G. K., Yang, M., Phoenix, J. L., Bolivar, V. J., and Crawley, J. N. (2008). Autism-like behavioral phenotypes in BTBR T+tf/J mice. *Genes Brain Behav.* 7, 152–163. doi: 10.1111/j.1601-183X.2007.00330.x

McTighe, S. M., Neal, S. J., Lin, Q., Hughes, Z. A., and Smith, D. G. (2013). The BTBR mouse model of autism spectrum disorders has learning and attentional impairments and alterations in acetylcholine and kynurenic acid in prefrontal cortex. *PLoS ONE* 8:e62189. doi: 10.1371/journal.pone.0062189

Meyza, K. Z., Defensor, E. B., Jensen, A. L., Corley, M. J., Pearson, B. L., Pobbe, R. L., et al. (2013). The BTBR T+ tf/J mouse model for autism spectrum disorders-in search of biomarkers. *Behav. Brain Res.* 251, 25–34. doi: 10.1016/j.bbr.2012.07.021

Miczek, K. A., Weerts, E. M., Vivian, J. A., and Barros, H. M. (1995). Aggression, anxiety and vocalizations in animals: GABAA and 5-HT anxiolytics. *Psychopharmacology (Berl.)* 121, 38–56. doi: 10.1007/BF02245590

Miller, V. M., Gupta, D., Neu, N., Cotroneo, A., Boulay, C. B., and Seegal, R. F. (2013). Novel inter-hemispheric white matter connectivity in the BTBR mouse model of autism. *Brain Res.* 1513, 26–33. doi: 10.1016/j.brainres.2013.04.001

Molenhuis, R. T., de Visser, L., Bruining, H., and Kas, M. J. (2014). Enhancing the value of psychiatric mouse models; differential expression of developmental behavioral and cognitive profiles in four inbred strains of mice. *Eur. Neuropsychopharmacol.* 24, 945–954. doi: 10.1016/j.euroneuro.2014.01.013

Moles, A., Kieffer, B. L., and D'Amato, F. R. (2004). Deficit in attachment behavior in mice lacking the mu-opioid receptor gene. *Science* 304, 1983–1986. doi: 10.1126/science.1095943

Moy, S. S., Nadler, J. J., Poe, M. D., Nonneman, R. J., Young, N. B., Koller, B. H., et al. (2008). Development of a mouse test for repetitive, restricted behaviours: relevance to autism. *Behav. Brain Res.* 188, 178–194. doi: 10.1016/j.bbr.2007.10.029

Moy, S. S., Nadler, J. J., Young, N. B., Perez, A. P., Holloway, L. P., Barbaro, R. P., et al. (2007). Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. *Behav. Brain Res.* 176, 4–20. doi: 10.1016/j.bbr.2006.07.030

Nastiti, K., Benton, D., and Brain, P. F. (1991). The effects of compounds acting at the benzodiazepine receptor complex on the ultrasonic calling of mouse pups. *Behav. Pharmacol.* 2, 121–128.

Oddi, D., Crusio, W. E., D'Amato, F. R., and Pietropaolo, S. (2013). Monogenic mouse models of social dysfunction: implications for autism. *Behav. Brain Res.* 251, 75–84. doi: 10.1016/j.bbr.2013.01.002

Onaivi, E. S., Benno, R., Halpern, T., Mehanovic, M., Schanz, N., Sanders, C., et al. (2011). Consequences of cannabinoid and monoaminergic system disruption in a mouse model of autism spectrum disorders. *Curr. Neuropharmacol.* 9, 209–214. doi: 10.2174/157015911795017047

Onore, C. E., Careaga, M., Babineau, B. A., Schwartzer, J. J., Berman, R. F., and Ashwood, P. (2013). Inflammatory macrophage phenotype in BTBR T+tf/J mice. *Front. Neurosci.* 7:158. doi: 10.3389/fnins.2013.00158

Oswalt, G. L., and Meier, G. W. (1975). Olfactory, thermal, and tactual influences on infantile ultrasonic vocalization in rats. *Dev. Psychobiol.* 8, 129–135. doi: 10.1002/dev.420080205

Pearson, B. L., Bettis, J. K., Meyza, K. Z., Yamamoto, L. Y., Blanchard, D. C., and Blanchard, R. J. (2012). Absence of social conditioned place preference in BTBR T+tf/J mice: relevance for social motivation testing in rodent models of autism. *Behav. Brain Res.* 233, 99–104. doi: 10.1016/j.bbr.2012.04.040

Pearson, B. L., Pobbe, R. L., Defensor, E. B., Oasay, L., Bolivar, V. J., Blanchard, D. C., et al. (2011). Motor and cognitive stereotypies in the BTBR T+tf/J mouse model of autism. *Genes Brain Behav.* 10, 228–235. doi: 10.1111/j.1601-183X.2010.00659.x

Pobbe, R. L., Pearson, B. L., Defensor, E. B., Bolivar, V. J., Blanchard, D. C., and Blanchard, R. J. (2010). Expression of social behaviors of C57BL/6J versus BTBR inbred mouse strains in the visible burrow system. *Behav. Brain Res.* 214, 443–449. doi: 10.1016/j.bbr.2010.06.025

Pobbe, R. L., Defensor, E. B., Pearson, B. L., Bolivar, V. J., Blanchard, D. C., and Blanchard, R. J. (2011). General and social anxiety in the BTBR T+tf/J mouse strain. *Behav. Brain Res.* 216, 446–451. doi: 10.1016/j.bbr.2010.08.039

Roullet, F. I., Wöhr, M., and Crawley, J. N. (2011). Female urine-induced male mice ultrasonic vocalizations, but not scent-marking, is modulated by social experience. *Behav. Brain Res.* 216, 19–28. doi: 10.1016/j.bbr.2010.06.004

Scattoni, M. L., Gandhy, S. U., Ricceri, L., and Crawely, J. N. (2008). Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. *PLoS ONE* 3:e3067. doi: 10.1371/journal.pone.0003067

Scattoni, M. L., Martire, A., Cartocci, G., Ferrante, A., and Ricceri, L. (2013). Reduced social interaction, behavioural flexibility and BDNF signalling in the BTBR T+ tf/J strain, a mouse model of autism. *Behav. Brain Res.* 251, 35–40. doi: 10.1016/j.bbr.2012.12.028

Scattoni, M. L., Ricceri, L., and Crawley, J. N. (2011). Unusual repertoire of vocalizations in adult BTBR T+tf/J mice during three types of social

encounters. *Genes Brain Behav.* 10, 44–56. doi: 10.1111/j.1601-183X.2010.00623.x

Schilbach, L., Eickhoff, S. B., Cieslik, E. C., Kuzmanovic, B., and Vogeley, K. (2012). Shall we do this together? Social gaze influences action control in a comparison group, but not in individuals with high-functioning autism. *Autism* 16, 151–162. doi: 10.1177/1362361311409258

Schwarting, R. K. W., and Wöhr, M. (2012). On the relationships between ultrasonic calling and anxiety-related behavior in rats. *Braz. J. Med. Biol. Res.* 45, 337–348. doi: 10.1590/S0100-879X2012007500038

Schwartzer, J. J., Careaga, M., Onore, C. E., Rushakoff, J. A., Berman, R. F., and Ashwood, P. (2013). Maternal immune activation and strain specific interactions in the development of autism-like behaviors in mice. *Transl. Psychiatry* 3, e240. doi: 10.1038/tp.2013.16

Sforazzini, F., Bertero, A., Dodero, L., David, G., Galbusera, A., Scattoni, M. L., et al. (2015). Altered functional connectivity networks in acallosal and socially impaired BTBR mice. *Brain Struct. Funct.* doi: 10.1007/s00429-014-0948-9. [Epub ahead of print].

Silverman, J. L., Babineau, B. A., Oliver, C. F., Karras, M. N., and Crawley, J. N. (2013a). Influence of stimulant-induced hyperactivity on social approach in the BTBR mouse model of autism. *Neuropharmacology* 68, 210–222. doi: 10.1016/j.neuropharm.2012.07.042

Silverman, J. L., Oliver, C. F., Karras, M. N., Gastrell, P. T., and Crawley, J. N. (2013b). AMPAKINE enhancement of social interaction in the BTBR mouse model of autism. *Neuropharmacology* 64, 268–282. doi: 10.1016/j.neuropharm.2012.07.013

Silverman, J. L., Smith, D. G., Rizzo, S. J., Karras, M. N., Turner, S. M., Tolu, S. S., et al. (2012). Negative allosteric modulation of the mGluR5 receptor reduces repetitive behaviors and rescues social deficits in mouse models of autism. *Sci. Transl. Med.* 4:131ra51. doi: 10.1126/scitranslmed.3003501

Silverman, J. L., Tolu, S. S., Barkan, C. L., and Crawley, J. N. (2010a). Repetitive self-grooming behavior in the BTBR mouse model of autism is blocked by the mGluR5 antagonist MPEP. *Neuropsychopharmacology* 35, 976–989. doi: 10.1038/npp.2009.201

Silverman, J. L., Turner, S. M., Barkan, C. L., Tolu, S. S., Saxena, R., Hung, A. Y., et al. (2011). Sociability and motor functions in Shank1 mutant mice. *Brain Res.* 1380, 120–137. doi: 10.1016/j.brainres.2010.09.026

Silverman, J. L., Yang, M., Turner, S. M., Katz, A. M., Bell, D. B., Koenig, J. I., et al. (2010b). Low stress reactivity and neuroendocrine factors in the BTBR T+tf/J mouse model of autism. *Neuroscience* 171, 1197–1208. doi: 10.1016/j.neuroscience.2010.09.059

Squillace, M., Dodero, L., Federici, M., Migliarini, S., Errico, F., Napolitano, F., et al. (2014). Dysfunctional dopaminergic neurotransmission in asocial BTBR mice. *Transl. Psychiatry* 4, e427. doi: 10.1038/tp.2014.69

Stephenson, D. T., O'Neill, S. M., Narayan, S., Tiwari, A., Arnold, E., Samaroo, H. D., et al. (2011). Histopathologic characterization of the BTBR mouse model of autistic-like behavior reveals selective changes in neurodevelopmental proteins and adult hippocampal neurogenesis. *Mol. Autism* 2:7. doi: 10.1186/2040-2392-2-7

Sungur, A. Ö., Vörckel, K. J., Schwarting, R. K. W., and Wöhr, M. (2014). Repetitive behaviors in the Shank1 knockout mouse model for autism spectrum disorder: developmental aspects and effects of social context. *J. Neurosci. Methods* 234, 92–100. doi: 10.1016/j.jneumeth.2014.05.003

Szentgyörgyi, H., Kapusta, J., and Marchlewska-Koj, A. (2008). Ultrasonic calls of bank vole pups isolated and exposed to cold or to nest odor. *Physiol. Behav.* 93, 296–303. doi: 10.1016/j.physbeh.2007.09.015

Takahashi, A., Yap, J. J., Bohager, D. Z., Faccidomo, S., Clayton, T., Cook, J. M., et al. (2009). Glutamatergic and GABAergic modulations of ultrasonic vocalizations during maternal separation distress in mouse pups. *Psychopharmacology (Berl.)* 204, 61–71. doi: 10.1007/s00213-008-1437-8

Tian, M., Broxmeyer, H. E., Fan, Y., Lai, Z., Zhang, S., Aronica, S., et al. (1997). Altered hematopoiesis, behavior, and sexual function in mu opioid receptor-deficient mice. *J. Exp. Med.* 185, 1517–1522. doi: 10.1084/jem.185.8.1517

Wahlsten, D., Metten, P., and Crabbe, J. C. (2003). Survey of 21 inbred mouse strains in two laboratories reveals that BTBR T/+ tf/tf has severely reduced hippocampal commissure and absent corpus callosum. *Brain Res.* 971, 47–54. doi: 10.1016/S0006-8993(03)02354-0

Wöhr, M. (2014). Ultrasonic vocalizations in Shank mouse models for autism spectrum disorders: detailed spectrographic analyses and developmental profiles. *Neurosci. Biobehav. Rev.* 43, 199–212. doi: 10.1016/j.neubiorev.2014.03.021

Wöhr, M., Dahlhoff, M., Wolf, E., Holsboer, F., Schwarting, R. K. W., and Wotjak, C. T. (2008). Effects of genetic background, gender, and early environmental factors on isolation-induced ultrasonic calling in mouse pups: an embryo-transfer study. *Behav. Genet.* 38, 579–595. doi: 10.1007/s10519-008-9221-4

Wöhr, M., Moles, A., Schwarting, R. K. W., and D'Amato, F. R. (2011a). Lack of social exploratory activation in male μ-opioid receptor KO mice in response to playback of female ultrasonic vocalizations. *Soc. Neurosci.* 6, 76–87. doi: 10.1080/17470911003765560

Wöhr, M., Roullet, F. I., and Crawley, J. N. (2011b). Reduced scent marking and ultrasonic vocalizations in the BTBR T+tf/J mouse model of autism. *Genes Brain Behav.* 10, 35–43. doi: 10.1111/j.1601-183X.2010.00582.x

Wöhr, M., and Scattoni, M. L. (2013). Behavioural methods used in rodent models of autism spectrum disorders: current standards and new developments. *Behav. Brain Res.* 251, 5–17. doi: 10.1016/j.bbr.2013.05.047

Yafai, A. F., Verrier, D., and Reidy, L. (2014). Social conformity and autism spectrum disorder: a child-friendly take on a classic study. *Autism* 18, 1007–1013. doi: 10.1177/1362361313508023

Yang, M., Abrams, D. N., Zhang, J. Y., Weber, M. D., Katz, A. M., Clarke, A. M., et al. (2012a). Low sociability in BTBR T+tf/J mice is independent of partner strain. *Physiol. Behav.* 107, 649–662. doi: 10.1016/j.physbeh.2011.12.025

Yang, M., Bozdagi, O., Scattoni, M. L., Wöhr, M., Roullet, F. I., Katz, A. M., et al. (2012b). Reduced excitatory neurotransmission and mild autism-relevant phenotypes in adolescent Shank3 null mutant mice. *J. Neurosci.* 32, 6525–6541. doi: 10.1523/JNEUROSCI.6107-11.2012

Yang, M., Clarke, A. M., and Crawley, J. N. (2009). Postnatal lesion evidence against a primary role for the corpus callosum in mouse sociability. *Eur. J. Neurosci.* 29, 1663–1677. doi: 10.1111/j.1460-9568.2009.06714.x

Yang, M., Loureiro, D., Kalikhman, D., and Crawley, J. N. (2013). Male mice emit distinct ultrasonic vocalizations when the female leaves the social interaction arena. *Front. Behav. Neurosci.* 7:159. doi: 10.3389/fnbeh.2013.00159

Yang, M., Scattoni, M. L., Zhodzishsky, V., Chen, T., Caldwell, H., Young, W. S., et al. (2007a). Social approach behaviors are similar on conventional versus reverse lighting cycles, and in replications across cohorts, in BTBR T+tf/J, C57BL/6J, and vasopressin receptor 1B mutant mice. *Front. Behav. Neurosci.* 1:1. doi: 10.3389/neuro.08.001.2007

Yang, M., Zhodzishsky, V., and Crawley, J. N. (2007b). Social deficits in BTBR T+tf/J mice are unchanged by cross-fostering with C57BL/6J mothers. *Int. J. Dev. Neurosci.* 25, 515–521. doi: 10.1016/j.ijdevneu.2007.09.008

Yee, N., Schwarting, R. K. W., Fuchs, E., and Wöhr, M. (2012). Increased affective ultrasonic communication during fear learning in adult male rats exposed to maternal immune activation. *J. Psychiatr. Res.* 46, 1199–1205. doi: 10.1016/j.jpsychires.2012.05.010

Yoshida, W., Dziobek, I., Kliemann, D., Heekeren, H. R., Friston, K. J., and Dolan, R. J. (2010). Cooperation and heterogeneity of the autistic mind. *J. Neurosci.* 30, 8815–8818. doi: 10.1523/JNEUROSCI.0400-10.2010

Zanettini, C., Carola, V., Lo Iacono, L., Moles, A., Gross, C., and D'Amato, F. R. (2010). Postnatal handling reverses social anxiety in serotonin receptor 1A knockout mice. *Genes Brain Behav.* 9, 26–32. doi: 10.1111/j.1601-183X.2009.00531.x

Zhang, W. Q., Smolik, C. M., Barba-Escobedo, P. A., Gamez, M., Sanchez, J. J., Javors, M. A., et al. (2014). Acute dietary tryptophan manipulation differentially alters social behavior, brain serotonin and plasma corticosterone in three inbred mouse strains. *Neuropharmacology* 90, 1–8. doi: 10.1016/j.neuropharm.2014.10.024

Zhang, Y., Gao, D., Kluetzman, K., Mendoza, A., Bolivar, V. J., Reilly, A., et al. (2013). The maternal autoimmune environment affects the social behavior of offspring. *J. Neuroimmunol.* 258, 51–60. doi: 10.1016/j.jneuroim.2013.02.019

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 25 March 2015
doi: 10.3389/fnins.2015.00099

Social buffering suppresses fear-associated activation of the lateral amygdala in male rats: behavioral and neurophysiological evidence

Felipe Fuzzo[1], *Jumpei Matsumoto*[1], *Yasushi Kiyokawa*[2], *Yukari Takeuchi*[2], *Taketoshi Ono*[1] *and Hisao Nishijo*[1]*

[1] System Emotional Science, University of Toyama, Sugitani, Toyama, Japan, [2] Laboratory of Veterinary Ethology, The University of Tokyo, Tokyo, Japan

OPEN ACCESS

Edited by:
Kathleen Dave, St. Luke's University Health Network, USA

Reviewed by:
Hiroaki Wagatsuma, Kyushu Institute of Technology, Japan
Jian Han, The Pennsylvania State University, USA

***Correspondence:**
Hisao Nishijo, System Emotional Science, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Sugitani 2630, Toyama 930-0194, Japan
nishijo@med.u-toyama.ac.jp

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *27 November 2014*
Accepted: *09 March 2015*
Published: *25 March 2015*

Citation:
Fuzzo F, Matsumoto J, Kiyokawa Y, Takeuchi Y, Ono T and Nishijo H (2015) Social buffering suppresses fear-associated activation of the lateral amygdala in male rats: behavioral and neurophysiological evidence. Front. Neurosci. 9:99. doi: 10.3389/fnins.2015.00099

In social mammals, the presence of an affiliative conspecific reduces stress responses, a phenomenon referred to as "social buffering." In a previous study, we found that the presence of a conspecific animal ameliorated a variety of stress responses to an aversive conditioned stimulus (CS), including freezing and Fos expression in the lateral amygdala (LA) of male rats. Although these findings suggest that the presence of a conspecific animal suppresses neural activity in the LA, direct neurophysiological evidence of suppressed activity in the LA during social buffering is still lacking. In the present study, we analyzed freezing behavior and local field potentials in the LA of fear-conditioned rats in response to the CS, in the presence or absence of a conspecific. After auditory aversive conditioning, the CS was presented to the conditioned rats in the presence or absence of a conspecific animal, on 2 successive days. The presence of a conspecific animal significantly decreased the mean peak amplitudes of auditory evoked field potentials, gamma oscillations (25–75 Hz) and high frequency oscillations (100–300 Hz) in the LA. Furthermore, magnitudes of these neural responses positively correlated with freezing duration of the fear-conditioned rats. The results provide the first electrophysiological evidence that social buffering suppresses CS-induced activation in the LA, which consequently reduces conditioned fear responses.

Keywords: social buffering, gamma oscillation, high frequency oscillation, lateral amygdala, male rat

Introduction

In social mammals, the presence of an affiliative conspecific reduces stress responses induced by a variety of stimuli. For example, the presence of an accompanying conspecific, or cues associated with a conspecific, reduces stress responses to a loud noise in rats (Taylor, 1981), and to a novel environment in rats (Latane, 1969; Terranova et al., 1999; Wilson, 2000; Kiyokawa et al., 2014b), sheep (da Costa et al., 2004), cows (Boissy and Le Neindre, 1990), and monkeys (Winslow et al., 2003). In addition, it ameliorates stress responses to a predator or predator-associated cues in rats (Bowen et al., 2013) and monkeys (Vogt et al., 1981). This phenomenon is called "social buffering" (Hennessy et al., 2009).

Social buffering phenomena have also been reported in experimental models using the fear-conditioning paradigm (Davitz and Mason, 1955; Stanton et al., 1985). After receiving simultaneous presentation of a conditioned stimulus (CS) and foot shock during the conditioning phase, the animal shows stress responses to the CS alone during a testing phase. We have previously reported that a variety of stress responses in adult male rats, including freezing behavior to an auditory CS, were reduced by the presence of another adult male rat (Kiyokawa et al., 2007, 2014a), suggesting that social buffering mitigates the conditioned fear response. This social buffering seems not to be induced by heterospecifics because the presence of a male guinea pig did not suppress stress responses in male rats (Kiyokawa et al., 2009). Because these effects persisted even if the dyad were separated by two wire mesh screens (Kiyokawa et al., 2009), the subject rat supposedly received non-somatosensory signals from the accompanying rat. On exploring this further, we found that when the main olfactory epithelium (MOE) of the subject rat was lesioned beforehand, the subject rat showed stress responses even if it was accompanied by a conspecific (Kiyokawa et al., 2009). In addition, the presence of olfactory signals alone induced social buffering of conditioned fear responses (Takahashi et al., 2013; Kiyokawa et al., 2014b). This evidence suggests that olfactory signals detected by the MOE play an important role in social buffering of conditioned fear responses.

In parallel with these studies, we investigated the neural mechanism underlying social buffering of conditioned fear responses. Because anatomical evidence indicates that all signals detected at the MOE are sent to the main olfactory bulb (Mombaerts et al., 1996), this would presumably also be true for olfactory signals responsible for social buffering. Indeed, anatomical and lesion studies have revealed that the signals responsible for social buffering are transmitted to the posteromedial region of the olfactory peduncle (pmOP) (Kiyokawa et al., 2012). Because activation of the lateral amygdala (LA) plays pivotal roles in aversive conditioning (Nishijo et al., 1988; LeDoux et al., 1990; Ono et al., 1995; LeDoux, 2000), we hypothesized that the olfactory signals responsible for social buffering suppress LA activation during social buffering of conditioned fear responses. Although our previous studies have found that the pmOP and amygdala are anatomically and functionally connected (Kiyokawa et al., 2012) and that social buffering suppresses Fos expression in the LA (Kiyokawa et al., 2007, 2014b; Takahashi et al., 2013), direct electrophysiological evidence that supports this hypothesis is lacking. It is noted that Fos expression could be induced without neuronal depolarization (e.g., Numan, 2014).

To test this hypothesis, we directly observed neuronal activity in the LA using neurophysiology. Briefly, fear-conditioned subjects were exposed to the CS either alone or with a conspecific rat separated by two wire mesh screens. Fear conditioning has been reported to enhance auditory evoked field potentials (AEFPs) in the LA (Rogan and LeDoux, 1995; Rogan et al., 1997). In addition, previous studies have reported that fear conditioning enhances neuronal responses to CS in the LA (Nishijo et al., 1988; Muramoto et al., 1993; Quirk et al., 1995; Repa et al., 2001), and that the power of gamma and high frequency (HF) oscillations of the local field potentials correlates with local neuronal activities (Ray and Maunsell, 2011; Buzsáki and Wang, 2012; Buzsáki and Silva, 2012). Based on these findings, we analyzed AEFPs, and power of gamma and HF oscillations of the local field potentials in the LA of rats in the presence or absence of a conspecific rat.

Materials and Methods

Animals

Forty-four experimentally naïve male Wistar rats (aged 6–7 weeks) were used (Charles River Laboratories, Kanagawa, Japan). The rats were initially housed two animals per cage, in an ambient temperature of 23 ± 1°C and under a 12-h light/12-h dark cycle (lights switched on at 07:00). Food and water were available *ad libitum*. The rats were assigned to two groups, either the subjects ($n = 22$) or conspecifics ($n = 22$) that received no fear-conditioning and were only exposed to the CS during the testing phase. Five days after their arrival, rats were housed individually. All rats were handled for 5 min per day for 3 days prior to testing in order to minimize the effects of inevitable handling during the experiments. All rats were treated in strict compliance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals, National Institutes of Health Guide for the Care and Use of Laboratory Animals, and Guidelines for the Care and Use of Laboratory Animals at the University of Toyama. All experimental procedures were approved by our institutional committee for experimental animal ethics.

Surgery

Five days after their arrival, that is, 7 days before conditioning, each subject was anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Then, an electrode assembly was implanted unilaterally (right side $n = 9$) or bilaterally ($n = 13$) aiming at the LA (2.9 mm caudal from the bregma, 4.3–5.3 mm lateral from the midline, and 6.1–6.8 mm below the brain surface) based on the brain atlas of Paxinos and Watson (2006). Each electrode assembly was composed of four tetrodes and a microdrive. Each tetrode comprised four tungsten microwires (20 μm in diameter; California Fine Wire, Grover Beach, CA), which were encased in stainless steel tubes (30 gauge; Hakko, Osaka, Japan). The tip impedance was around 200 kΩ at 1 kHz.

Fear Conditioning

Fear conditioning was performed in an illuminated room between 09:00 and 13:00, and has been described in our previous studies (Kiyokawa et al., 2009, 2012, 2013). During conditioning, each subject was placed in an acrylic conditioning box with a punctured ceiling and metal grid floor [28 × 20 × 20 (height) cm] for 20 min, where seven repetitions of a 3-s tone (800 Hz, 85 dB) that terminated concurrently with a foot shock (0.5 s, 0.8 mA) were presented. The intertrial interval randomly varied between 30 and 180 s. After the conditioning, the subject was returned to its home cage.

Fear-expression Tests and Neurophysiological Recordings

The apparatus for the fear-expression test consisted of two rectangular enclosures [25 × 25 × (height) 45 cm] placed on an acrylic

board (45 × 60 cm) (**Figure 1A**). Each enclosure comprised three acrylic walls and one removable wire mesh wall. Clean bedding was spread to cover the floor. The wire mesh wall (height, 45 cm) consisted of a 1-cm^2 grid mesh in the lower part (height, 20 cm) and vertical bars spaced at 1-cm intervals in the upper part (height, 25 cm), which prevented the rats from climbing up. Two enclosures were placed side-by-side so that the wire mesh walls of both were adjacent to each other at a distance of 5 cm.

Two fear-expression tests were conducted over 2 successive days. The first test was conducted one day after conditioning between 09:00 and 13:00 in an illuminated room as described in our previous studies (Kiyokawa et al., 2009, 2012, 2013) with a slight modification. After the cables had been connected to their heads, the subjects were tested in one of the two conditions: in "Alone" ($n = 10$) or "Social" ($n = 12$) conditions. In the Alone condition (**Figure 1A**, left), the subject was placed in one enclosure while the other enclosure was left vacant. In the Social condition (**Figure 1A**, right), the subject was placed in one enclosure, and the conspecific was placed in the other. After an acclimation period of 2–3 min, subject rats performed the first fear-expression test, wherein the CS was presented for a duration of 3 s at 1-min intervals, for 5 min. On the second day, each subject underwent the second fear-expression test in a condition different from that experienced in the first fear-expression test. Thus, all rats were tested in both conditions. Each subject similarly underwent the fear-expression test, although the second fear-expression test had a duration of 20 min, and so the CS was presented 20 times.

Subject behavior during the fear-expression tests was recorded with a CCD camera. The analog signals of neuronal activities were digitized and stored in a computer via Omniplex (Plexon, Dallas, TX). For the subjects implanted with two electrode assemblies bilaterally, the electrode assembly that detected spontaneous neural activity at a higher S/N ratio was selected and used for recording. The amplified neuronal signals were digitized at a 40 kHz sampling rate. The signals were low pass-filtered (300 Hz) and stored on a computer at a 1-kHz sampling rate for the analysis of field potentials.

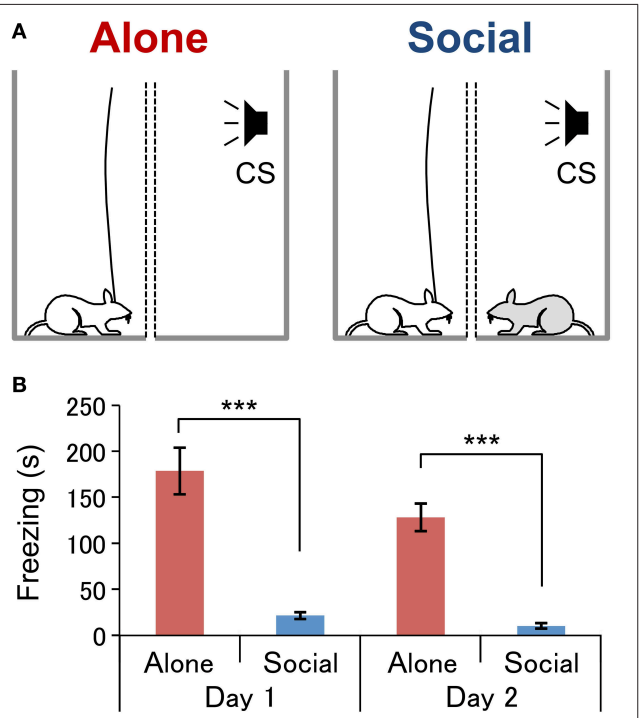


FIGURE 1 | Experimental conditions (A) and behavioral results (B). (A) Experimental conditions under which neurophysiological recordings were conducted. In the Alone condition (left, Alone), a subject rat (white) was exposed to an auditory conditioned stimulus (CS) without the presence of an associate. In the Social condition (right, Social), a subject rat was exposed to the CS in the presence of an conspecific rat (gray). Two wire mesh walls (dotted lines) were installed in order to prevent physical contact between the rats. **(B)** Comparison of the freezing duration between the conditions on each day of the fear-expression test. $^{***}P < 0.001$, Student's *t*-test.

Behavioral Data Analysis

A researcher who was blind to the experimental conditions recorded the duration of freezing behavior (immobile posture with cessation of skeletal and vibrissae movement except in respiration) based on visual observation of the video recordings. The data are expressed as means ± standard error of means, and significance was set at $P < 0.05$ for all statistical tests. The mean duration of freezing during the first and second fear-expression tests, as well as during the first 5-min of the second fear-expression test, were analyzed with the Student's *t*-test.

Neurophysiological Data Analysis

In the present study, we analyzed AEFPs and event-related spectral perturbation (ERSP), and spectral power to assess neural activity in the LA. AEFPs elicited by the CS in the second fear-expression test were measured against a ground-reference electrode (stainless screw) on the skull over the cerebellum, and were averaged across the 20 CS presentations. These averaged AEFPs were corrected for the baseline during 50 ms before the CS onset. The mean amplitudes of the averaged AEFPs around the peak latencies of the AEFPs (21–23 ms after the CS onset) were compared between the Alone and Social condition using the Student's *t*-test.

To analyze localized neural activity in the LA, local field potentials, recorded by bipolar recording from two electrodes selected from different tetrodes in the LA that were separated by around 600 μm, were subjected to ERSP and spectral power analyses. ERSPs in individual CS trials in the second fear-expression test were computed by the Matlab function "newtimef.m," a time-frequency decomposition function in the EEGLAB toolbox (Delorme and Makeig, 2004). The time-frequency decomposition was performed using Morlet wavelets with a constant three-cycle length. ERSP values were normalized against the spectral power during the 50-ms pre-CS period. The grand mean ERSPs were computed separately for the two conditions (Alone/Social) by averaging mean ERSPs of individual rats in the second fear-expression test. To estimate latencies of neural responses to the CS in gamma (25–75 Hz) and HF (100–300 Hz) bands, mean normalized ERSP values in gamma and HF bands within a 100 ms window (from −50 to 50 ms around the CS onset) were analyzed with a Two-Way repeated measures ANOVA: two conditions

(Alone/Social) × 100 time bins (1-ms bins from -50 to 50 ms around the CS onset). Subsequent multiple *post-hoc* comparisons were performed with simple main effect analyses. Response latency was defined as the time of the first significant difference between the two conditions after the CS onset.

In the spectral power analysis, power changes after the CS onset in two frequency bands [gamma (25–75 Hz) and HF (100–300 Hz)] were compared between the two conditions. First, power spectrums in two windows of 80 ms (−80 ms before the CS onset; 10–90 ms after the CS onset) in individual CS trials for each condition were computed by Welch's method (Matlab function pWelch) using a single Hamming window taper and 50% overlapping 40-ms time windows. Total powers in the gamma and HF bands were then calculated for individual CS trials in each condition for each rat. Power changes were calculated by subtracting the total powers in the two frequency bands measured within the two windows of interest (−80–0 ms; 10–90 ms). In this data processing, power changes were computed using two data sets; one data set consisting of power change data derived from the first five CS trials in the first and second fear-expression tests, and another consisting of the power change data derived from the 20 CS trials of the second fear-expression test. In the first data set, the power changes in gamma and HF bands were compared between the two conditions using the Friedman test. In the second data set, the power changes in gamma and HF bands were compared between the two conditions using the Wilcoxon rank-sum test.

To investigate the relationship between neural and behavioral responses in the second fear-expression test, the correlation between mean AEFP amplitudes and freezing duration was analyzed using Pearson's correlation analysis. The correlation between the power changes in each frequency band and freezing duration were also analyzed using Spearman's correlation analysis since the data did not show a normal distribution.

Histology

After the experiments, all subjects were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and received a 20-μA negative current through the recording electrodes for 30 s. The subject rats were then transcardially perfused with 0.9% saline followed by 10% buffered formalin containing 2% potassium ferricyanide. The brain was removed and fixed in formalin for at least 48 h. Serial sections of 50 μm were cut on a freezing microtome and stained with Cresyl Violet. Electrode locations were

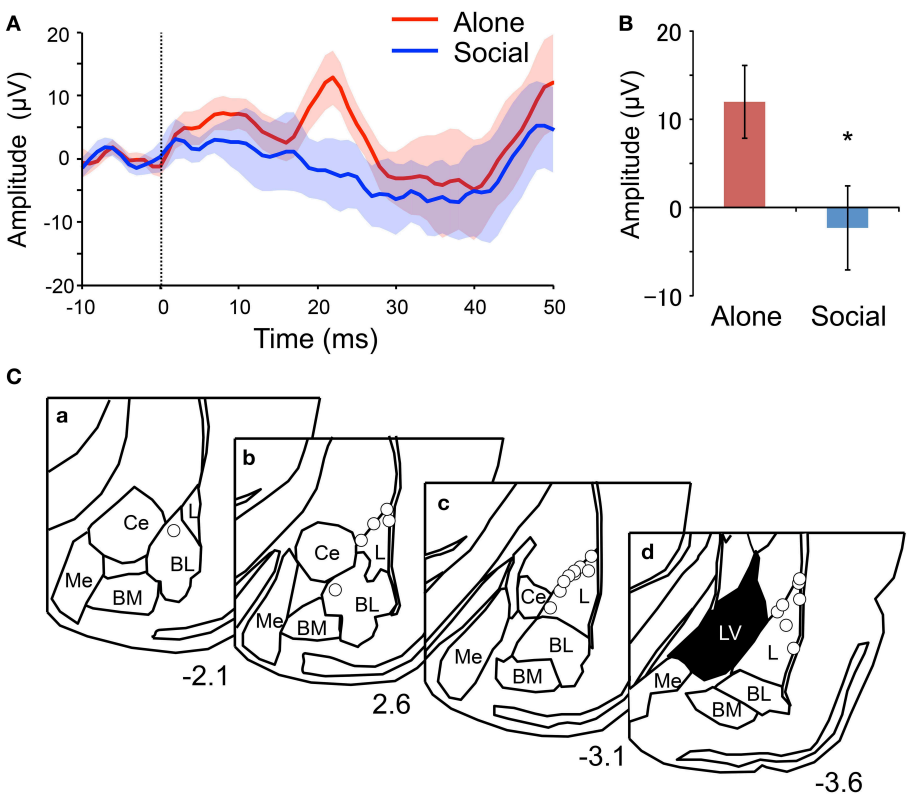


FIGURE 2 | Reduction of mean peak amplitude of the auditory evoked field potentials (AEFPs) in the Social condition. (A) Averaged AEFPs in the Alone (red) and Social (blue) conditions. The dotted line indicates CS onset. The solid lines and translucent areas indicate the means and SEMs, respectively. **(B)** Comparison of the mean peak amplitudes (averaged voltages between 21 and 23 ms after CS onset) between the conditions. $^{*}P < 0.05$, unpaired *t*-test. **(C)** Locations of AEFP-recording electrodes. Circles indicate the locations. The value below each section indicates distance (mm) from the bregma. L, lateral amygdala; BL, basolateral amygdala; BM, basomedial amygdala; Me, medial amygdala; Ce, central amygdala; LV, lateral ventricle.

verified microscopically and mapped onto the appropriate tissue sections with reference to the atlas of Paxinos and Watson (2006).

Results

Behavioral Analysis

In the present study, fear-conditioned subjects underwent two fear-expression tests, one alone (Alone condition, **Figure 1A**, left) and the other with an a conspecific (Social condition, **Figure 1A**, right). The mean duration of freezing was significantly shorter in the Social than in the Alone condition (**Figure 1B**) in both the first fear-expression test (Student's t-test, $P < 0.001$) and first 5-min of the second fear-expression test (Student's t-test, $P < 0.001$). A collective analysis of data from both tests revealed freezing duration to be significantly shorter in the Social than in the Alone condition (Social, 17 ± 3 s; Alone, 151 ± 15; Student's t-test, $P < 0.001$).

AEFP Analysis

AEFPs recorded during the presentation of 20 CS in the second fear-expression test were analyzed. For this test, freezing duration was significantly shorter in the Social than in the Alone condition

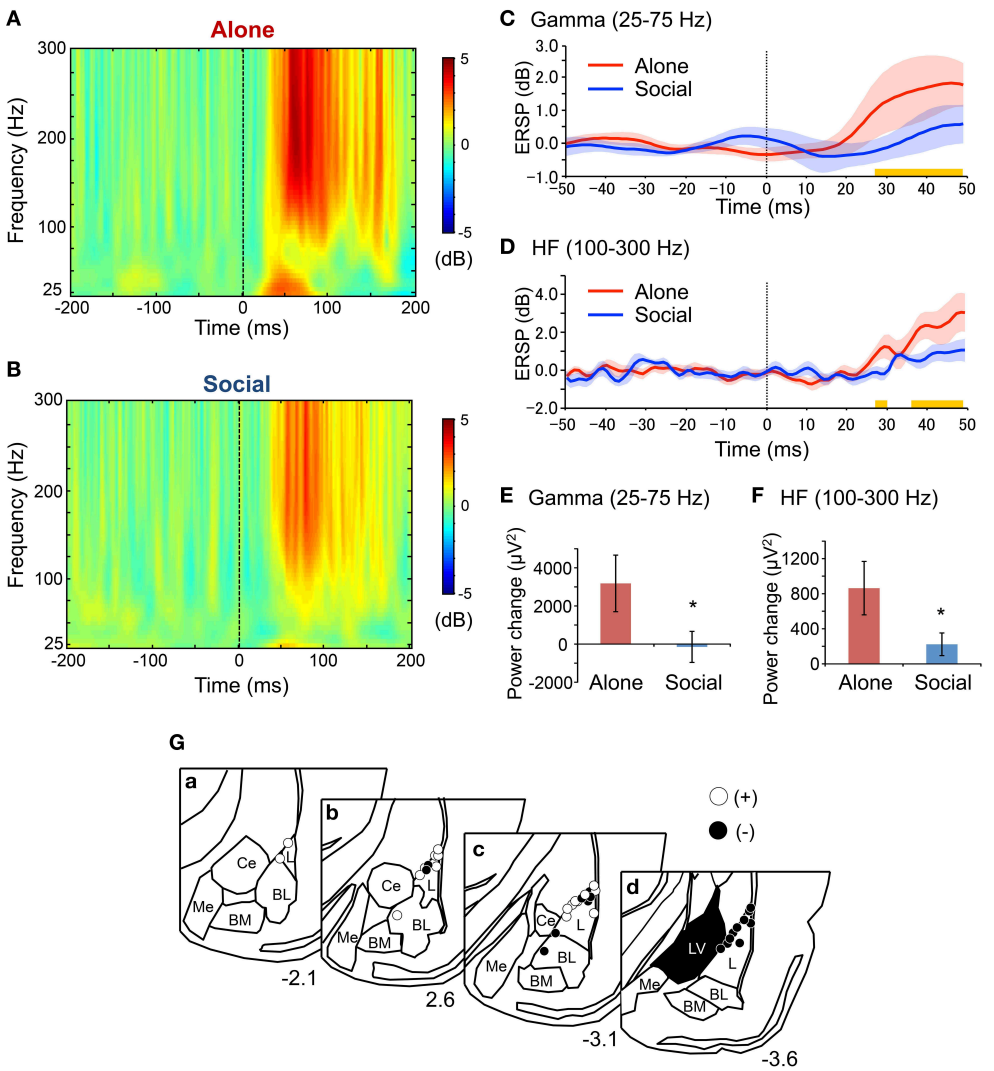


FIGURE 3 | Reduction of gamma and high frequency (HF) oscillations after the CS in the Social condition in the second fear-expression test. (A,B) Averaged event-related spectral perturbations (ERSPs) in the Alone **(A)** and the Social **(B)** conditions. The dotted lines indicate CS onset. Each ERSP value was corrected for a log power spectrum of the −50–0 ms pre-tone period (in dB). **(C,D)** The time courses of averaged ERSP of gamma **(C)** and HF **(D)** oscillations. The red and blue solid lines indicate the mean ERSPs in the Alone and Social conditions, respectively. The corresponding translucent areas indicate the SEMs. The dotted line indicates the CS onset. Yellow bars indicate the latency windows in which there were significant differences between the conditions ($P < 0.05$, simple main effect analysis). **(E,F)** Comparison of power changes after the CS in gamma **(E)** and HF **(F)** ranges between the conditions. $^{*}P < 0.05$, Wilcoxon rank-sum test. **(G)** Locations of the electrodes recording local oscillations in bipolar measurement. Open and filled circles indicate positive and negative poles, respectively. Other conventions are the same as those of **Figure 2C**.

(Social, 49 ± 10 s; Alone, 422 ± 55; Student's *t*-test, $P < 0.001$). **Figure 2A** shows grand averaged AEFPs across all rats, and indicates a clear peak at 22 ms after CS onset in the Alone condition, but not in the Social condition. In addition, the mean peak amplitudes (averaged voltages between 21 and 23 ms after CS onset) were significantly smaller in the Social than in the Alone condition (Student's *t*-test, $P < 0.05$) (**Figure 2B**). Tip locations of the electrodes recording AEFPs are shown in **Figure 2C**. All electrode tips were located within the basolateral amygdala, most of which were located in the lateral nucleus (L) of the amygdala.

ERSP and Spectral Power Analyses

ERSPs recorded during the presentation of 20 CS in the second fear-expression test were analyzed. **Figure 3** shows the grand averaged ERSPs across all rats in the Alone (**Figure 3A**) and Social (**Figure 3B**) conditions. Gamma and HF oscillation was more prominent after CS onset in the Alone compared with the Social condition. Response latencies in the two frequency bands were estimated by analyzing at what point after CS onset differences in ERSP values between the two conditions became significant (**Figures 3C,D**). The first significant differences between the two conditions were noted 28 ms after CS onset in both gamma (simple main effect analysis, $P < 0.05$) and HF (simple main effect analysis, $P < 0.05$) bands. Tip locations of the bipolar electrodes recording ERSPs are shown in **Figure 3G**.

Figures 3E,F show results of the spectral power analysis of the data derived from 20 CS trials in the second fear-expression tests in gamma (**Figure 3E**) and HF (**Figure 3F**) bands. Power changes in gamma (Wilcoxon rank-sum test, $P < 0.05$) and HF oscillation (Wilcoxon rank-sum test, $P < 0.05$) were significantly smaller in the Social than in the Alone condition. Furthermore, analysis of data from the initial 5 CS trials in the first and second fear-expression test revealed similar results; power changes in the HF band were significantly smaller in the Social than in the Alone condition (Social, 280 ± 174 μV^2; Alone, 792 ± 239; Friedman test, $P < 0.05$), although there were no significant differences in power changes in the gamma band (Social, 5654 ± 4721 μV^2; Alone, 3057 ± 1838; Friedman test, $P > 0.05$).

Correlational Analysis

Figure 4 shows the correlation between behavioral and neural responses to the CS. Freezing duration significantly and positively correlated with the peak mean amplitudes of the AEFPs (**Figure 4A**) ($P < 0.01$), power change of gamma oscillations (**Figure 4B**) ($P < 0.01$), and power change of HF oscillations (**Figure 4C**) ($P < 0.05$).

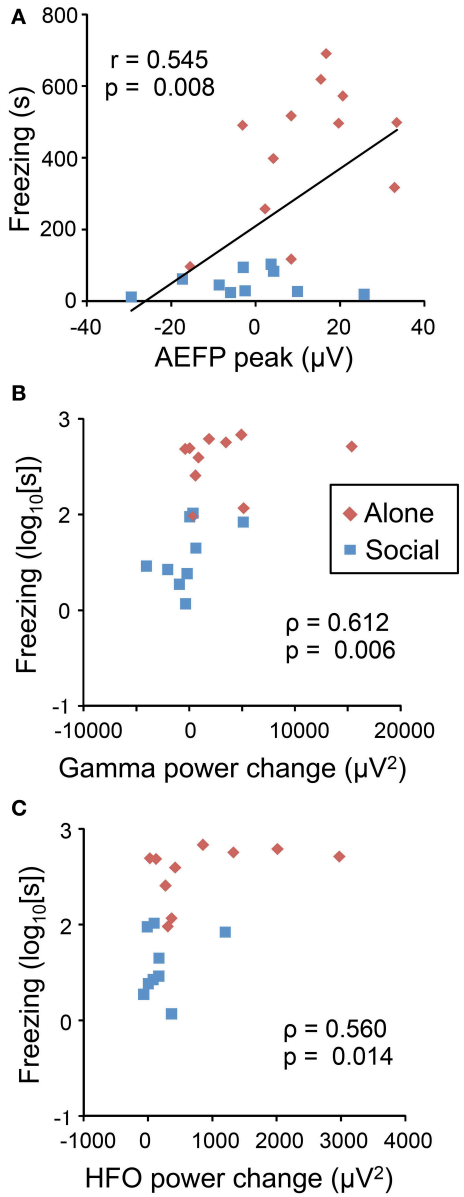


FIGURE 4 | Correlation between neurophysiological responses to the CS and freezing behaviors. Freezing duration positively correlated with AEFP peak amplitude **(A)**, power change of gamma oscillations **(B)**, and power change of HF oscillations **(C)**. Red and blue dots indicate the data from individual rats in the Alone or Social condition, respectively. Values in the figures indicate Pearson's (r, **A**) and Spearman's correlation coefficients (ρ, **B**,**C**), and the corresponding *p*-values.

Discussion

The Role of the LA in Social Buffering

Previous two studies also reported suppression of the expressions of the immediate early genes in the LA during social buffering in sheep and rats (da Costa et al., 2004; Kiyokawa et al., 2007). However, the expressions of the immediate early genes are indirect measures of neural activity (see Introduction). On the other hand, local field potentials recorded in the present study more directly reflects neural activity and enables to analyze precise time-courses of neuronal activity in response to the CS. Thus, the present results provide the first direct electrophysiological evidence that CS-induced LA activation was suppressed during social buffering of conditioned fear responses.

Consistent with previous studies (Kiyokawa et al., 2007, 2014a), the present study found that the presence of a conspecific animal suppressed freezing behavior of rat subjects during presentation of an auditory CS. These results indicate that social

buffering mitigates conditioned fear responses. Consistent with these behavioral results, the peak mean amplitudes of AEFPs and power changes elicited by the CS in gamma and HF bands in the LA were suppressed in the Social condition. These results thus provide direct electrophysiological evidence for the suppression of LA activation during social buffering of conditioned fear responses. Furthermore, behavioral responses (freezing duration) positively correlated with the peak mean amplitudes of AEFPs and power changes in gamma and HF bands, which suggests that behavioral responses and LA activation were positively correlated. Considering that LA lesions block freezing responses to CS (LeDoux et al., 1990), we can hypothesize that in the present study, social buffering was mediated through the suppression of neural activity in the LA in male rats.

The CS-induced activation in the LA observed in the alone condition had several similar characteristics to those reported in the previous studies. The peak latency of AEFPs observed in the present study was 22 ms after the onset of the CS, which was consistent with the previous studies reporting the peak latency around 10–30 ms after the onset of the CS (Rogan and LeDoux, 1995; Rogan et al., 1997). Furthermore, as in the previous study (Schafe et al., 2005), the peak amplitudes were correlated with the intensity of freezing behavior in response to the CS. In addition, the response latencies of gamma and HF oscillations were 28 ms in this study. Although, to the best of our knowledge, this is the first study that measured the change of gamma and HF oscillations in response to the CS, these oscillations has been suggested to correlate with local neuronal activities (Ray and Maunsell, 2011; Buzsáki and Silva, 2012; Buzsáki and Wang, 2012). In previous studies, single neuronal responses to the CS were observed around 10–30 ms after the onset of the CS (Muramoto et al., 1993; Quirk et al., 1995; Repa et al., 2001), suggesting that the response latencies of gamma and HF oscillations in this study were consistent with the neuronal activities reported in previous studies. Taken together, these characteristics suggest that CS-induced activation in the LA observed in the present study was consistent with those reported in previous literatures.

Neural Pathways for Social Buffering

Because olfactory signals responsible for social buffering are transmitted from the MOE to the pmOP, which is then activated (see Introduction), the pmOP is supposedly responsible for this suppression of LA activation, perhaps via a direct suppression. We have previously found that the pmOP directly projects to the LA, and that these two structures are functionally connected (Kiyokawa et al., 2012). The pmOP could therefore suppress principal (pyramidal-like) neurons in the LA if its direct projections are inhibitory. The second possibility is that the pmOP suppresses activation of principal neurons in the LA via an activation of GABAergic interneurons in the LA. Finally, that the pmOP might indirectly suppresses principal neurons in the LA, whereby intercalated cells might serve as the relay site for LA suppression. The intercalated cells located as clusters in the fiber bundles surrounding the basolateral complex of the amygdala (BLA) include GABAergic neurons, and send projections to the LA and the central amygdala (CeA) (Duvarci and Pare, 2014). It has been reported that the extinction of conditioned fear depends on activation of the intercalated cells that suppress CS-induced CeA activation (Duvarci and Pare, 2014). In addition, the pmOP not only projects to the LA, but also seems to project to the fiber bundles surrounding the BLA (Kiyokawa et al., 2012). Considering this, it is possible that the pmOP activates the intercalated cells, which in turn suppress CS-induced activation of principal neurons in the LA in response to the CS during social buffering of conditioned fear responses. Further studies are required to investigate these possibilities.

In conclusion, we have shown that LA activation following an auditory CS is suppressed during social buffering of conditioned fear responses. Since social buffering is considered one of the key characteristics for a species to be gregarious, the present findings provide insights for future studies that aim to investigate the neurobiology of gregariousness and sociability.

Acknowledgments

This work was supported partly by the Japan Society for the Promotion of Science Asian Core Program, the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Scientific Research (B) (25290005), and Grant-in-Aid for Young Scientists (A) (23688035) from the Japan Society for the Promotion of Science.

References

Boissy, A., and Le Neindre, P. (1990). Social influences on the reactivity of heifers: implications for learning abilities in operant conditioning. *Appl. Anim. Behav. Sci.* 25, 149–165. doi: 10.1016/0168-1591(90)90077-Q

Bowen, M. T., Kevin, R. C., May, M., Staples, L. G., Hunt, G. E., and McGregor, I. S. (2013). Defensive aggregation (huddling) in *Rattus Norvegicus* toward predator odor: individual differences, social buffering effects and neural correlates. *PLoS ONE* 8:e68483. doi: 10.1371/journal.pone.0068483

Buzsáki, G., and Silva, F. L. (2012). High frequency oscillations in the intact brain. *Prog. Neurobiol.* 98, 241–249. doi: 10.1016/j.pneurobio.2012.02.004

Buzsáki, G., and Wang, X. J. (2012). Mechanisms of gamma oscillations. *Annu. Rev. Neurosci.* 35, 203–225. doi: 10.1146/annurev-neuro-062111-150444

da Costa, A. P., Leigh, A. E., Man, M. S., and Kendrick, K. M. (2004). Face pictures reduce behavioural, autonomic, endocrine and neural indices of stress and fear in sheep. *Proc. R. Soc. Lond. B Biol. Sci.* 271, 2077–2084. doi: 10.1098/rspb.2004.2831

Davitz, J. R., and Mason, D. J. (1955). Socially facilitated reduction of a fear response in rats. *J. Comp. Physiol. Psychol.* 48:149. doi: 10.1037/h0046411

Delorme, A., and Makeig, S. (2004). EEGLAB: an open source toolbox for analysis of single-trial EEG dynamics including independent component analysis. *J. Neurosci. Methods* 134, 9–21. doi: 10.1016/j.jneumeth.2003.10.009

Duvarci, S., and Pare, D. (2014). Amygdala microcircuits controlling learned fear. *Neuron* 82, 966–980. doi: 10.1016/j.neuron.2014.04.042

Hennessy, M. B., Kaiser, S., and Sachser, N. (2009). Social buffering of the stress response: diversity, mechanisms, and functions. *Front. Neuroendocrinol.* 30, 470–482. doi: 10.1016/j.yfrne.2009.06.001

Kiyokawa, Y., Hiroshima, S., Takeuchi, Y., and Mori, Y. (2014a). Social buffering reduces male rats' behavioral and corticosterone responses to a conditioned stimulus. *Horm. Behav.* 65, 114–118. doi: 10.1016/j.yhbeh.2013.12.005

Kiyokawa, Y., Honda, A., Takeuchi, Y., and Mori, Y. (2014b). A familiar conspecific is more effective than an unfamiliar conspecific for social buffering of conditioned fear responses in male rats. *Behav. Brain Res.* 267, 189–193. doi: 10.1016/j.bbr.2014.03.043

Kiyokawa, Y., Kodama, Y., Takeuchi, Y., and Mori, Y. (2013). Physical interaction is not necessary for the induction of housing-type social buffering of conditioned hyperthermia in male rats. *Behav. Brain Res.* 256, 414–419. doi: 10.1016/j.bbr.2013.08.037

Kiyokawa, Y., Takeuchi, Y., and Mori, Y. (2007). Two types of social buffering differentially mitigate conditioned fear responses. *Eur. J. Neurosci.* 26, 3606–3613. doi: 10.1111/j.1460-9568.2007.05969.x

Kiyokawa, Y., Takeuchi, Y., Nishihara, M., and Mori, Y. (2009). Main olfactory system mediates social buffering of conditioned fear responses in male rats. *Eur. J. Neurosci.* 29, 777–785. doi: 10.1111/j.1460-9568.2009.06618.x

Kiyokawa, Y., Wakabayashi, Y., Takeuchi, Y., and Mori, Y. (2012). The neural pathway underlying social buffering of conditioned fear responses in male rats. *Eur. J. Neurosci.* 36, 3429–3437. doi: 10.1111/j.1460-9568.2012.08257.x

Latane, B. (1969). Gregariousness and fear in laboratory rats. *J. Exp. Soc. Psychol.* 5, 61–69. doi: 10.1016/0022-1031(69)90006-7

LeDoux, J. E. (2000). Emotion circuits in the brain. *Annu. Rev. Neurosci.* 23, 155–184. doi: 10.1146/annurev.neuro.23.1.155

LeDoux, J. E., Cicchetti, P., Xagoraris, A., and Romanski, L. M. (1990). The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. *J. Neurosci.* 10, 1062–1069.

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., et al. (1996). Visualizing an olfactory sensory map. *Cell* 87, 675–686. doi: 10.1016/S0092-8674(00)81387-2

Muramoto, K., Ono, T., Nishijo, H., and Fukuda, M. (1993). Rat amygdaloid neuron responses during auditory discrimination. *Neuroscience* 52, 621–636. doi: 10.1016/0306-4522(93)90411-8

Nishijo, H., Ono, T., and Nishino, H. (1988). Single neuron responses in amygdala of alert monkey during complex sensory stimulation with affective significance. *J. Neurosci.* 8, 3570–3583.

Numan, M. (2014). *Neurobiology of Social Behavior: Toward an Understanding of the Prosocial and Antisocial Brain*. London: Academic Press.

Ono, T., Nishijo, H., and Uwano, T. (1995). Amygdala role in conditioned associative learning. *Prog. Neurobiol.* 46, 401–422. doi: 10.1016/0301-0082(95)00008-J

Paxinos, G., and Watson, C. (2006). *The Rat Brain in Stereotaxic Coordinates, 6th Edn*. New York, NY: Academic.

Quirk, G. J., Repa, J. C., and LeDoux, J. E. (1995). Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: parallel recordings in the freely behaving rat. *Neuron* 15, 1029–1039. doi: 10.1016/0896-6273(95)90092-6

Ray, S., and Maunsell, J. H. (2011). Different origins of gamma rhythm and high-gamma activity in macaque visual cortex. *PLoS Biol.* 9:e1000610. doi: 10.1371/journal.pbio.1000610

Repa, J. C., Muller, J., Apergis, J., Desrochers, T. M., Zhou, Y., and LeDoux, J. E. (2001). Two different lateral amygdala cell populations contribute to the initiation and storage of memory. *Nat. Neurosci.* 4, 724–731. doi: 10.1038/89512

Rogan, M. T., and LeDoux, J. E. (1995). LTP is accompanied by commensurate enhancement of auditory-evoked responses in a fear conditioning circuit. *Neuron* 15, 127–136. doi: 10.1016/0896-6273(95)90070-5

Rogan, M. T., Stäubli, U. V., and LeDoux, J. E. (1997). Fear conditioning induces associative long-term potentiation in the amygdala. *Nature* 390, 604–607. doi: 10.1038/37601

Schafe, G. E., Doyère, V., and LeDoux, J. E. (2005). Tracking the fear engram: the lateral amygdala is an essential locus of fear memory storage. *J. Neurosci.* 25, 10010–10014. doi: 10.1523/JNEUROSCI.3307-05.2005

Stanton, M. E., Patterson, J. M., and Levine, S. (1985). Social influences on conditioned cortisol secretion in the squirrel monkey. *Psychoneuroendocrinology* 10, 125–134. doi: 10.1016/0306-4530(85)90050-2

Takahashi, Y., Kiyokawa, Y., Kodama, Y., Arata, S., Takeuchi, Y., and Mori, Y. (2013). Olfactory signals mediate social buffering of conditioned fear responses in male rats. *Behav. Brain Res.* 240, 46–51. doi: 10.1016/j.bbr.2012.11.017

Taylor, G. T. (1981). Fear and affiliation in domesticated male rats. *J. Comp. Physiol. Psychol.* 95, 685. doi: 10.1037/h0077817

Terranova, M. L., Cirulli, F., and Laviola, G. (1999). Behavioral and hormonal effects of partner familiarity in periadolescent rat pairs upon novelty exposure. *Psychoneuroendocrinology* 24, 639–656. doi: 10.1016/S0306-4530(99)00019-0

Vogt, J. L., Coe, C. L., and Levine, S. (1981). Behavioral and adrenocorticoid responsiveness of squirrel monkeys to a live snake: is flight necessarily stressful? *Behav. Neural Biol.* 32, 391–405. doi: 10.1016/S0163-1047(81)90826-8

Wilson, J. H. (2000). A conspecific attenuates prolactin responses to open-field exposure in rats. *Horm. Behav.* 38, 39–43. doi: 10.1006/hbeh.2000.1600

Winslow, J. T., Noble, P. L., Lyons, C. K., Sterk, S. M., and Insel, T. R. (2003). Rearing effects on cerebrospinal fluid oxytocin concentration and social buffering in rhesus monkeys. *Neuropsychopharmacology* 28, 910–918. doi: 10.1038/sj.npp.1300128

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 29 April 2015
doi: 10.3389/fnins.2015.00152

Consequences of temporary inhibition of the medial amygdala on social recognition memory performance in mice

Julia Noack, Rita Murau and Mario Engelmann *

Center for Behavioral Brain Sciences, Institut für Biochemie und Zellbiologie, Otto-von-Guericke-Universität Magdeburg, Magdeburg, Germany

OPEN ACCESS

Edited by:
Yasushi Kiyokawa, The University of Tokyo, Japan

Reviewed by:
Sabine M. Hölter, Helmholtz Zentrum München, German Research Center for Environmental Health, Germany
Heather Caldwell, Kent State University, USA

***Correspondence:**
Mario Engelmann, AG Neuroendokrinologie and Verhalten, Institut für Biochemie und Zellbiologie, Otto-von-Guericke-Universität Magdeburg, Leipziger Str. 44/Haus 1, D-39120 Magdeburg, Germany
mario.engelmann@med.ovgu.de

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *27 January 2015*
Accepted: *14 April 2015*
Published: *29 April 2015*

Citation:
Noack J, Murau R and Engelmann M (2015) Consequences of temporary inhibition of the medial amygdala on social recognition memory performance in mice. Front. Neurosci. 9:152. doi: 10.3389/fnins.2015.00152

Different lines of investigation suggest that the medial amygdala is causally involved in the processing of information linked to social behavior in rodents. Here we investigated the consequences of temporary inhibition of the medial amygdala by bilateral injections of lidocaine on long-term social recognition memory as tested in the social discrimination task. Lidocaine or control NaCl solution was infused immediately before learning or before retrieval. Our data show that lidocaine infusion immediately before learning did not affect long-term memory retrieval. However, intra-amygdalar lidocaine infusions immediately before choice interfered with correct memory retrieval. Analysis of the aggressive behavior measured simultaneously during all sessions in the social recognition memory task support the impression that the lidocaine dosage used here was effective as it—at least partially—reduced the aggressive behavior shown by the experimental subjects toward the juveniles. Surprisingly, also infusions of NaCl solution blocked recognition memory at both injection time points. The results are interpreted in the context of the importance of the medial amygdala for the processing of non-volatile odors as a major contributor to the olfactory signature for social recognition memory.

Keywords: male C57BL/6JOlaHsd mice, social long-term recognition memory, medial amygdala, olfaction, lesion, lidocaine, behavior, learning and memory

Introduction

Individual recognition in socially living rodents is primarily based on the acquisition and processing of the conspecifics' body odors. They are thought to contain information about age, sex, reproductive state and the health status and, thus, differ individually and they are often referred to as the "olfactory signature." Physico-chemically, body odors are composed of volatile and non-volatile fractions. Once acquired by the individual, the neuronal processing of such "olfactory signatures" involves distinct brain areas linked to both the main and the accessory olfactory system. One of the most important brain areas involved in the processing of olfactory signals is the medial amygdala (MeA) which has been suggested to play an important role in olfactory social stimulus processing in rodents (Lehman et al., 1980; Ferguson et al., 2001; Broad et al., 2002; Curtis and Wang, 2003; Gobrogge et al., 2007; Gutierrez-Castellanos et al., 2014). Not only the analysis of c-Fos synthesis (Ferguson et al., 2001; Richter et al., 2005; Noack et al., 2010), but also genetic and pharmacological (Ferguson et al., 2001) studies revealed that this brain area may play a key

role for social recognition in mice. In this context the MeA was shown to act not only as a relay station from the olfactory bulb to deeper brain areas, but also to signal back to the accessory olfactory bulb thereby controlling the impact of the non-volatile fraction of the conspecific's "olfactory signature" on approach-avoidance behavior (Martel and Baum, 2009). However, it is unclear what might be the consequences of temporarily blocking the information processing in the MeA during acquisition/consolidation of olfactory memory vs. its recall. Therefore, we investigated the effects of bilateral temporary suppression of action potential propagation in the MeA by injecting Lidocaine into the MeA prior to both learning and recall of the individual olfactory information.

Some of the results were previously published in abstract form (Noack et al., 2012).

Materials and Methods

Experimental Subjects

Male C57BL/6JOlaHsd mice (originally obtained from Harlan-Winkelmann, Bochern, Germany and subsequently bred in the animal facility of the Medical faculty of the Otto-von-Guericke-Universität Magdeburg), aged 9–16 weeks, were kept in groups of 5 under a constant 12 h:12 h light-dark cycle (light starting at 6 a.m.) with food and water available *ad libitum*.

Juvenile C57BL/6JOlaHsd mice of both sexes (age 25–35 days) were used as social stimuli. The suitability of these social stimuli for the behavioral test used was previously proven in intensive studies (Engelmann et al., 2011).

All procedures and manipulations were approved by the local governmental body and according to the European Communities Council Directive recommendations for the care and use of laboratory animals (2010/63/EU).

Social Discrimination Procedure

Experimental subjects were separated 2 h before testing. The test procedure consisted of two sessions (4 min each) separated by a 24 h exposition interval (**Figure 1A**). During the first session (sampling), a conspecific juvenile (J1) was presented to the experimental subject, allowing the adult to acquire the juvenile's "olfactory signature." During the second session (choice) J1 was re-exposed to the adult together with a second, previously not encountered juvenile (J2). A significantly longer investigation duration of J2 vs. J1 during choice was taken as an evidence for an intact long-term social recognition memory (LTsrM) (Engelmann et al., 2011). In addition, sexual (e.g., attempts of mounting) and aggressive behavior [e.g., chasing and biting the juvenile(s)] were separately monitored (see Engelmann et al., 2011 for more details).

Surgery

Animals were briefly anesthetized with isoflurane (2%, Baxter, Unterschleißheim, Germany, applied via an anesthesia system (MLW, Leipzig, Germany) by a constant flow of 1.2 l/min and then injected with a mixture of ketamine and xylazine (i.p., 2 ml Ketavet® (Pfizer Pharmacia, Berlin, Germany), 0.5 ml Rompun® (Bayer Vital, Leverkusen, Germany), 7.5 ml 0.9% NaCl solution).

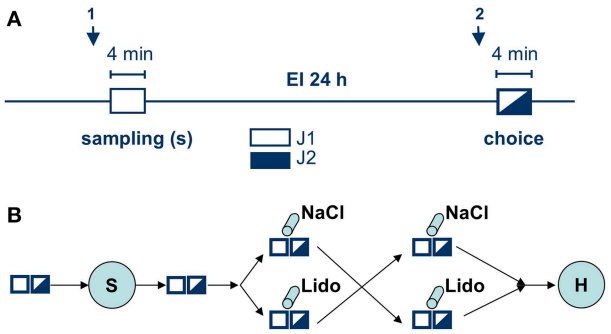


FIGURE 1 | Experimental protocol. (A) shows the social discrimination procedure. Arrows indicate time points for intra-amygdalar injections either directly before sampling (1) or before choice (2); both sampling (white rectangle) and choice (black-white rectangle) lasted 4 min and were separated by a 24 h exposure interval (EI). During sampling a given Juvenile (J1, white) was exposed to the experimental subject. During choice J1 (encountered during sampling) together with a novel, previously not encountered juvenile (J2, black) were exposed to the experimental subject and the given behavioral parameters toward each juvenile measured. **(B)** shows the whole experimental series involving all manipulations and illustrating the cross-over design. White and black-white rectangles illustrate the two sessions in social discrimination, S, surgery to implant the guide cannulas, cylinders indicate intra-amygdalar injections of NaCl or lidocaine (Lido) during behavioral testing; H, histological analysis.

Deeply anesthetized animals were fixed into a stereotaxic frame (TSE Systems, Bad Homburg, Germany). Coordinates for bilateral implantation of the guide cannulas (stainless surgical steel 0.55 × 0.08 × 9.00 mm; Injecta, Klingenthal, Germany) were selected according to a stereotaxic mouse brain atlas (Franklin and Paxinos, 1997). The ventral tip of each guide cannula was placed 1 mm above the target area, the MeA, at the following coordinates starting from Bregma: lateral ± 2.5 mm, anterior 0.3 mm, ventral 4.5 mm. The guide cannulas were secured to the skull and to two stainless steel screws (1.0 × 2.0 mm, Paul Korth, Lüdenscheid, Germany) inserted into the bone with light curling dental cement (Ivoclar Vivadent, Schaan, Lichtenstein). Stylets were made from insect pins and inserted into the guide cannulas to keep them patent. Animals received an injection of Meloxicam (s.c., 0.05 mg/kg b. wt., Metacam® Boehringer Ingelheim Vetmedica, Ingelheim, Germany) to temper post-operative pain.

Experimental subjects were allowed to recover from surgery for at least 7 days before the experiment. Pilot studies revealed that at this time point both body weight and social behavior reached pre-surgery levels (**Figure 2**).

Acute Injections, Substances, and Treatment

For simultaneous acute bilateral injections animals were removed from the cage, anesthetized with isoflurane (see above) and placed on a table. Stylets were removed and injection cannulas gently inserted into the guide cannulas. Substances were infused via a constant flow of 0.5 µl/min using a microinfusion pump (CMA Microdialysis, Stockholm, Sweden). The proper application was controlled by watching the movement of an airbubble within a scaled tubing connecting the microinfusion pump

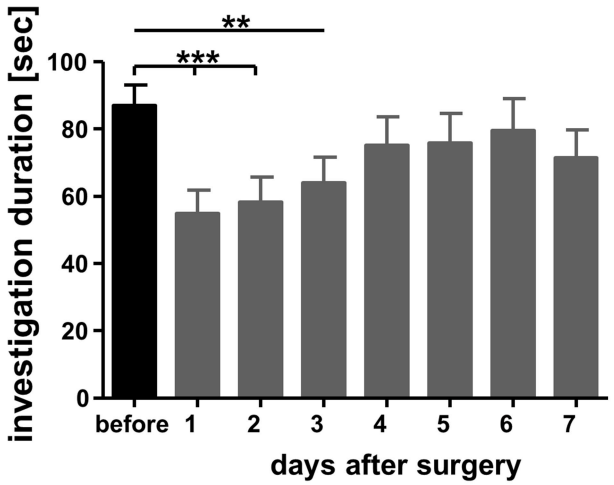


FIGURE 2 | Investigation durations measured a day before bilateral implantation of guide cannulas targeting the medial amygdala and on the subsequent days. Adult male mice were exposed in their home cages for 4 min toward a previously not encountered conspecific juvenile similar to sampling in the social discrimination procedure (for more details see text). A trained observed measured the duration spent by the experimental subjects investigating the juveniles. $^{**}p < 0.01$ and $^{***}p < 0.01$ compared with the investigation duration measured during "before." Repeated measures ANOVA followed by Dunnett's-test.

with the injection cannula. We infused a volume of 0.5 µl into each hemisphere. After the infusion injection cannulas were left in place for an additional minute to allow for complete delivery. Subsequently, anesthesia was stopped, the injection cannulas were carefully replaced by the stylets and the animals were returned to their experimental cages. Given the dosage of Lidocaine (Xylocitin-loc®, Mibe, Jena, Germany; 20 µg/µl diluted in 0.9% NaCl solution, Braun, Melsungen, Germany) used we calculated an inhibition of action potential generation and propagation by blocking voltage-gated sodium channels, duration of effect: <60 min (Malpeli and Schiller, 1979; Boehnke and Rasmusson, 2001).

Experimental Design

Before undergoing surgery experimental subjects used in this study were tested for their intact long-term social discrimination abilities. Seven days after surgery the animals were tested for their social discrimination abilities without any further treatment to confirm that the surgery and the implantation of the guide cannulas did not cause effects (e.g., by lesions) that *per se* interfered with a successful long-term recognition memory.

Recent reports suggest interfering effects of both isoflurane anesthesia (Pearce et al., 2012) and transport of animals between rooms (Moura et al., 2011) on olfactory memory performance. Therefore, we tested the impact of the manipulations linked to the acute intra-amygdalar injections on the behavioral parameters as measured in the present study. Our data revealed that the anesthesia procedure *per se* did not produce interfering effects for LTsrM if applied according to our experimental protocol (Noack, unpublished observations, Camats Perna, unpublished observations). This is in line with previous reports that the brief isoflurane-anesthesia performed at different time intervals before and after sampling fails to corrupt long-term juvenile recognition abilities in male mice of the mouse strain under study *per se* (Richter et al., 2005; Wanisch et al., 2008; Engelmann, 2009; Engelmann et al., 2011).

As shown in **Figure 1B**, infusions of lidocaine or NaCl were performed in a double-blind, cross-over approach either (1) directly before sampling or (2) directly before choice. The code for the substances was randomly assigned to each experiment separately by a co-worker not involved in the infusion and behavioral testing. The blinding was broken at the end of the histological verification of the placement of the injection side. Recovery time between infusion and the onset of the respective sampling and choice session respectively was ∼ 6 min. The same group of animals ($n = 15$, divided into two subgroups according to the protocol shown in **Figure 1B**) received the infusion of lidocaine and NaCl (cross-over) before sampling. Another group of animals ($n = 17$, also divided into two subgroups and treated according to **Figure 1B**) received the infusions (cross-over) before choice. Thus, each animal in the respective group received both lidocaine and NaCl. The interval between the two successive treatments (NaCl followed by lidocaine or lidocaine followed by NaCl; **Figure 1B**) was 7 days.

Statistics

Data are presented as means + SEM. For the analysis of the aggressive/sexual behavior data were submitted in a non-parametric Friedmans ANOVA followed by Wilcoxon corrected for repeated measures. For the analysis of the investigation duration measured during sampling data were analyzed using paired student's *t*-tests. Statistics were performed using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, U.S.A.). A $p < 0.05$ was considered to indicate statistical significance.

Results

Aggressive/Sexual Behavior

We observed that the durations spent in aggressive behavior were—at least under treatment conditions—too low to allow for a reliable analysis (average duration of an investigation bout <0.5 s). Therefore, we analyzed the number of bouts instead since it has been shown to provide a more reliable measure in cases of low durations of individual investigation bouts (Engelmann et al., 2011). Our data show that—compared to implanted, but otherwise untreated conditions—both NaCl and lidocaine infusions significantly reduced the number of investigation bouts of aggressive/sexual behavior (Friedmans ANOVA: $Fs = 26.53$, $p < 0.01$; **Figure 3**). Furthermore, under lidocaine treatment aggressive/sexual behavior decreased almost completely reaching statistical significance—compared to NaCl treatment—if the animals were injected immediately before choice (Friedmans ANOVA: $Fs = 24.14$, $p < 0.01$; **Figure 3**).

Social Discrimination

After injection of NaCl-solution immediately before sampling there was no significant difference in the investigation durations

of the experimental subjects toward both juveniles J1 and J2 during choice (paired T-test, $t = 1.099$, $df = 14$, $p = 0.29$; **Figure 4**). In contrast, the same animals investigated J2 significantly longer than J1 during choice after receiving the injection of lidocaine directly before sampling (paired T-test, $t = 2.648$, $df = 14$, $p = 0.019$).

After receiving injections of NaCl or lidocaine immediately before choice, the experimental subjects showed no significant differences in the duration of investigation behavior toward J1 and J2 during choice (NaCl: paired T-test, $t = 1.270$, $df = 16$, $p = 0.222$; lidocaine: paired T-test, $t = 1.050$, $df = 16$, $p = 0.309$; **Figure 4**).

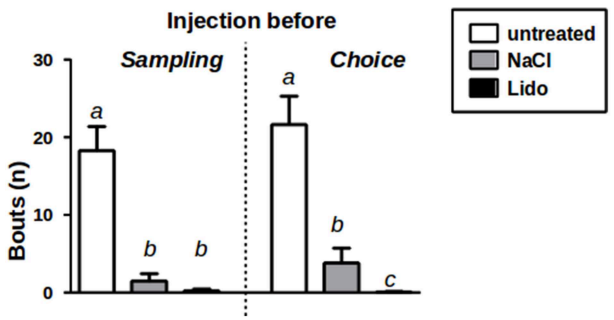


FIGURE 3 | Aggression/Sexual behavior toward J1 during sampling w/o injection (untreated) and after injection of NaCl or Lidocaine (Lido) directly before sampling (left panel, $n = 15$). Aggression/Sexual behavior toward J1+J2 during choice w/o injection and after injection of NaCl or Lido directly before choice (right panel, $n = 17$). a = $p < 0.01$ vs. b and c; c = $p < 0.05$ vs. b, Friedman-test followed by Wilcoxon corrected for repeated measures.

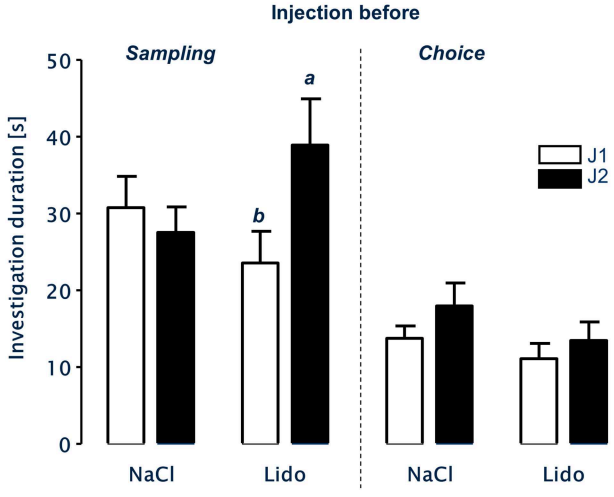


FIGURE 4 | LTsrM in mice after injection of NaCl or Lidocaine (Lido) immediately before either sampling (left panel, $n = 15$) or choice (right panel, $n = 17$). Mice treated with NaCl before sampling or choice failed to recognize the previously encountered juvenile. Injection of Lidocaine directly before sampling did not affect LTsrM. In contrast, injection of Lidocaine directly before choice impaired LTsrM. b = $p < 0.05$ vs. a, paired student's t-test.

Discussion

The present study was designed to investigate the impact of temporary inhibition of the medial amygdala on LTsrM. Our data suggest that the combination of isoflurane anesthesia with direct bilateral infusions of different drugs is suitable to investigate the impact for example of temporary lesions of distinct brain areas for LTsrM in mice. More specifically, the protocol used here seems to avoid the interfering effects of isoflurane anesthesia reported by other authors (Pearce et al., 2012).

Previous studies have shown that permanent lesions of the medial amygdala significantly reduced aggressive behavior not only in rats (Vochteloo and Koolhaas, 1987), but also in C57Bl/6 mice (Wang et al., 2013). Aggressive behavior is also seen—albeit in comparably low intensity—during the social discrimination testing where 13–35% of the total social behavior was considered to be aggressive and of a sexual nature (Engelmann et al., 2011). The almost complete lack of aggressive/sexual behavior toward the stimulus juveniles after administration of lidocaine (see **Figure 3**) implies that lidocaine administration inhibited local neurosignaling that in turn resulted in a suppression of aggressive/sexual behavior. This observation is important with respect to the effects observed for recognition memory: Lidocaine administration failed to interfere with LTsrM if administered immediately before sampling and blocked the memory performance if administered immediately before choice (**Figure 4**). Thus, temporarily lesioning the medial amygdala during acquiring the olfactory signature does not affect juvenile recognition.

Interestingly, intra-amygdalar injection of the NaCl-solution alone (originally thought to act as "neutral" control) reduced aggressive behavior (**Figure 3**) and impaired LTsrM (**Figure 4**). This implies that the NaCl-solution triggered behavioral effects that tended to be similar to that of lidocaine. Indeed, there is some evidence that infusion of the NaCl-solution also lowers electrophysiological activity in a short-term manner (Malpeli and Schiller, 1979; Tehovnik and Sommer, 1997). Nevertheless, as shown in **Figure 3** the behavioral suppressive effect of NaCl solution treatment was not as pronounced as that of lidocaine. This may have resulted not only in the reduced aggressive activity toward the juveniles (**Figure 3**), but also in an improper processing of the "olfactory signature" during sampling. This is in line with results of a study investigating among other contextual fear conditioning that revealed that injections of NaCl-solutions into the lateral amygdala mimicked partially effects of lidocaine when compared with sham injections (Calandreau et al., 2005). It may be speculative, but we propose that the resulting modified "olfactory signature" was artificial and could not match with the correctly processed "olfactory signature" obtained during the choice session, resulting in an inability to recognize the originally encountered juvenile, and thus the reported lack of an intact LTsrM.

Lidocaine should completely block electrical activity in the MeA, beyond any abovementioned effect of NaCl, and this is supported by the aggression/sexual behavioral data. We observed that lidocaine injection before sampling did not interfere with LTsrM. However, when injected immediately before choice

successful recognition of the previously encountered juvenile was blocked (**Figure 4**). These observations were surprising and led us to the conclusion that information processing within the MeA can be blocked without interfering with the successful acquisition of olfactory information important for LTsrM (electrical block during sampling). According to our results however, the MeA is likely to act as an essential relay station for information during recall after being activated during learning (electrical block only during choice). In addition, an even more detailed interpretation of the different consequences of lidocaine treatment for LTsrM "before sampling" vs. "before choice" may relate to the fact that the MeA is thought to be primarily involved in the processing of non-volatile olfactory cues (for review see Baum and Bakker, 2013). Previously it was shown that the non-volatile fraction of the "olfactory signature" is not essential for a correct social memory retrieval, if not available during sampling, but it must be available for successful LTsrM during choice when it was accessible during sampling (Noack et al., 2010). Our present results seem to mirror these findings by blocking the information processing of the non-volatile fraction of the olfactory signature in the MeA. Blocking the transfer/processing of information in this brain area by lidocaine, but not NaCl, during learning does not affect LTsrM when both fractions are accessible during retrieval. In contrast, when both fractions of the olfactory signature were processed successfully during sampling (including the non-volatile fraction in the MeA) the blockade (lidocaine) and the modulation (NaCl) of the non-volatile odor processing in the MeA interfered with LTsrM. This hypothesis might be an attractive target to further investigate the phenomenon of interference in further studies.

Taken together, the data of the present study suggest that the MeA seems to play an—albeit dispensable—role in the processing of the non-volatile-fraction of the "olfactory signature" for LTsrM. Blocking the generation of action potentials in the MeA during retrieval also blocks LTsrM. Therefore, our data point toward a contribution of the MeA in the processing of complete "olfactory signatures" for social behavior. These observations illustrate the plasticity of the neuronal substrate for processing stimuli relevant for social recognition memory in order to compensate for the temporary lesion of an otherwise essential brain area.

Author Contributions

ME and JN planned the experiments and wrote the manuscript. JN and RM performed the experiments.

Acknowledgments

The authors wish to thank PD Dr. Carsten T. Wotjak and Anna Mederer (MPI for Psychiatry Munich) for their supervision with the intracerebral injection procedure in mice and Carole Torsney (Edinburgh) for correcting the English.

References

Baum, M. J., and Bakker, J. (2013). Roles of sex and gonadal steroids in mammalian pheromonal communication. *Front. Neuroendocrinol.* 34:268–284. doi: 10.1016/j.yfrne.2013.07.004

Boehnke, S. E., and Rasmusson, D. D. (2001). Time course and effective spread of lidocaine and tetrodotoxin delivered via microdialysis: an electrophysiological study in cerebral cortex. *J. Neurosci. Methods* 105, 133–141. doi: 10.1016/S0165-0270(00)00348-4

Broad, K. D., Mimmack, M. L., Keverne, E. B., and Kendrick, K. M. (2002). Increased BDNF and trk-B mRNA expression in cortical and limbic regions following formation of a social recognition memory. *Eur. J. Neurosci.* 16, 2166–2174. doi: 10.1046/j.1460-9568.2002.02311.x

Calandreau, L., Desmedt, A., Decorte, L., and Jaffard, R. (2005). A different recruitment of the lateral and basolateral amygdala promotes contextual or elemental conditioned association in Pavlovian fear conditioning. *Learn. Mem.* 12, 383–388. doi: 10.1101/lm.92305

Curtis, J. T., and Wang, Z. (2003). Forebrain c-fos expression under conditions conducive to pair bonding in female prairie voles (Microtus ochrogaster). *Physiol. Behav.* 80, 95–101. doi: 10.1016/S0031-9384(03)00226-9

Engelmann. (2009). Competition between two memory traces for long-term recognition memory. *Neurobiol. Learn. Mem.* 91, 58–65. doi: 10.1016/j.nlm.2008.08.009

Engelmann, M., Hädicke, J., and Noack, J. (2011). Testing declarative memory in laboratory rats and mice using the non-conditioned social discrimination procedure. *Nat. Protoc.* 6, 1152–1162. doi: 10.1038/nprot.2011.353

Ferguson, J. N., Aldag, J. M., Insel, T. R., and Young, L. J. (2001). Oxytocin in the medial amygdala is essential for social recognition in the mouse. *J. Neurosci.* 21, 8278–8285.

Franklin, K. B. J., and Paxinos, G. (1997). *The Mouse Brain in Stereotaxic Coordinates.* San Diego, CA: Academic Press.

Gobrogge, K. L., Liu, Y., Jia, X., and Wang, Z. (2007). Anterior hypothalamic neural activation and neurochemical associations with aggression in pair-bonded male prairie voles. *J. Comp. Neurol.* 502, 1109–1122. doi: 10.1002/cne.21364

Gutierrez-Castellanos, N., Pardo-Bellver, C., Martinez-Garcia, F., and Lanuza, E. (2014). The vomeronasal cortex—afferent and efferent projections of the posteromedial cortical nucleus of the amygdala in mice. *Eur. J. Neurosci.* 39, 141–158. doi: 10.1111/ejn.12393

Lehman, M. N., Winans, S. S., and Powers, J. B. (1980). Medial nucleus of the amygdala mediates chemosensory control of male hamster sexual behavior. *Science* 210, 557–560. doi: 10.1126/science.7423209

Malpeli, J. G., and Schiller, P. H. (1979). A method of reversible inactivation of small regions of brain tissue. *J. Neurosci. Methods* 1, 143–151. doi: 10.1016/0165-0270(79)90011-6

Martel, K. L., and Baum, M. J. (2009). A centrifugal pathway to the mouse accessory olfactory bulb from the medial amygdala conveys gender-specific volatile pheromonal signals. *Eur. J. Neurosci.* 29, 368–376. doi: 10.1111/j.1460-9568.2008.06564.x

Moura, P. J., Venkitaramani, D. V., Tashev, R., Lombroso, P. J., and Xavier, G. F. (2011). Transport of animals between rooms: a little-noted aspect of laboratory procedure that may interfere with memory. *Behav. Processes* 88, 12–19. doi: 10.1016/j.beproc.2011.06.008

Noack, J., Murau, R., and Engelmann, M. (2012). "The medial amygdala in learning and recall of long-term social recognition memory in mice: a temporary lesion study," in *FENS Forum Abstr* 2012 (Barcelona).

Noack, J., Richter, K., Laube, G., Haghgoo, H. A., Veh, R. W., and Engelmann, M. (2010). Different importance of the volatile and non-volatile fractions of an olfactory signature for individual social recognition in rats versus mice and short-term versus long-term memory. *Neurobiol. Learn. Mem.* 94, 568–575. doi: 10.1016/j.nlm.2010.09.013

Pearce, R. A., Duscher, P., Van Dyke, K., Lee, M., Andrei, A. C., and Perouansky, M. (2012). Isoflurane impairs odour discrimination learning in rats: differential effects on short- and long-term memory. *Br. J. Anaesth.* 108, 630–637. doi: 10.1093/bja/aer451

Richter, K., Wolf, G., and Engelmann, M. (2005). Social recognition memory requires two stages of protein synthesis in mice. *Learn. Mem.* 12, 407–413. doi: 10.1101/lm.97505

Tehovnik, E. J., and Sommer, M. A. (1997). Effective spread and timecourse of neural inactivation caused by lidocaine injection in monkey cerebral cortex. *J. Neurosci. Methods* 74, 17–26. doi: 10.1016/S0165-0270(97)02229-2

Vochteloo, J. D., and Koolhaas, J. M. (1987). Medial amygdala lesions in male rats reduce aggressive behavior: interference with experience. *Physiol. Behav.* 41, 99–102. doi: 10.1016/0031-9384(87)90137-5

Wang, Y., He, Z., Zhao, C., and Li, L. (2013). Medial amygdala lesions modify aggressive behavior and immediate early gene expression in oxytocin and vasopressin neurons during intermale exposure. *Behav. Brain Res.* 245, 42–49. doi: 10.1016/j.bbr.2013.02.002

Wanisch, K., Wotjak, C. T., and Engelmann, M. (2008). Long-lasting second stage of recognition memory consolidation in mice. *Behav. Brain Res.* 186, 191–196. doi: 10.1016/j.bbr.2007.08.008

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 25 August 2015
doi: 10.3389/fnbeh.2015.00223

The olfactory hole-board test in rats: a new paradigm to study aversion and preferences to odors

Kerstin E. A. Wernecke[1,2]* *and Markus Fendt*[1,2]

[1] *Institute for Pharmacology and Toxicology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany,* [2] *Center for Behavioral Brain Sciences, Magdeburg, Germany*

Odors of biological relevance (e.g., predator odors, sex odors) are known to effectively influence basic survival needs of rodents such as anti-predatory defensiveness and mating behaviors. Research focused on the effects of these odors on rats' behavior mostly includes multi-trial paradigms where animals experience single odor exposures in subsequent, separated experimental sessions. In the present study, we introduce a modification of the olfactory hole-board test that allows studying the effects of different odors on rats' behavior within single trials. First, we demonstrated that the corner holes of the hole-board were preferentially visited by rats. The placement of different odors under the corner holes changed this hole preference. We showed that holes with carnivore urine samples were avoided, while corner holes with female rat urine samples were preferred. Furthermore, corner holes with urine samples from a carnivore, herbivore, and omnivore were differentially visited indicating that rats can discriminate these odors. To test whether anxiolytic treatment specifically modulates the avoidance of carnivore urine holes, we treated rats with buspirone. Buspirone treatment completely abolished the avoidance of carnivore urine holes. Taken together, our findings indicate that the olfactory hole-board test is a valuable tool for measuring avoidance and preference responses to biologically relevant odors.

Keywords: approach, avoidance, buspirone, carnivore urine, innate fear, female rat urine, hole-board test, predator

OPEN ACCESS

Edited by:
Benno Roozendaal, Radboud University Nijmegen Medical Centre, Netherlands

Reviewed by:
Barbara Ferry, Center of Research in Neurosciences Lyon, UMR 5292 – INSERM U 1028 – UCBL1, France
Newton Sabino Canteras, University of São Paulo, Brazil

***Correspondence:**
Kerstin E. A. Wernecke, Institute for Pharmacology and Toxicology, Otto-von-Guericke University Magdeburg, Leipziger Strasse 44, D-39120 Magdeburg, Germany
kerstin.wernecke@med.ovgu.de

Received: *22 May 2015*
Accepted: *07 August 2015*
Published: *25 August 2015*

Citation:
Wernecke KEA and Fendt M (2015) The olfactory hole-board test in rats: a new paradigm to study aversion and preferences to odors. Front. Behav. Neurosci. 9:223. doi: 10.3389/fnbeh.2015.00223

General Introduction

Challenged by the large diversity of natural odor blends in their environment, most mammalian species have developed highly sensitive olfactory systems to identify and discriminate biologically relevant odors. In rodents, the detection of some odors is of critical importance because they trigger different basic behaviors essential for their survival. In particular, odors transmitted between individuals of the same species (pheromones) are used to communicate information on the gender, reproductive state, social status, and subject identity. Thus, pheromones have been highly associated with the mediation of, e.g., mate choice, parental care and territorial behaviors (Brennan and Kendrick, 2006; Fortes-Marco et al., 2013). Predator odors present a different group of biologically relevant odors called kairomones (Fortes-Marco et al., 2013; Rajchard, 2013). Kairomones are odors that damage the interests of the releaser while being beneficial for the receiving animal (of another species). In this context, predator odors warn prey animals of a potential confrontation with a predator. For example, odors derived from cats or other carnivorous

species (e.g., urine samples from foxes, bobcats, pumas, and coyotes) elicit a range of innate defensive behaviors in rodents including avoidance and hiding behavior, an increase in risk assessment behaviors and the suppression of non-defensive behaviors such as foraging, sexual behavior and overall locomotor activity (Blanchard and Blanchard, 1989; Apfelbach et al., 2005; Endres et al., 2005; Masini et al., 2005; Fendt, 2006; Wernecke et al., 2015).

Research focused on the effects of odors on rats' behaviors often include multi-trial paradigms where animals experience a sequence of single odor exposures (e.g., Wallace and Rosen, 2000; Fendt, 2006; Ferrero et al., 2011; Rivard et al., 2014). In the current set of experiments, we have used a modified version of the olfactory hole-board test (Moy et al., 2008) to study behavioral effects of different odors within single trials. In this procedure, rats are placed in a standard hole-board apparatus with automated recording of nose pokes, also called head dips. Previous work has shown that the hole-board test offers a simple method for measuring exploratory behavior of animals in an unfamiliar environment (Takeda et al., 1998; Brown and Nemes, 2008). Whether an animal prefers or avoids a hole results from an inner conflict between the natural drive of rodents to explore and the potential aversive properties of the hole. Thus, according to this hypothesis, a general decrease in head dipping behavior is interpreted to reflect increased anxiety in animals, while high levels of head dipping behavior are defined as a decline in anxiety (Crawley, 1985; Lister, 1990; Saitoh et al., 2006).

In the present study, a series of four experiments has been conducted to investigate if the hole-board test can be used to investigate behavioral responses of rats to different odors within single trials. In *Experiment 1*, rats were tested for hole preference in the classical 16-hole configuration. *Experiments 2* and *3* were conducted to assess whether rats display a shift in hole preference when both aversive and attractive odors were presented in the preferred corner holes. *Experiment 4* tested if avoidance behavior to holes with carnivore urine samples can be reduced by treating the rats with the anxiolytic compound buspirone.

General Materials and Methods

Subjects

Testing was carried out using 64 experimentally naive male Sprague-Dawley rats (2–3 months-old) weighing 200–350 g at the time of testing. Rats were bred and reared at the local animal facility (original breeding stock: Taconic, Denmark). They were housed in groups of 5–6 animals in standard Macrolon Type IV cages with water and food available *ad libitum*. Cages were kept in temperature and humidity-controlled rooms (22 ± 2°C, 50–55%) under a 12 h light/dark cycle with lights on at 6:00 am. Behavioral testing was conducted during the light phase between 8:00 am and 3:00 pm.

All experiments were carried out in accordance with international ethical guidelines for the care and use of laboratory animals for experiments (2010/63/EU), and were approved by the local authorities (Landesverwaltungsamt Sachsen-Anhalt, Az. 42505-2-1172 UniMD).

Testing Apparatus

All experiments were conducted in a computer-controlled hole-board apparatus (ActiMot2 Hole-Board System, TSE Systems, Bad Homburg, Germany) consisting of three testing boxes constructed from transparent Plexiglas (51.5 cm× 51.5 cm× 41 cm) and a height-adjustable frame with infrared detectors (sample rate: 100 Hz). A removable hole-board with 16 holes (3 cm diameter) in a grid-pattern was placed on the floor of the testing box. Holes were categorized into four corner holes (holes 1, 4, 13, 16; see **Figure 1B** Inlay), four back wall holes (holes 2, 3, 5, 8), four front wall holes (holes 9, 12, 14, 15) and four center holes (holes 6, 7, 10, 11). Supplier-specific lids were used to close particular holes, meaning that

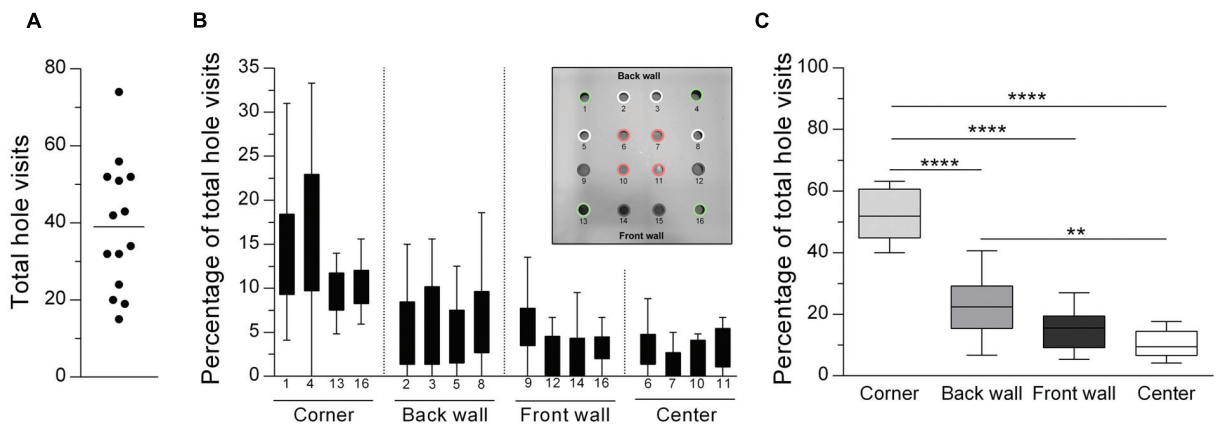


FIGURE 1 | General distribution of hole visits (median) during a hole-board test showing a clear preference for corner holes. (A) Total numbers of hole visits for individual animals (n = 14) are displayed as a scatter dot plot with the horizontal line describing the mean. Number of hole visits (% of total) are depicted for **(B)** all 16 holes and **(C)** summed up for each hole category. **p < 0.01; ****p < 0.0001 comparisons as indicated (Holm-Sidak's multiple comparison test after significant main effects in an ANOVA).

the number and/or the location of the holes could be modified as required for each experiment. The apparatus was located in a small testing room with dimmed illumination (illumination: ∼30 lx).

General Testing Procedure and Odor Presentation

For *Experiment 1*, the hole-board was used in its 16-hole configuration. Rats were individually placed into the testing box and tested for 20 min. Rats head-dipping behavior was monitored by the infrared detectors. The software automatically measured the total number of head dips (hole visits) for each single hole. More specifically, a head dip was counted when the animal placed its head into a hole for at least 300 ms with the ears even with the floor of the hole-board. A minimal time interval of 300 ms had to elapse after a head dip before a new hole visit was counted. For the experiments with odor presentations (*Experiments 2–4*), the holes of the center region were covered (12-hole configuration). Then, animals were individually placed into the testing box and exposed to four odors simultaneously. For this, 1 ml odor samples (described in detail below) were pipetted into small glass bowls (4 cm outer diameter, 2.5 cm height) and placed underneath the hole of each corner prior to testing. The animals were not able to touch the odor samples. For each test session, one corner hole contained only water which served as a control odor. The location of the different types of odor samples was pseudo-randomly changed across individuals (*Experiments 2–4*) and tests (*Experiment 4*). The wall holes were always left empty. Head dips into the wall holes were used to assess baseline levels of exploration behavior and to control for individual differences in the total number of head dips. After each test, the testing boxes were thoroughly cleaned with soapy water and ventilated with clean air, before the next rats were tested.

Odors

Urine samples from foxes, bobcats, pumas and coyotes were purchased from Maine Outdoor Solutions Inc., (Hermon, ME, USA). We previously demonstrated that urine samples of these carnivores induce avoidance behavior in an open-field experiment (Fendt, 2006). Urine samples from elks and mona monkeys were obtained from the local zoo (Zoologischer Garten Magdeburg, Magdeburg, Germany). Female rat urine was self-collected by placing adult female Sprague-Dawley rats ($n = 12$, 3–6 months-old) individually in a metabolic cage (Tecniplast, Hohenpeißenberg, Germany) for ∼30 min on consecutive days. Female urine samples of individual animals were mixed up to ensure that urine from all estrus cycle phases were present. All urine samples were aliquoted into 1 ml portions and stored at – 18°C until usage.

Descriptive and Statistical Analysis

Hole visits were expressed as percentages of total hole visits. In all figures, behavioral data are shown as box-and-whisker plots. The horizontal line represents the median and the box the lower and upper quartiles. The whiskers were calculated with the Tukey method (GraphPad Prism 6.00, GraphPad Software Inc., La Jolla, CA, USA).

For statistical analysis, data were first tested on normal distribution (D'Agostino and Pearson omnibus test). For normally distributed data, analyses of variance (ANOVA) and *post hoc* comparisons by Holm-Sidak's test were used. Non-normally distributed data were analyzed using the Friedman test followed by Dunn's multiple comparisons test. Either hole location (*Experiment 1*) or odor (*Experiments 2–4*) was used as within-subject factor. A $p < 0.05$ was considered statistically significant. All analyses were carried out using GraphPad Prism.

Pilot tests revealed that the hole visit behavior of animals that are extremely active or extremely inactive is only marginally modulated by odors (floor/ceiling effects; see also discussion of *Experiment 1*). In *Experiments 2–4*, we therefore excluded animals with more than 65 or less than 15 total hole visits from further analysis.

Experiment 1

The first experiment was conducted to determine whether rats display a specific exploration pattern in the hole-board test when no odors are present. From other exploration-based tasks (e.g., open-field) it is known that rodents prefer to remain in the periphery of the apparatus (thigmotaxis), whereas the bright and unprotected areas are usually avoided (Lister, 1990; Wallace and Rosen, 2000; Litvin et al., 2008). Therefore, we expected that our rats would show preference (i.e., a high number of visits) to the holes in the corners and along the side walls, and avoidance (i.e., a low number of visits) to the four holes in the center of the box. This was also observed in the mouse version of the olfactory hole-board test (Moy et al., 2008).

Subjects and Procedure

Fourteen male Sprague-Dawley rats were tested. They were put into the middle of the hole-board (all 16 holes open, no odors) and their hole visits were recorded for 20 min.

Results

Figure 1 illustrates the local distribution of hole visits (16 holes, no odor). The total number of hole visits ranged between 15 and 74 head dips (**Figure 1A**), with a mean of 39 head dips. The subsequent analysis revealed that there were clear differences in the percentage number of total hole visits according to the position of the holes [corner vs. center vs. front wall vs. back wall: ANOVA: $F_{(3,39)} = 66.21$; $p < 0.0001$; **Figures 1B,C**]. Corner holes were visited significantly more often than holes with other locations (Holm-Sidak's tests: ps < 0.0001). Furthermore, the back wall holes were visited more often than the center holes ($p = 0.001$). There were no significant differences in the hole visits within the different hole categories [corner holes: Friedman test: $Q = 3.24$; $p = 0.36$; wall holes: ANOVA: $F_{(7,91)} = 2.21$; $p = 0.075$; center holes: Friedman test: $Q = 6.49$; $p = 0.09$].

Discussion

The behavior of animals in the hole-board test, originally described by Boisser and Simon (1962, 1964), is determined by a conflict between curiosity-based exploration and fear-based

avoidance from novel, unknown locations (Hughes, 2007; Brown and Nemes, 2008). Thus, altered head dipping activity is often interpreted as changes in the anxiety state of the animals (Takeda et al., 1998; Brown and Nemes, 2008). In *Experiment 1*, rats were tested in the 16-hole configuration of the hole-board without any odors. Our results indicate that rats showed the highest rate of head dips for corner holes, and the lowest rate of head dips for center holes (**Figures 1B,C**). This finding is in line with the results of previous hole-board and related exploration-based rodent models and can be explained by thigmotaxis (Lister, 1990; Lamprea et al., 2008; Moy et al., 2008).

Interestingly, Moy et al. (2008) also established an olfactory hole-board test, however, to model repetitive behavior, a core symptom of autism, in mice. In their study, different appetitive odor samples (e.g., familiar cage bedding, food items) were presented in the less-preferred center holes and the ability of mice to shift their hole preference was assessed. In contrast to this, we wanted to mainly investigate how hole visits are influenced by aversive odors. Based on previous studies from our laboratory (Fendt, 2006; Ferrero et al., 2011; Wernecke et al., 2015), we expected that holes with aversive odors will be visited less often, i.e., avoided. Such avoidance is much easier to observe when holes are very often visited under control conditions. Therefore, our approach was to place the test odors under the corner holes. To further increase the number of corner hole visits, we closed the four center holes. Given that the hole visit activity was very different for individual animals, we also decided that this individual variance should be included into the analyses of odor effects on hole visits. Therefore, hole visits were presented as the percentage of total head dips (cf. Moy et al., 2008).

To avoid floor or ceiling effects we further excluded animals from the behavioral analysis when these rats were either too inactive (i.e., few total hole visits) or too active (i.e., many total hole visits). Based on these thoughts, the testing protocols of the following studies were designed and the exclusion criteria were defined.

Experiment 2

It is well-established that aversive odors, such as predator odors, innately induce a variety of defensive responses including avoidance and escape behavior (Dielenberg and McGregor, 2001; Apfelbach et al., 2005; Masini et al., 2005). On the other hand, attractive odors, such as the odors of female conspecifics, are approached (Liberles, 2014). To investigate whether these behaviors can also be observed in the olfactory hole-board test for rats, we placed urine samples of carnivores, female conspecifics and a water control sample under the corner holes of the hole-board. We expected that holes with aversive odors will be avoided (i.e., less hole visits) and holes with attractive odors will be preferred (i.e., more hole visits).

Subjects and Procedure

Fourteen male Sprague-Dawley rats were used in this experiment. The following odor samples were presented: fox urine, bobcat urine, female rat urine, and water. The locations of the odor samples were pseudo-randomized.

Results

The percentage of total hole visits for each corner hole of the present experiment is shown in **Figure 2**. The different odor samples significantly affected the corner hole visits [ANOVA: $F_{(3,39)} = 54.85$; $p < 0.0001$]. *Post hoc* pairwise comparisons with the water control indicated a strong increase of visits to the holes with female rat urine (Holm-Sidak's test: $p < 0.0001$), while holes with fox urine ($p = 0.03$) or bobcat urine ($p = 0.02$) were visited less often. The mean number of total hole visits was 39 (data not shown).

Discussion

Experiment 2 investigated whether simultaneous presentation of both aversive and attractive odors led to changes in hole visit behavior. We showed that holes with carnivore urine samples were clearly visited less often than the hole with water, i.e., carnivore urine was avoided. These results support findings from previous studies showing avoidance behavior to carnivore urine. For instance, Osada et al. (2013) observed that mice similarly avoided the short arm of a Y-maze when it contained wolf urine. Using an open-field test we previously showed that rats avoid the quadrant or corner of the testing arena containing carnivore urine, e.g., from foxes, bobcats, pumas, coyotes, or lions (Fendt, 2006; Ferrero et al., 2011; Wernecke et al., 2015). This is confirmed by field studies demonstrating that carnivore urine samples (e.g., dingo, coyote, bobcat, wolf) are effective repellents

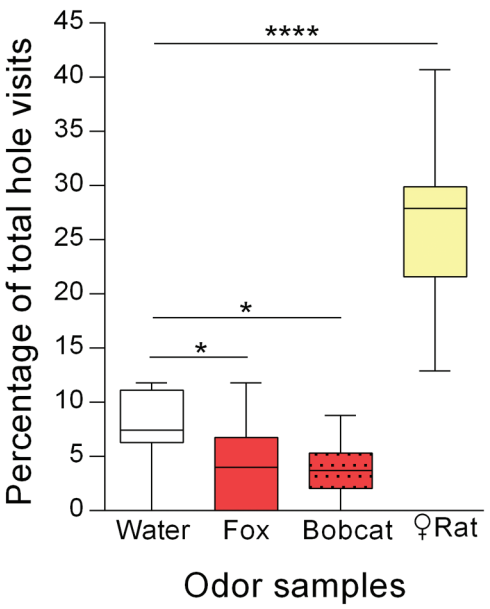


FIGURE 2 | Rats ($n = 14$) avoided holes with carnivore urine whereas holes with female rat urine were preferred. Percentages of total hole visits (median) for the different corner holes are shown. *$p < 0.05$; ****$p < 0.0001$ comparisons as indicated (Holm-Sidak's multiple comparison test after significant main effects in an ANOVA).

protecting forestry and agricultural areas from feeding-related damage (Nolte et al., 1994; Bramley and Waas, 2001; Parsons et al., 2007).

Our second observation is that rats were attracted to the hole containing urine from female rats. Sexually naive male mice similarly preferred to investigate female urine over water in a Y-maze test (Pankevich et al., 2006). In the present experiment, the female urine sample was presented simultaneously with aversive carnivore urine samples. Since we were able to measure these appetitive effects of the female urine samples, we suggest that the different odor samples did not strongly diffuse within the hole-board testing apparatus and that avoidance/preference responses were most likely restricted to the holes containing the particular odor sample. Otherwise, an increase in general anxiety due to the recognition of aversive carnivore odors should be detectable. This would most probably reduce sexually motivated behaviors like approach to female urine samples (cf. Retana-Marquez et al., 1996; Rhees et al., 2001; Kobayashi et al., 2013).

Taken together, the present experiment is in agreement with the rats' natural motivation to approach odors of potential mating partners (Liberles, 2014) and to avoid odors of carnivores (Apfelbach et al., 2005; Masini et al., 2005). Importantly, using the olfactory hole-board test, we are able to study olfactory avoidance and preference behavior to different types of odors presented on the same hole-board in the same test session.

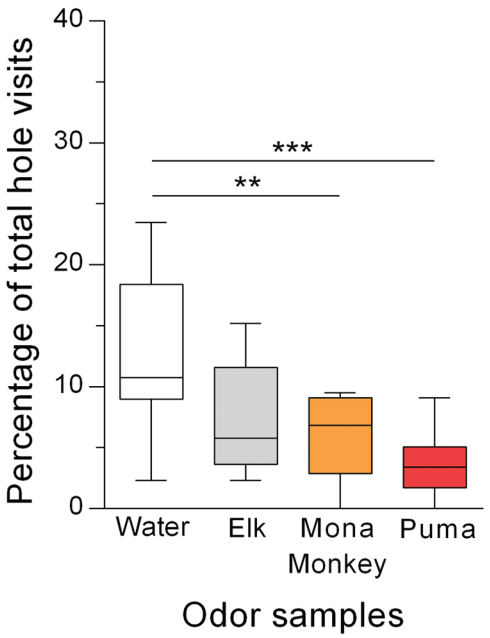


FIGURE 3 | Rats ($n = 22$) avoided holes with omnivore and carnivore urine but not holes with herbivore urine. Percentages of total hole visits (median) for the different corner holes are shown. $^{**}p < 0.01$; $^{***}p < 0.001$ comparisons as indicated (Dunn's multiple comparison test after significant main effects in the Friedman test).

Experiment 3

The previous experiment showed that rats avoid holes with carnivore urine and preferred holes with female rat urine. However, this phenomenon could also be explained by a simple avoidance of odors from other species, whereas odors from conspecifics are preferred. To exclude this possibility, we exposed rats to urine samples from an herbivorous species (elk), an omnivorous species (mona monkey) and a carnivorous species (puma). Based on previous studies (Fendt, 2006; Ferrero et al., 2011), we would expect that carnivore but not herbivore urine samples will be avoided, whereas omnivore urine may lead to an intermediate response.

Subjects and Procedure

Twenty two male Sprague-Dawley rats were included in this experiment. Rats were exposed to carnivore urine (Puma, *Puma concolor*), herbivore urine (Elk, *Cervus canadensis*), omnivore urine (Mona monkey, *Cercopithecus mona*) and water as a control odor.

Results

Again, the holes with the different odor samples were differently visited by the rats (**Figure 3**), as indicated by a significant odor effect (Friedman test: $Q = 20.98$; $p = 0.0001$). *Post hoc* pairwise comparisons of the percentages of corner hole visits with the percentage of water control hole visits showed that the holes with the urine from mona monkeys and pumas were avoided [Dunn's test: $p = 0.009$ (mona monkey urine); $p = 0.0001$ (puma urine)], whereas the holes with elk urine were not differently visited ($p = 0.14$) than the water control hole. The mean number of total hole visits was 31 (data not shown).

Discussion

Rats were exposed to urine samples from an elk, mona monkey and puma, as representatives for herbivore, omnivore, and carnivore species, respectively. Rats avoided the holes containing urine from either the puma or the mona monkey, whereas the holes with elk urine appeared to be neutral (**Figure 3**). These findings as well as similar findings from literature (Ramp et al., 2005; Fendt, 2006; Du et al., 2012) suggest that prey animals are able to discriminate between urine of harmless herbivore species and urine of omnivore or carnivore species, both being potential predators. This would be an important evolutionary adaptation since rats would only invest energy for the defense from potential predators but would not waste energy with defensive responses to odors of herbivore species which are no threat to rats. The question now is by which mechanisms do rats innately recognize urine from potential predators? One possibility is that rats detect predators through common metabolites derived from a carnivorous diet (Nolte et al., 1994; Berton et al., 1998; Ferrero et al., 2011). Such a metabolite could be 2-phenylethylamine (PEA), a component of most carnivore species' urine and also of some omnivore species' urine (Ferrero et al., 2011). Only moderate concentrations of PEA have been identified in urine samples of omnivores or smaller carnivores (e.g., ferret, fox, cat, human), while higher amounts of PEA are present in urine samples of larger feline carnivores

(e.g., tiger, lion, jaguar). These different PEA levels in the urine may be responsible for the intensity of the expressed avoidance behaviors.

Experiment 4

The aim of the present experiment was to test whether anxiolytic treatments specifically modulate the avoidance of carnivore urine holes without affecting the preference of rats to female rat urine.

Benzodiazepines are highly effective anxiolytic substances in both humans and animals (Gelfuso et al., 2014). When tested in predator odor exposure tests, different benzodiazepines (e.g., midazolam) have been reported to change defensive responsiveness to cat odor leading to decreased hiding behavior and increased approach behavior (Blanchard et al., 1998; Dielenberg et al., 1999; McGregor and Dielenberg, 1999; Siviy et al., 2010). However, we observed that treatment of rats with midazolam (0, 0.19, 0.38 mg/kg) had sedative effects and strongly dose-dependently reduced the number of total hole visits (Friedman test: $Q = 20.46$; $p < 0.0001$; Supplementary Figure S1). This makes it very difficult to evaluate whether midazolam treatment affects the avoidance response to carnivore urine.

An established anxiolytic compound with only minor sedative properties is the 5-HT_{1A} receptor agonist buspirone (Kehne et al., 1988; Carli et al., 1989; Moser, 1989). Therefore, rats were treated with buspirone and the effects on olfactory hole-board performance were tested.

Subjects and Procedure

Experiment 4 included 14 male Sprague-Dawley rats. 20 min prior to testing, each animal was pretreated with the vehicle (saline) or the 5-HT_{1A} receptor agonist buspirone (0.1, 1 mg/kg). Injections were given intraperitoneal (i.p.) and were administered at a volume of 1 ml/kg. Each rat received each of the three treatment conditions in a pseudo-randomized order with 24 h between each test. Rats were exposed to fox urine, coyote urine, female rat urine, and water as control odor.

Results

The analysis of the total numbers of hole visits confirmed that buspirone has only minor sedative properties and did not significantly affect the total number of hole visits [ANOVA: $F_{(2,26)} = 2.28$; $p = 0.129$, **Figure 4A**].

The percentages of corner hole visits after treatment with saline or buspirone (0.1 mg/kg; 1 mg/kg) are illustrated in **Figure 4B**. We performed separate ANOVAs for each treatment. In saline-treated rats, there was a significant main effect of odor (Friedman test: $Q = 29.10$; $p < 0.0001$). *Post hoc* comparisons with the water control indicated that rats significantly avoided the holes with fox (Dunn's test: $p = 0.016$) or coyote urine ($p = 0.039$). However, there was no effect of female rat urine ($p = 0.237$). After treatment with 0.1 mg/kg buspirone, there was still a significant main effect of odor [ANOVA: $F_{(3,39)} = 14.81$; $p < 0.0001$] with holes with fox urine (Holm-Sidak's test: $p = 0.005$) or coyote urine ($p = 0.042$) being avoided. The holes with female rat urine were not visited more often by rats than the

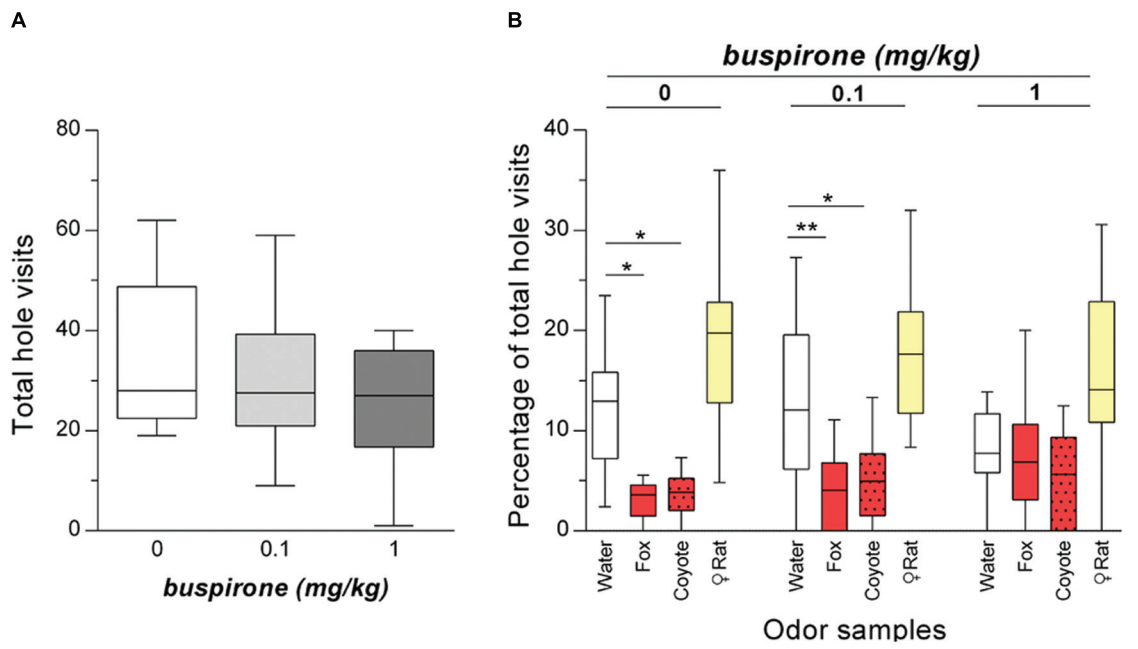


FIGURE 4 | Buspirone treatment specifically blocked the avoidance of holes with carnivore urine without sedating the rats ($n = 14$). **(A)** Total number of hole visits (median) of rats after treatment with buspirone (0; 0.1; 1 mg/kg buspirone). **(B)** Percentages of total hole visits (median) for the different corner holes and for each treatment are shown. $^*p < 0.05$; $^{**}p < 0.01$ comparison as indicated (Holm-Sidak's/Dunn's multiple comparison test after significant main effects in an ANOVA/Friedman test).

water hole ($p = 0.105$). Notably, different effects were observed after treatment with 1 mg/kg buspirone. Although there was again a main effect of odor (Friedman test: $Q = 12.05$; $p = 0.007$), the percentages of total hole visits for the different corner holes were not different from water [Dunn's tests: $p > 0.999$ (fox urine); $p = 0.563$ (coyote urine); $p = 0.171$ (female rat urine)].

Discussion

Treatment with the 5-HT_{1A} receptor agonist buspirone dose-dependently blocked the avoidance of holes with carnivore urine in the olfactory hole-board test. Importantly, buspirone did not affect the total hole visits and the visits of holes with attractive female rat urine (**Figures 4A,B**) indicating a specific anxiolytic effect on behavioral changes induced by carnivore urine. We further showed that the olfactory hole-board test is inappropriate for testing compounds with strong sedative effects such as midazolam (Supplementary Figure S1).

Our finding that buspirone reduced predator odor-induced defensive behavior supports previous findings showing that treatment with 8-OHDPAT [(±)-8-hydroxy-2-(di-*n*-propylamino) tetralin], another 5-HT_{1A} receptor agonist, decreases freezing and increases approach behavior to TMT (2,4,5 trimethylthiazoline), a synthetic predator odor (Shields and King, 2008). Similarly, it has been demonstrated that buspirone effectively reduces anxiety in other rodent anxiety models, such as the elevated-plus-maze test and the black/white exploration test (Moser, 1989; Hendrie et al., 1997).

General Discussion

Rodents, as most other mammals, are predominantly olfactory oriented and largely depend on olfactory cues for operating in their environment (Sotnikov et al., 2011; Galliot et al., 2012). Therefore, odors are of considerable significance in guiding nearly every class of animal behavior (Doty, 1986) and their perception and discrimination are believed to be crucial for survival and reproduction. In this sense, the recognition of predator odors and odors of the sexual counterpart is critically important. The former induces defensive behaviors, whereas the latter induces attraction behavior in rats.

The aim of the present study was to assess whether the olfactory hole-board test can be used as a behavioral paradigm for investigating olfactory preference and avoidance to biologically relevant odors, as well as whether such a preference or avoidance can be selectively modulated by pharmacological treatments. We made use of the rats' natural preference for corner holes in the hole-board test and examined whether this pattern of hole preference could be manipulated by placing both appetitive and aversive odors under these holes. Using the innate preference for corner holes allowed us to circumvent a floor effect since odor-induced avoidance responses are more easily detected when the holes are frequently visited under control conditions. The key advantage of the olfactory hole-board test is that it allows testing animals' responses to four odors in a single test session. Moreover, since appetitive odor samples can be presented simultaneously with aversive odor samples under different corner holes, we were further able to test preference and avoidance responses at the same time. This is unique, since most research focused on the effects of biologically-relevant odors on the behaviors of rats used multi-trial paradigms (e.g., olfactory habituation/dishabituation task) with sequential presentations of different odors (Mandairon et al., 2009; Silverman et al., 2010; Lehmkuhl et al., 2014). Moreover, because rodents in nature are not exposed to only one pure odor but to several odors at the same time, the olfactory hole-board may present also a more natural test situation.

As mentioned in *Experiment 1,* Moy et al. (2008) had also developed an olfactory version of the hole-board test. In contrast to our study, they made use of the mice's natural aversion of the center holes and tested whether the placement of novel, appetitive odors in these holes may modify this innate aversion. Consequently, a lack of a hole preference shift to the center holes has been interpreted to reflect the resistance to adapt their behavioral responses in regard to environmental factors (Moy et al., 2008). This suggests that the hole-board test in association with the presentation of odor samples is versatile allowing the study of multiple research issues. While the mouse olfactory hole-board was only used to test appetitive odor samples, in our version, rats were exposed to both appetitive and aversive odor samples.

The quality of the olfactory hole-board test in testing aversion and preferences in the same test session was shown by *Experiments 2* and *3*. Rats avoided visiting corner holes with urine of potential predators (fox, bobcat, puma, coyote, mona monkey). Simultaneously, rats visited holes with urine from female rats more often indicating attraction behavior. Importantly, the odor-induced avoidance response was specific to the urine of omnivore and carnivore species. In contrast, the number of visits to holes with urine of an herbivore species was indistinguishable to that of the control holes. This supports the idea that rats are able to discriminate between urine of different species, both predator and non-threatening species (Ramp et al., 2005; Fendt, 2006; Ferrero et al., 2011; Du et al., 2012). Regarding the experiments with female rat urine presentation (*Experiments 2 and 4*), a variable efficiency to induce hole preference was recognizable. It has been shown that male rats are more attracted to odors of estrous females than that of non-estrus females (Hosokawa and Chiba, 2005; Achiraman and Archunan, 2006; Achiraman et al., 2010). We collected urine regardless of the female's estrus cycle stage. Therefore, different amounts of estrus urine in the different odor samples may serve as a likely explanation for the varying effectiveness of female rat urine to attract male rats.

The present study further tested whether the avoidance of carnivore urine holes can be reduced by treating the rats with the anxiolytic compounds midazolam or buspirone (*Experiment 4*). Treatment with buspirone specifically abolished the avoidance response to holes with carnivore urine. Notably, such effects are difficult to detect when the anxiolytic compound has strong sedative effects, as was the case with midazolam since too few hole visits were observed after midazolam treatment. The finding that the olfactory hole-board test provides direct measures of olfactory responses in rats that can be specifically pharmacologically manipulated further makes it possible to use

this test to examine, for instance, the specificity of anxiolytic treatment effects.

Conclusion

The present study demonstrates that the olfactory hole-board test may provide an appropriate tool for the assessment of olfactory aversion and preferences in rats. In contrast to many other testing paradigms, this paradigm allows testing of up to four odors simultaneously in single trials. Furthermore, the olfactory hole-board test is applicable to test anxiolytic treatments without sedating properties indicating predictive validity.

Acknowledgments

The authors thank Timothy French for language editing and Kathrin Freke for her help in animal care. We gratefully acknowledge the testing assistance of Sinthyia Ahmed. This study was funded by the federal state of Saxony-Anhalt, Project: Center for Behavioral Brain Sciences (CBBS).

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnbeh.2015.00223

References

Achiraman, S., and Archunan, G. (2006). 1-Iodo-2methylundecane, a putative estrus-specific urinary chemo-signal of female mouse (*Mus musculus*). *Theriogenology* 66, 1913–1920. doi: 10.1016/j.theriogenology.2006.05.010

Achiraman, S., Ponmanickam, P., Ganesh, D. S., and Archunan, G. (2010). Detection of estrus by male mice: synergistic role of olfactory-vomeronasal system. *Neurosci. Lett.* 477, 144–148. doi: 10.1016/j.neulet.2010.04.051

Apfelbach, R., Blanchard, C., Blanchard, R., Hayes, R., and McGregor, I. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neurosci. Biobehav. Rev.* 29, 1123–1144. doi: 10.1016/j.neubiorev.2005.05.005

Berton, F., Vogel, E., and Belzung, C. (1998). Modulation of mice anxiety in response to cat odor as a consequence of predators diet. *Physiol. Behav.* 65, 247–254. doi: 10.1016/S0031-93849800126-7

Blanchard, D. C., Griebel, G., Rodgers, R. J., and Blanchard, R. J. (1998). Benzodiazepine and serotonergic modulation of antipredator and conspecific defense. *Neurosci. Biobehav. Rev.* 22, 597–612. doi: 10.1016/S0149-76349700054-7

Blanchard, R., and Blanchard, D. (1989). Antipredator defensive behaviors in a visible burrow system. *J. Comp. Psychol.* 103, 70–82. doi: 10.1037/0735-7036.103.1.70

Boisser, J., and Simon, P. (1962). La reaction dexploration chez la souris. *Therapie* 17, 1225–1232.

Boisser, J., and Simon, P. (1964). Dissociation de deux composantes dans le compartment dinvestigation de la souris. *Arch. Int. Pharmadyn.* 147, 372–387.

Bramley, G., and Waas, J. (2001). Laboratory and field evaluation of predator odors as repellents for kiore (*Rattus exulans*) and ship rats (*R. rattus). J. Chem. Ecol.* 27, 1029–1047. doi: 10.1023/A:1010399322861

Brennan, P. A., and Kendrick, K. M. (2006). Mammalian social odours: attraction and individual recognition. *Philos. Trans. R. Soc. B* 361, 2061–2078. doi: 10.1098/rstb.2006.1931

Brown, G. R., and Nemes, C. (2008). The exploratory behaviour of rats in the hole-board apparatus: is head-dipping a valid measure of neophilia? *Behav. Proc.* 78, 442–448. doi: 10.1016/j.beproc.2008.02.019

Carli, M., Prontera, C., and Samanin, R. (1989). Evidence that central 5-hydroxytryptaminergic neurones are involved in the anxiolytic activity of buspirone. *Br. J. Pharmacol.* 96, 829–836. doi: 10.1111/j.1476-5381.1989.tb11891.x

Crawley, J. N. (1985). Exploratory behavior models of anxiety in mice. *Neurosci. Biobehav. Rev.* 9, 37–44. doi: 10.1016/0149-7634859 0030-2

Dielenberg, R. A., Arnold, J. C., and McGregor, I. S. (1999). Low-dose midazolam attenuates predatory odor avoidance in rats. *Pharmacol. Biochem. Behav.* 62, 197–201. doi: 10.1016/S0091-30579800064-1

Dielenberg, R. A., and McGregor, I. S. (2001). Defensive behavior in rats towards predatory odors: a review. *Neurosci. Biobehav. Rev.* 25, 597–609. doi: 10.1016/S0149-76340100044-6

Doty, R. L. (1986). Odor-guided behavior in mammals. *Experientia* 42, 257–271. doi: 10.1007/BF01942506

Du, Y., Huang, Y., Zhang, H., Li, D., Yang, B., Wei, M., et al. (2012). Innate predator recognition in giant pandas. *Zool. Sci.* 29, 67–70. doi: 10.2108/zsj.29.67

Endres, T., Apfelbach, R., and Fendt, M. (2005). Behavioral changes induced in rats by exposure to trimethylthiazoline, a component of fox odor. *Behav. Neurosci.* 119, 1004–1010. doi: 10.1037/0735-7044.119.4.1004

Fendt, M. (2006). Exposure to urine of canids and felids, but not of herbivores, induces defensive behavior in laboratory rats. *J. Chem. Ecol.* 32, 2617–2627. doi: 10.1007/s10886-006-9186-9

Ferrero, D. M., Lemon, J. K., Fluegge, D., Pashkovski, S. L., Korzan, W. J., Datta, S. R., et al. (2011). Detection and avoidance of a carnivore odor by prey. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11235–11240. doi: 10.1073/pnas.1103317108

Fortes-Marco, L., Lanuza, E., and Martinez-Garcia, F. (2013). Of pheromones and kairomones: what receptors mediate innate emotional responses? *Anat. Rec.* 296, 1346–1363. doi: 10.1002/ar.22745

Galliot, E., Laurent, L., Hacquemand, R., Pourié, G., and Millot, J. L. (2012). Fear-like behavioral responses in mice in different odorant environments: trigeminal versus olfactory mediation under low doses. *Behav. Proc.* 90, 161–166. doi: 10.1016/j.beproc.2012.01.002

Gelfuso, E. A., Rosa, D. S., Fachin, A. L., Mortari, M. R., Cunha, A. O. S., and Beleboni, R. O. (2014). Anxiety: a systematic review of neurobiology, traditional pharmaceuticals and novel alternatives from medicinal plants. *CNS Neurol. Disord. Drug Targerts* 13, 150–165. doi: 10.2174/18715273113129990102

Hendrie, C. A., Eilam, D., and Weiss, S. M. (1997). Effects of diazepam and buspirone on the behaviour of wild voles (*Microtus socialis*) in two models of anxiety. *Pharmacol. Biochem. Behav.* 58, 573–576. doi: 10.1016/S0091-3057(97)00278-5

Hosokawa, N., and Chiba, A. (2005). Effects of sexual experience on conspecific odor preference and estrous odor-induced activation of the vomeronasal projection pathway and the nucleus accumbens in male rats. *Brain Res.* 1066, 101–108. doi: 10.1016/j.brainres.2005.10.036

Hughes, R. N. (2007). Neotic preferences in laboratory rodents: issues, assessment and substrates. *Neurosci. Biobehav. Rev.* 31, 441–464. doi: 10.1016/j.neubiorev.2006.11.004

Kehne, J. H., Cassella, J. V., and Davis, M. (1988). Anxiolytic effects of buspirone and gepirone in the fear-potentiated startle paradigm. *Psychopharmacology* 94, 8–13. doi: 10.1007/BF00735872

Kobayashi, T., Kiyokawa, Y., Arata, S., Takeuchi, Y., and Mori, Y. (2013). c-Fos expression during the modulation of sexual behavior by an alarm pheromone. *Behav. Brain Res.* 237, 230–237. doi: 10.1016/j.bbr.2012.09.042

Lamprea, M. R., Cardenas, F. P., Setem, J., and Morato, S. (2008). Thigmotactic responses in an open-field. Braz. *J. Med. Biol. Res.* 41, 135–140. doi: 10.1590/S0100-879X2008000200010

Lehmkuhl, A. M., Dirr, E. R., and Fleming, S. M. (2014). Olfactory assays for mouse models of neurodegenerative disease. *J. Vis. Exp.* 90:e51804. doi: 10.3791/51804

Liberles, S. D. (2014). Mammalian pheromones. *Annu. Rev. Physiol.* 76, 151–175. doi: 10.1146/annurev-physiol-021113-170334

Lister, R. G. (1990). Ethologically-based animal models of anxiety disorders. *Pharmacol. Therapeut.* 46, 321–340. doi: 10.1016/0163-7258(90)90021-S

Litvin, Y., Pentkowski, N. S., Pobbe, R. L., Blanchard, D. C., and Blanchard, R. J. (2008). "Unconditioned models of fear and anxiety," in *Handbook of Anxiety and Fear*, eds R. J. Blanchard, C. D. Blanchard, G. Griebel, and D. Nutt (Amsterdam, NL: Elsevier E.V.), 81–99. doi: 10.1016/S1569-7339(07)00006-9

Mandairon, N., Sultan, S., Rey, N., Kermen, F., Moreno, M., Busto, G., et al. (2009). A computer-assisted odorized hole-board for testing olfactory perception in mice. *J. Neurosci. Methods* 180, 296–303. doi: 10.1016/j.jneumeth.2009.04.008

Masini, C. V., Sauer, S., and Campeau, S. (2005). Ferret odor as a processive stress model in rats: neurochernical, behavioral, and endocrine evidence. *Behav. Neurosci.* 119, 280–292. doi: 10.1037/0735-7044.119.1.280

McGregor, I. S., and Dielenberg, R. A. (1999). Differential anxiolytic efficacy of a benzodiazepine on first versus second exposure to a predatory odor in rats. *Psychopharmacology* 147, 174–181. doi: 10.1007/s002130051158

Moser, P. C. (1989). An evaluation of the elevated plus-maze test using the novel anxiolytic buspirone. *Psychopharmacology* 99, 48–53. doi: 10.1007/BF00634451

Moy, S. S., Nadler, J. J., Poe, M. D., Nonneman, R. J., Young, N. B., Koller, B. H., et al. (2008). Development of a mouse test for repetitive, restricted behaviors: relevance to autism. *Behav. Brain Res.* 188, 178–194. doi: 10.1016/j.bbr.2007.10.029

Nolte, D. L., Mason, J. R., Epple, G., Aronov, E., and Campbell, D. L. (1994). Why are predator urines aversive to prey? *J. Chem. Ecol.* 20, 1505–1516. doi: 10.1007/BF02059876

Osada, K., Kurihara, K., Izumi, H., and Kashiwayanagi, M. (2013). Pyrazine analogues are active components of wolf urine that induce avoidance and freezing behaviours in mice. *PLoS ONE* 8:e61753. doi: 10.1371/journal.pone.0061753

Pankevich, D. E., Cherry, J. A., and Baum, M. J. (2006). Effect of vomeronasal organ removal from male mice on their preference for and neural Fos responses to female urinary odors. *Behav. Neurosci.* 120, 925–936. doi: 10.1037/0735-7044.120.4.925

Parsons, M. H., Lamont, B. B., Vacs, B. R., and Davis, S. J. J. F. (2007). Effects of novel and historic predator urines on semi-wild western grey kangaroos. *J. Wildl. Manage.* 71, 1225–1228. doi: 10.2193/2006-096

Rajchard, J. (2013). Kairomones - important substances in interspecific communication in vertebrates: a review. *Vet. Med.* 58, 561–566.

Ramp, D., Russell, B. G., and Croft, D. B. (2005). Predator scent induces differing responses in two sympatric macropodids. *Aust. J. Zool.* 53, 73–78. doi: 10.1071/ZO04053

Retana-Marquez, S., Salazar, E. D., and Velazquez-Moctezuma, J. (1996). Effect of acute and chronic stress on masculine sexual behavior in the rat. *Psychoneuroendocrinology* 21, 39–50. doi: 10.1016/0306-4530(95)00029-1

Rhees, R. W., Lephart, E. D., and Eliason, D. (2001). Effects of maternal separation during early postnatal development on male sexual behavior and female reproductive function. *Behav. Brain Res.* 123, 1–10. doi: 10.1016/S0166-43280000381-8

Rivard, G. F., Moser, E. G., D'Ambrose, S. P., and Lin, D. M. (2014). Lack of fear response in mice (*Mus musculus*) exposed to human urine odor. *J. Am. Assoc. Lab. Anim. Sci.* 53, 141–145.

Saitoh, A., Hirose, N., Yamada, M., Yamada, M., Nozaki, C., Oka, T., et al. (2006). Changes in emotional behavior of mice in the hole-board test after olfactory bulbectomy. *J. Pharmacol. Sci.* 102, 377–386. doi: 10.1254/jphs.FP0060837

Shields, J., and King, J. A. (2008). The role of 5-HT 1A receptors in the behavioral responses associated with innate fear. *Behav. Neurosci.* 122, 611–617. doi: 10.1037/0735-7044.122.3.611

Silverman, J. L., Yang, M., Lord, C., and Crawley, J. N. (2010). Behavioural phenotyping assays for mouse models of autism. *Nat. Rev. Neurosci.* 11, 490–502. doi: 10.1038/nrn2851

Siviy, S. M., Steets, C., and DeBrouse, L. M. (2010). Effects of chlordiazepoxide on predator odor-induced reductions of playfulness in juvenile rats. *Behav. Brain Res.* 206, 254–262. doi: 10.1016/j.bbr.2009.09.026

Sotnikov, S. V., Markt, P. O., Umriukhin, A. E., and Landgraf, R. (2011). Genetic predisposition to anxiety-related behavior predicts predator odor response. *Behav. Brain Res.* 225, 230–234. doi: 10.1016/j.bbr.2011.07.022

Takeda, H., Tsuji, M., and Matsumiya, T. (1998). Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. *Eur. J. Pharmacol.* 350, 21–29. doi: 10.1016/S0014-29999800223-4

Wallace, K. J., and Rosen, J. B. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behav. Neurosci.* 114, 912–922. doi: 10.1037/0735-7044.114.5.912

Wernecke, K. E. A., Vincenz, D., Storsberg, S., D'Hanis, W., Goldschmidt, J., and Fendt, M. (2015). Fox urine exposure induces avoidance behavior in rats and activates the amygdalar olfactory cortex. *Behav. Brain Res.* 279, 76–81. doi: 10.1016/j.bbr.2014.11.020

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 09 September 2015
doi: 10.3389/fnins.2015.00318

Impaired sense of smell and altered olfactory system in RAG-1$^{-/-}$ immunodeficient mice

Lorenza Rattazzi[1†], Anna Cariboni[2,3†], Ridhika Poojara[1], Yehuda Shoenfeld[4] and Fulvio D'Acquisto[1]*

[1] William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, UK, [2] Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy, [3] Department of Cell Biology, Institute of Ophthalmology, University College London, London, UK, [4] Zabludowicz Center for Autoimmune Diseases, Sheba Medical Centre, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Immune deficiencies are often associated with a number of physical manifestations including loss of sense of smell and an increased level of anxiety. We have previously shown that T and B cell-deficient recombinase activating gene (*RAG-1*)$^{-/-}$ knockout mice have an increased level of anxiety-like behavior and altered gene expression involved in olfaction. In this study, we expanded these findings by testing the structure and functional development of the olfactory system in *RAG-1*$^{-/-}$ mice. Our results show that these mice have a reduced engagement in different types of odors and this phenotype is associated with disorganized architecture of glomerular tissue and atrophy of the main olfactory epithelium. Most intriguingly this defect manifests specifically in adult age and is not due to impairment in the patterning of the olfactory neuron staining at the embryo stage. Together these findings provide a formerly unreported biological evidence for an altered function of the olfactory system in *RAG-1*$^{-/-}$ mice.

Keywords: immunosuppression, immunodeficiency, anxiety, olfactory dysfunction, main and accessory olfactory system (MOS and AOS), main olfactory epithelium (MOE)

OPEN ACCESS

Edited by:
Yasushi Kiyokawa, The University of Tokyo, Japan

Reviewed by:
Oreste Gualillo, Santiago University Clinical Hospital, Spain
Makoto Yokosuka, Nippon Veterinary and life Science University, Japan

***Correspondence:**
Fulvio D'Acquisto, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK
f.dacquisto@qmul.ac.uk

† First authorship.

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Neuroscience

Received: *24 June 2015*
Accepted: *27 August 2015*
Published: *09 September 2015*

Citation:
Rattazzi L, Cariboni A, Poojara R, Shoenfeld Y and D'Acquisto F (2015) Impaired sense of smell and altered olfactory system in RAG-1$^{-/-}$ immunodeficient mice. Front. Neurosci. 9:318. doi: 10.3389/fnins.2015.00318

Introduction

Many vertebrates, including most mammals and reptiles utilize the sense of smell for their survival since it is essential for finding nutritious food, a suitable mate and to escape predators (Mombaerts, 2004). Odor perception is a finely regulated process that occurs through a substantial and yet finite number of odorant receptors. Indeed, mammals have 347 genes coding for functional odor receptors among a total of 1000 genes and each olfactory receptor is activated by a specific odorant (Hoover, 2010; Glatz and Bailey-Hill, 2011).

Similar to the olfactory system, the immune system helps us to relate to the external environment and to differentiate harmful or innocuous agents. Recent clinical evidence have suggested that these two systems share more than just a similarity and potentially communicate with each other. Indeed, clinical studies on patients suffering from a wide range of immune disorders such as autoimmune pathologies like multiple sclerosis (Lutterotti et al., 2011; Erb et al., 2012; Garcia-Gonzalez et al., 2013), systemic lupus erythematous (Shoenfeld, 2007; Shoenfeld et al., 2009; Cavaco et al., 2012; Perricone et al., 2013) and Sjögren's syndrome (Midilli et al., 2013) or immunodeficiencies like HIV/AIDS (Graham et al., 1995; Mueller et al., 2002; Vance, 2004, 2007; Vance and Burrage, 2006) have often reported a reduction in threshold of discrimination of different odors (TDI) or olfactory dysfunction typically at the onset of disease. Hence either the absence of adaptive

immune cells (as in the case of immunodeficiencies) or the uncontrolled activation of these cells (as in autoimmune diseases) can cause a loss or reduced ability to smell i.e., anosmia or hyposmia.

Over the past few years, we have been investigating the crosstalk between emotions and immune system. Our recent characterization of the emotional behavior of immunodeficient recombination activation gene (*RAG*)-1 knockout mice has revealed an increased level of anxiety-like behavior in these animals. Most interestingly, these changes in behavior were accompanied with changes in the gene expression profile of the brain including a reduced expression of genes involved in olfactory transduction. This was a rather interesting finding considering that the same immunological conditions that cause anosmia have also been associated with increased incidence of mental disorders and stress. Most intriguingly, studies using both pharmacological and surgical impairment of the olfactory system have also described a significant increase in anxiety-like behavior thus suggesting the possibility that smell, anxiety and immune response might share a common molecular pathway.

To test this hypothesis, in this study we investigated if the increased anxiety—like behavior of *RAG-1*$^{-/-}$ mice was also associated with an impaired sense of smell. In addition to this, because *RAG-1* is expressed in olfactory sensory neurons (OSN) and epithelium (OE) we also investigated if the absence of this gene would affect the development of the olfactory system. Our results provide the first experimental evidence for a specific role of *RAG-1* in the conservation of the olfactory system in adult mice rather than its development at the embryo stage. Most importantly, our studies provide further evidence for a possible role of *RAG-1* as molecular link between emotions, immunity and sense of smell.

Materials and Methods

Mice

RAG-1$^{-/-}$ mice on C57/BL6 background were kindly provided by Prof. Hans Stauss, (University College London, UK) while control C57BL/6 mice were purchased from Charles River. Both strains were bred in our animal facility. We used 7 week-old male mice for all the behavioral tests and timed-embryos were obtained by mating mice in the evening. The presence of a vaginal plug in the morning indicates successful mating and the resulting embryo would be considered 0.5 day old (E0.5). Mice were housed in groups of maximum 6 animals per cage under specific-pathogen-free conditions and with free access to food and water. All the behavioral experiments were performed during the light phase of the light-dark cycle and no more than 2 tests per day were performed. All tests were conducted in a blinded fashion and according to the UK Animals (Scientific Procedures) Act, 1986. The local biological service unit at Queen Mary University of London approved all experimental protocols.

Buried Food Test

First described in the early 1970s (Alberts and Galef, 1971), the buried food test has been adapted under various names and a range of palatable food have been used (e.g., cookies, cereals and food pellets). The purpose of this experimental test is to measure an animal's ability to smell volatile odors and its natural tendency to use olfactory cues for foraging. The main parameters measured in this test are the latency to find the hidden food and the time spent eating it (Yang and Crawley, 2009). The testing protocol of 3 days consists of an odor familiarization exercise on day 1, food deprivation on day 2 and testing on day 3. On day 1, 7-week male C57BL/6 and *RAG-1*$^{-/-}$ mice were placed in a clean mouse plastic cage (25 × 42 × 12 cm) containing 3 cm of fresh cage bedding. Three Teddy Grahams cookies (Nabisco Inc.; 1 cookie for every 2 mice) where placed in each cage and left overnight. Cages were inspected on day 2 to verify that the cookies were consumed to make sure that the bait is a highly palatable food. On day 2 at approximately 4 pm (1 day before the test), food pellets were removed from the cages and testing mice fasted overnight. The test was performed on day 3 at approximately 11 am after 1-h acclimatization in the testing room. Mice were then individually introduced into a clean cage containing 3 cm deep of clean bedding and allowed to acclimate to the cage for 5 min to reduce the interference of novel environment exploration during the test. A cookie was buried beneath 1 cm of bedding in a random corner of the cage and the mouse introduced into the cage. The site of animal placement and the site at which the cookie was buried remained constant. Time necessary for the animal to retrieve the cookie with its front paws was measured in seconds (latency) up to a maximum parameter of 15 min (900 s was the maximum score; Yang and Crawley, 2009).

Olfactory Habituation/Cross-habituation Test

The capability of mice to detect and differentiate various odors (social and non-social odors) was examined with the olfactory habituation/cross-habituation test (Luo et al., 2002; Yang and Crawley, 2009). The main aim of this test is to measure an animal's tendency to investigate novel smells and presenting the mice with a sequence of different odors assesses this. A common sequence is (1) water; (2) two non-social odors; and (3) two social odors. Habituation is defined by a decrease in time spent sniffing the same odor. Cross-habituation is represented by a reinstatement of olfactory investigation when a novel odor is presented (Woodley and Baum, 2003; Wrenn et al., 2003; Wersinger et al., 2007). Prior testing, the mice were allowed to acclimate for 30 min to a clean food- and water-deprived testing cage with a dry cotton-tipped applicator inserted through the water bottle hole. This is a necessary practice because it can reduce the interference of novel environment exploration during the olfactory test. Non-social odors were prepared in the morning of the same day of the test, they included: (1) distilled water; (2) solution with almond extract; (3) solution with banana extract (McCormick Inc. brand). Almond and banana are standard non-social odors because they are distinctly different and mildly attractive natural food odors, but unrelated to the food with which laboratory rodents are familiar (Huckins et al., 2013). The solutions were prepared by adding 10 µl of almond or banana extract to 990 µl of distilled water (1:1000 dilution). For the social odors, a cotton-tipped applicator was swiped in a zigzag fashion 5 times on the bottom of a cage. We used 5 day-old dirty cages of female mice for unfamiliar social cage 1 and male mice of the

same age for unfamiliar social cage 2. Stimuli were presented in the following order: water × 3, almond × 3, banana × 3, social odor × 3. A trial period of 2 min was given for each stimulus presented, and thus the time spent sniffing the tip for each stimulus was recorded in seconds using a silent stopwatch.

Immunohistochemistry

Immunofluorescence was applied to formaldehyde fixed cryosections as previously described (Cariboni et al., 2011). Briefly, coronal sections were blocked with serum free protein block (DAKO) and immunostained with goat anti–OMP (1:500; DAKO), rabbit anti-Tuj1 (1:500; Covance), followed by cy3- and 488-conjugated donkey antigoat/rabbit Fab fragment secondary antibodies (Jackson ImmunoResearch). Nuclei were counterstained with DAPI (Sigma). For immunoperoxidase staining, formaldehyde-fixed sections were processed as described previously. Briefly, coronal adjacent sections of formaldehyde-fixed embryo heads of 25 m were incubated with hydrogen peroxide to quench endogenous peroxidase activity and then blocked and incubated with rabbit anti-peripherin (1:1000, Chemicon) or rabbit anti-GnRH (1:1000, ImmunoStar) primary antibodies and followed by biotinylated goat anti-rabbit antibody (Vector Laboratories). Immunoreactivity was visualized with the ABC kit (Vector Laboratories) and 3,3-diaminobenzidine (Sigma). The analysis was performed on at least 3 samples for each genotype. We measured the pixel intensity of OMP staining in 20 μm coronal sections through the olfactory glomeruli of 3 mice for each genotype. Haematoxylin and eosin staining (H&E) was applied to paraffin sections of whole heads as previously described for other tissues (Maione et al., 2009, 2010; Paschalidis et al., 2009). Briefly, whole heads were obtained from 7-week old wild-type C57BL/6 and *RAG-1*$^{-/-}$ mice. Prior fixation with 4% paraformaldehyde (pH 7.4) and decalcification in 10% EDTA (pH 7.2–7.4), the heads were embedded in paraffin wax. Sagittal sections were deparaffinized and stained with haematoxylin and eosin. Digital images were taken using the Image Pro image analysis software package.

Data Analysis

All the statistical analysis was performed using GraphPad Prism software. The buried food test was analyzed using the nonparametric Mann–Whitney *U*-test. Statistical significance was set at $p \leq 0.05$ and all data are presented as mean ± SEM as previously described (Dawson et al., 2005; Fleming et al., 2008). For the habituation/dishabituation test, One-Way repeated measured ANOVA within each group was used to compare the time that subjects spent investigating the stimulus upon the different exposure. All data are presented as mean ± SEM.

The total number of GnRH neurons/head was quantified as previously described (Cariboni et al., 2011). To compare the abundance of OMP^{+}-neurons we measured the pixel intensity of OMP staining in 20-μm coronal sections through the OB of 3 mice for each genotype, by using ImageJ software (NIH). To compare the area of glomeruli, we measured the area of each glomerulus in 20-μm coronal sections through the OB of adult mice for each genotype, at the same anatomical level, by using ImageJ software. To compare the OE thickness, we measured the thickness of OE in 10-μm sagittal sections through the nasal region for each genotype, at the same anatomical level, by using ImageJ software. To determine statistical significance, we used the unpaired *t*-test. A *P*-value of less than 0.05 was considered statistically significant. For all experiments, data are expressed as the mean ± SEM.

Results

RAG-1$^{-/-}$ Immunodeficient Mice Have an Altered Sense of Smell

The buried food test is a reliable protocol that relies on the natural tendency of the mouse to use olfactory cues for foraging. The main parameter is the latency to uncover a small piece of palatable food such as a cookie, hidden beneath a layer of sawdust, within an established length of time. We first tested the palatability of the bait leaving the cookie with mice overnight (see Materials and Methods Section) and observed no difference between *RAG-1*$^{-/-}$ and control C57/BL6, i.e., both strains consumed the whole cookie. However, when we performed the test of the buried cookie, we observed a significant five-fold increase in the latency to find the bait (203 s ± 77.7 vs. 42 s ± 18.9; $p < 0.001$) in the *RAG-1*$^{-/-}$ compared to control C57/BL6 mice (**Figure 1A**).

To further assess the olfactory function of *RAG-1*$^{-/-}$ mice, we used the habituation/cross-habituation test, which relies on the animal's tendency to explore novel smells and is also used to evaluate its ability to distinguish between different odors (Yang and Crawley, 2009). When presented with different stimuli (water, almond, banana, and social odor), control C57BL/6 mice (wild-type) showed the expected increase in time sniffing (compare number 1 bar in the water group with number 1 bar in the other groups) every time a new odor was introduced (cross-habituation). They also showed habituation to the same stimuli since the time spent sniffing the same stimuli was significantly reduced upon the second and the third exposure (compare white bar 1 with bars 2 and 3 in each group) (**Figure 1B**). In contrast, *RAG-1*$^{-/-}$ mice did not follow this pattern and showed an overall reduction in the time of sniffing. More specifically, the investigation rate was so low that both habituation and cross–habituation were difficult to be assessed. Interestingly, however, *RAG-1*$^{-/-}$ mice showed an increase in the time spent exploring the social stimulus compared to unfamiliar ones (almond and banana) and showed a trend toward a normal pattern of habituation (although the differences between the 3 exposure were not significant) suggesting a preserved function of the vomeronasal organ and an impaired activity of the main olfactory system (see Discussion).

Histological Assessment of the Olfactory and Vomeronasal Systems in *RAG-1*$^{-/-}$ Embryos

The development of the olfactory system is strictly linked to the development of the gonadotropin-releasing-hormone neurons, which regulate reproductive function (Wray, 2010; Forni and Wray, 2014). These neuroendocrine cells originate in the nasal placode, the embryonic structure that gives rise to

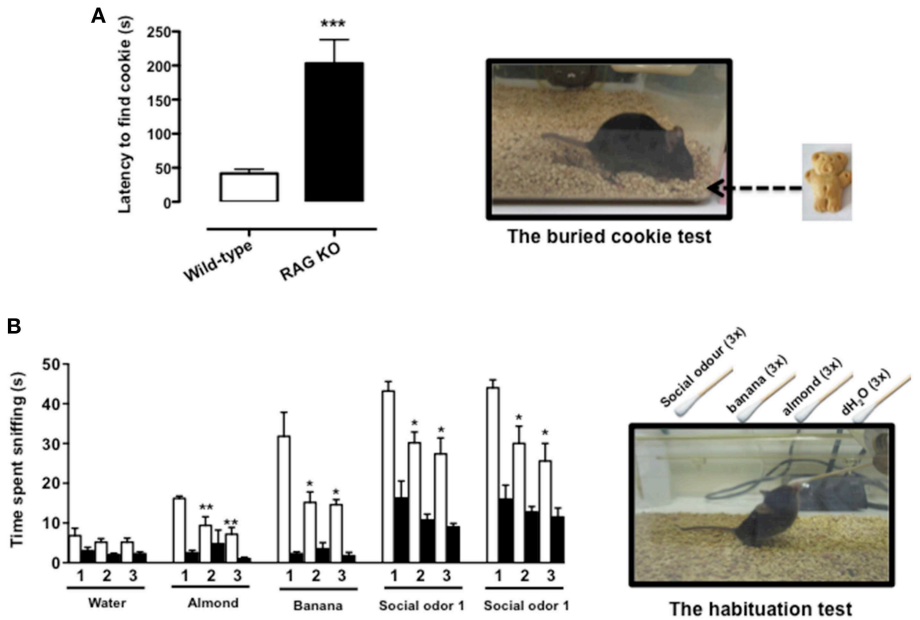


FIGURE 1 | *RAG-1*$^{-/-}$ mice show an impaired sense of smell. Adult 7 week-old *RAG-1*$^{-/-}$ and control C57/BL6 mice were tested with the buried cookie test (top panels) or the habituation/dishabituation test (bottom panels) as described in Materials and methods. The bar graph in **(A)** represents the time expressed in seconds required to find the buried cookie. Values are mean ± SEM obtained from a single experiment with $n = 5$ mice and representative on $n = 4$ experiments with similar results. ***p <0.005 vs. C57BL/6 control mice. The graph in **(B)** shows the time expressed in seconds spent sniffing the stimuli (water, almond, banana, and social odor). The numbers on the x-axes (1, 2, and 3) indicate the order of the repetitive exposure i.e., 1st, 2nd, and 3rd. Values are mean ± SEM obtained from a single experiment with $n = 5$ mice and representative on $n = 3$ experiments with similar results. *$p < 0.05$; **$p < 0.01$ vs. the 1st exposure. The left top and bottom pictures show a schematic representation of the buried cookie test (top) and the habituation/cross-habituation test (bottom) described in details Materials and Methods Section.

the OE and VNO, and migrate toward the brain apposed to olfactory (OLF) and vomeronasal (VN) axons. To investigate the mechanisms behind the olfactory deficits observed in the *RAG-1*$^{-/-}$ mice, we first analyzed the development of the main and accessory olfactory systems at day 14.5 (E14.5) by staining coronal head sections with an anti-peripherin and anti-GnRH antibodies as previously described (Cariboni et al., 2011). As shown in **Figure 2**, we did not observe any defects in either the fasciculation or the targeting of the olfactory nerves toward the olfactory bulbs (OB) between wild-type and *RAG-1*$^{-/-}$ embryos (**Figures 2A,D**, respectively). In addition, the vomeronasal nerves, responsible for pheromone detection in adulthood, were normal and comparable between the two genotypes (**Figures 2B,E**) as it was the migration and the number of the gonadotropin-releasing hormone neurons responsible for reproduction (**Figures 2C,F,G**; total number of GnRH neurons: wild-types 1317 ± 27.55 vs. *RAG-1*$^{-/-}$ 1303 ± 33.37, $p = 0.76$). Consistent with this, the size of the gonads in 7 week-old mice showed no gross difference between wild-type and *RAG-1*$^{-/-}$ (**Supplementary Figure 1**, respectively).

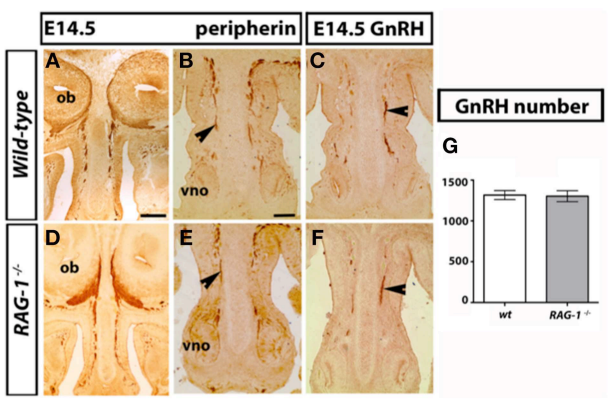


FIGURE 2 | Normal GnRH neuron development in embryonic *RAG-1*$^{-/-}$ mice. Coronal sections of embryonic day E14.5 mouse heads *RAG-1*$^{-/-}$ and control C57/BL6 mice were stained for peripherin **(A,B,D,E)** or GnRH **(C,F)** as described in Material and Methods. Black arrowheads indicate examples of extending olfactory/vomeronasal axons **(B,E)** and of migrating GnRH neurons in the nasal area **(C,F)**. Quantitation of total GnRH neuron number is displayed in **(G)**. Pictures are representative of $n = 4$–6 mice of each genotypes. Scale bars: 150 μm **(A,D)**; 100 μm **(B,C,E,F)**.

Histological Analysis of the Olfactory Bulbs of Newborn *RAG-1*$^{-/-}$ Mice

We next conducted a histological assessment of the olfactory system at birth (day 21) just before the pups are exposed to external and social stimuli. Analysis of the size and gross morphology of the olfactory bulbs in newborn *RAG-1*$^{-/-}$ mice showed no differences compared to control wild-type mice (**Figures 3A,B**, respectively). Immunostaining of the same tissues

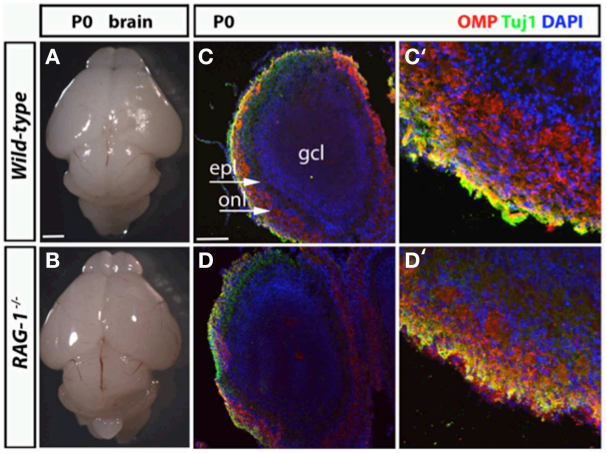


FIGURE 3 | Histological analysis of the olfactory system in newborn *RAG-1*$^{-/-}$ mice. (A,B) Brains from male postnatal age (P) 0 newborn *RAG-1*$^{-/-}$ and control C57/BL6 mice were photographed side-by-side to demonstrate no differences in the size and gross morphology of the brain and of the olfactory bulbs. **(C,D)** Coronal sections of the olfactory bulb from the same mice immunostained with antibodies against OMP and Tuj1 revealed no differences in the intensity and morphology of the projecting ORNs. DAPI was used to counterstain the nuclei. **(C′,D′)** Show higher magnifications of the same tissue sections. The organization of the olfactory bulb displayed in **(C,D)** are labeled as following: gcl, granule cell layer; epl, external plexiform epithelial; onl, olfactory nerve layer. Pictures are representative of $n = 3$ mice of each genotypes. Scale bars: 1 mm **(A,B)**; 100 μm **(C,D)**.

for the olfactory marker protein OMP and the pan-neuronal precursor marker Tuj1 confirmed these results and showed no difference in the localization or level of expression of these two markers (**Figures 3C,D**).

Impaired Olfactory System in Adult *RAG-1*$^{-/-}$ Mice

Morphological analysis of the olfactory bulb of fully developed (7 week-old) *RAG-1*$^{-/-}$ adult mice did not present any differences in size compared to control age-matched wild-type (**Figures 4A,B**, respectively). However, immunofluorescene staining of the olfactory bulb for OMP showed disorganized glomeruli (which are the initial sites for synaptic processing of odor information coming from the nose, Zou et al., 2009; Sakano, 2010) (**Figures 4C,E**) as well reduced expression of this marker (**Figures 4D,F**). Quantitative analysis of OMP staining over different sections confirmed these results and showed a significant ($p < 0.01$; $n = 3$) reduction of about 50% of the pixel intensity in *RAG-1*$^{-/-}$ mice (15.9 ± 1.7 mean pixel intensity/area) compared to wild-type control (28.8 ± 2.3 mean pixel intensity/area). No differences were observed in the size of each glomerulus, expressed as mean of the area (wild type: 0.0224 ± 0.0017 vs. *RAG-1*$^{-/-}$ 0.0255 ± 0.0022, $p = 0.72$; Area expressed as square mm).

On the opposite site of the glomeruli, olfactory neurons innervate the olfactory epithelium (Leinwand and Chalasani, 2011; Murthy, 2011; Takeuchi and Sakano, 2014). These tissues present in the turbinates of the nose act as "platform" for the olfactory neurons and undergo continuous regeneration. Given that olfactory bulbectomy has been shown to severely affect olfactory epithelium regeneration (Suzuki et al., 1998; Makino et al., 2009), we reasoned that the absence of fully functional olfactory neurons would impact the status of OE in *RAG-1*$^{-/-}$. Consistent with our expectation, staining of sagittal paraffin sections with haematoxylin and eosin showed reduced cellularity and thickness of the MOE in *RAG-1*$^{-/-}$ tissues compared to wild-type control (**Figures 5A,B**, respectively; **Figure 5C**, OE thickness: wild type 0.1900 ± 0.01581 vs. *RAG-1*$^{-/-}$ 0.1000 ± 0.01558; $p < 0.005$) further supporting the idea that the absence of immune cells may cause histological changes in olfactory neurons and an impairment of olfaction mainly in adult mice.

Discussion

Immunodeficiencies have long being associated with a number of physical manifestations that are not generally linked to immune functions including anxiety and anosmia. Performing a study on the behavioral profile of T and B cell-deficient *RAG-1*$^{-/-}$ mice we have observed an increased level of anxiety-like and surprisingly found significant changes in brain gene expression profiles of these mice when compared with their wild-type littermates. Pathways analysis of these genes revealed a number of interesting links to different diseases and unexpectedly a defect in the signaling pathways involved in the olfactory system. This was a rather interesting finding since it suggested that *RAG-1*$^{-/-}$ mice could represent an ideal experimental system to study the simultaneous occurrence of anxiety and anosmia that has been described in clinical cases of immunodeficiency.

To verify our hypothesis, we first tested the olfactory function of *RAG-1*$^{-/-}$ mice using classical behavioral models based on the ability of the mouse to recognize the odor of "palatable" baits. Our results from the buried cookie tests showed an increase in the latencies to find the buried food and recognizing the stimulus in *RAG-1*$^{-/-}$ mice compared to control. These differences in odor recognition were further confirmed with the habituation/cross-habituation test where the *RAG-1*$^{-/-}$ mice showed an impaired pattern of habituation and cross-habituation to distinct and yet volatile odors.

As common feature of both tests, *RAG-1*$^{-/-}$ mice showed an overall difficulty in preforming the expected task provided (finding the cookie or being interested in different odor stimulations) and this might be linked to their increased level of anxiety-like behavior that makes them distracted from the task. Consistent with this, other authors have shown an increased locomotor and exploratory activity and degree of anxieties in chemically induced anosmic mice (Kudyakova et al., 2007; Glinka et al., 2012).

Aiming to further understand the cellular mechanism behind the impaired sense of smell of *RAG-1*$^{-/-}$ mice, we investigated whether there were any defects in the cellular structure of the olfactory organs at three key time points: at the embryo stage, soon after birth and at adult age (6–8 weeks). Our results show that *RAG-1*$^{-/-}$ had no differences in the development of the olfactory nerves at embryonic day 14.5, which is the stage of development that follows the establishment of the first

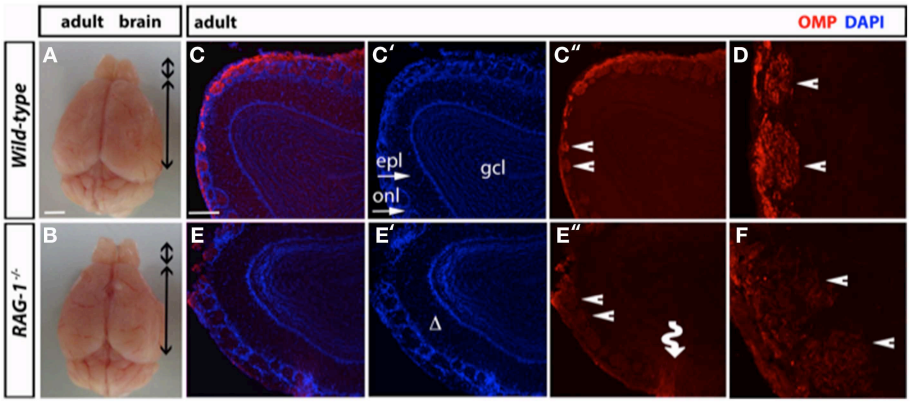


FIGURE 4 | Impaired tissue structure of the olfactory system of adult *RAG-1*$^{-/-}$ mice. (A,B) Brains from male postnatal age (P) 21 *RAG-1*$^{-/-}$ and control C57/BL6 mice were photographed side-by-side to demonstrate no differences in the size and gross morphology of the brain and of the olfactory bulbs (OB). **(C,D)** Coronal sections of the olfactory bulb from the same mice immunostained with an anti-OMP antibody showed a reduced OMP signal in the mutant mice compared to controls. DAPI was used to stain the nuclei. The funny arrow **(E″)** highlights the disorganized structure of the glomerulus in the mutant OB. **(D,F)** are higher magnification of the areas pointed by the arrows in **(C″,E″)**. Pictures are representative of $n = 3$ mice of each genotypes. Scale bars: 1 mm **(A,B)**; 100 μm **(C,E)**.

olfactory sensory link between the olfactory epithelium and the olfactory bulb. Same results were obtained in the expression of key olfactory markers OMP and Tuj1 in the olfactory bulb at day 0 suggesting no involvement of *RAG-1*$^{-/-}$ in the development of the olfactory system at this stage. Indeed, although we did not perform any behavioral test for olfaction in newborn mice, at observational level we did not see any difference in the ability of the newborn *RAG-1*$^{-/-}$ to recognize the nipples of the lactating mother. Nor we observed any difference in the weight of these pups that would suggest an impaired ability to feed themselves because of olfactory defects (data not shown).

Histological analysis of the MOE in adult mice provided us with a completely different scenario featured by a significant reduction in thickness and cellularity of the epithelium and a disorganized architecture of the glomerular tissue of the olfactory bulb in *RAG-1*$^{-/-}$ mice compared to control C57BL/6 mice. These structural differences might explain the increased time needed to track the volatile odor released by the chocolate chip cookie in *RAG-1*$^{-/-}$ mice. The MOE is largely tasked with smelling inherently "neutral odors" (Munger, 2009; Huckins et al., 2013) and its dysfunction can be readily observed in anosmic mice that are known to typically display a significant reduction in the latency to identify an odor stimulus. Most interestingly, the changes across the MOE might provide an explanation for the increased anxiety behavior of *RAG-1*$^{-/-}$ mice that we have previously reported (Rattazzi et al., 2013). Congruently, recent studies have suggested that "*functional activation of the MOE but not the VNO causes elevated levels of anxiety*" (Glinka et al., 2012). The reverse might also be true since studies in humans have also suggested that the induction of a state of anxiety provoke a shift in the perception of a neutral odor (that becomes unpleasant) and to an increase in time needed to detect it (Krusemark et al., 2013).

We did not find any defect in the structure of the VNO (data not shown) and this might also explain the unperturbed response of *RAG-1*$^{-/-}$ mice to social odor. These results are consistent with previous studies by Mcgowan et al. (2011) where the authors described an intact ability of *RAG-1*$^{-/-}$ mice to recognize social odor. The same study, however, differs from our as the authors have found no changes in the recognition of non-social odors by *RAG-1*$^{-/-}$ mice. The differences in the results are most likely due to the different experimental settings they used i.e., high volatile odors (lemon and peppermint instead of almond and banana) and much older mice (3–5 months as opposed to 7–8 week old). In addition to this, in this study the authors showed no difference in anxiety-like behavior in the open field test while previous results from our and other research groups showed significant difference in open field, marble burying and light/dark box. We do not know how to explain these discrepancies except with possible differences in the housing conditions and gut microbiota that could account for difference in behavior.

The current study does not establish if the defects in olfaction of the *RAG-1*$^{-/-}$ are due to the lack of immune system or to an intrinsic role of *RAG-1* gene in the development of the olfactory system. Previous studies have shown that RAG-1 is expressed in the olfactory neurons as well as in other brain regions such as cerebellum and the hippocampal of mice (Chun et al., 1991)—in the olfactory neurons contained in the two placodes located anterior and dorsal to the eyes in zebrafish (Jessen et al., 1999, 2001)—and in a subpopulation of zebrafish olfactory neurons projecting to the lateral olfactory bulb (Feng et al., 2005). Consistent with our findings, the study performed in zebrafish showed that depletion of RAG-1 by morpholino-mediated knockdown or mutation, did not affect axon targeting. If we combine these observations together, it is possible to exclude that RAG-1 plays a key role in OSN development during the embryo stage.

Looking at the adult stage, our results differ from those obtained in zebrafish since in these animals there was no changes of odorant receptor expression or response of OSNs to amino

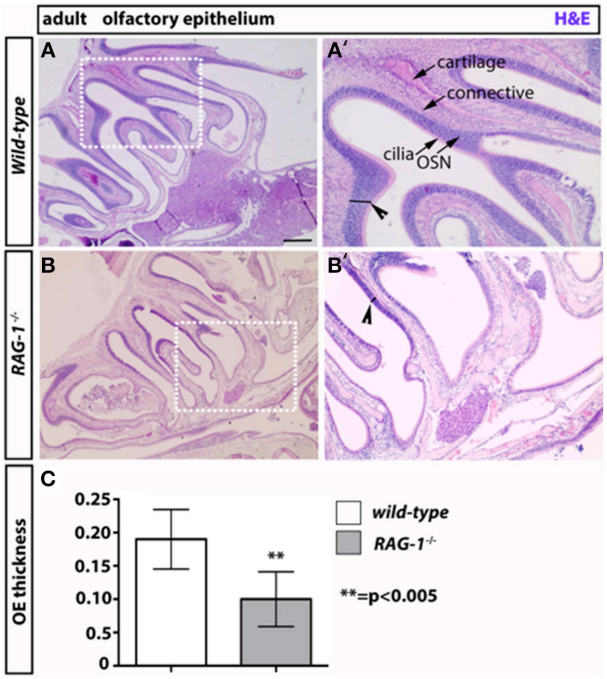


FIGURE 5 | Decreased thickness and cellularity of the main olfactory epithelium of *RAG-1*$^{-/-}$ mice. The pictures in **(A,B)** show the hematoxilin and eosin staining of the MOE of 7 week-old *RAG-1*$^{-/-}$ and control C57/BL6 mice. The pictures in **(A′,B′)** are higher magnification of the boxed areas in **(A,B)** and highlight the differences in thickness (black segments) between the two tissues (arrowheads). The different cellular layers of MOE are indicated with arrows and correspond to: cilia, chemosensory cilia; OSN, olfactory sensory neurons; connective, connective tissue and cartilage. Quantitation if OE thickness is displayed in **(C)**. Pictures are representative of $n = 3$ mice of each genotypes. Scale bars: 150 μm **(A,B).**

acids. We do not know the reason behind this discrepancy and we are tempted to think that T cells might be responsible for the changes in odor perception. The full validation of this hypothesis would require a full new set of investigations addressing a number of specific questions. Nevertheless, we think that there are considerations that can be taken into account in support of this idea.

First of all, our previous study on the emotional behavior of *RAG-1*$^{-/-}$ mice showed that *RAG-1*$^{-/-}$*/OT-II* but not *RAG-1*$^{-/-}$*/OT-I* could "rescue" the gene expression profile and behavior of the former (Rattazzi et al., 2013). This suggested to us that T cells rather than B cells, and CD4^{+} T cells (present in *RAG-1*$^{-/-}$*/OT-II*) rather CD8^{+} T cells (present in *RAG-1*$^{-/-}$*/OT-I* mice), had a significant impact on the observed impaired emotional behavior of *RAG-1*$^{-/-}$ mice. Looking at the changes in olfactory transduction pathway of these very same mice, a similar patter of regulation could be observed i.e., *RAG-1*$^{-/-}$*/OT-II* were similar to wild type supporting the idea that CD4^{+} T cells might rescue the olfactory defect of *RAG-1*$^{-/-}$ mice(Rattazzi et al., 2013).

In terms of the mechanism by which CD4^{+} T cells would control the healthy state of the MOE, we speculate that this might be linked to the impaired development of nasal-associated lymphoid tissue (NALT) (Bienenstock and Mcdermott, 2005; Ruddle and Akirav, 2009). Like all other mucosal-associated lymphoid tissues, these are organized clusters of T and B cells that act as patrolling stations and local reservoir of the immune system in the mucosal districts. Studies on the origin of NALT have shown these structures develop before the other secondary lymphoid organs and start at postneonatal age continuing till weaning (Bienenstock and Mcdermott, 2005; Drayton et al., 2006; Ruddle and Akirav, 2009; Brandtzaeg, 2011). Most interesting, the structural differentiation of the NALT has been proposed to be completed after 6 weeks of age (which is the age of the mice we have used in our tests) and to be influenced by environmental stimuli. Given that the presence of these structures has been shown to be important for the release of factors regulating olfactory epithelium proliferation, differentiation, and maturation (Schwob, 2002), it would be tempting to assume that their absence is one of the main causes of atrophy (reduced thickness and cellularity) of the MOE of the *RAG-1*$^{-/-}$ mice and, as consequence of that, of the impaired organization of the glomeruli in the olfactory bulb.

In conclusion, the results of this study provide first evidence for an impaired olfactory function in adult *RAG-1*$^{-/-}$ mice. Future studies using this animal model might help to identify new therapeutic targets or experimental approaches to investigate possible link between immunodeficiency, anxiety and anosmia.

Author Contributions

LR and RP performed the behavioral experiments and the collection of tissues. LR and AC performed the histological analyses and helped writing and revising the manuscript. FD and YS designed the study, analyzed the data, and wrote the manuscript.

Acknowledgments

We would like to thank Prof. Mauro Perretti for carefully reading this manuscript. AC is supported by funding from Telethon GP13142.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2015.00318

Supplementary Figure 1 | Normal morphology of the gonads of adult *RAG-1*$^{-/-}$ mice. Testes pairs from adult littermate males of the indicated genotypes were photographed side-by-side to show no differences in the size and gross morphology of the gonads of 7 week-old *RAG-1*$^{-/-}$ and control C57/BL6 mice. Pictures are representative of $n = 4$–6 mice of each genotypes. Scale bar: 500 μm.

References

Alberts, J. R., and Galef, B. G. Jr. (1971). Acute anosmia in the rat: a behavioral test of a peripherally-induced olfactory deficit. *Physiol. Behav.* 6, 619–621. doi: 10.1016/0031-9384(71)90218-6

Bienenstock, J., and Mcdermott, M. R. (2005). Bronchus- and nasal-associated lymphoid tissues. *Immunol. Rev.* 206, 22–31. doi: 10.1111/j.0105-2896.2005.00299.x

Brandtzaeg, P. (2011). Immune functions of nasopharyngeal lymphoid tissue. *Adv. Otorhinolaryngol.* 72, 20–24. doi: 10.1159/000324588

Cariboni, A., Davidson, K., Rakic, S., Maggi, R., Parnavelas, J. G., and Ruhrberg, C. (2011). Defective gonadotropin-releasing hormone neuron migration in mice lacking SEMA3A signalling through NRP1 and NRP2: implications for the aetiology of hypogonadotropic hypogonadism. *Hum. Mol. Genet.* 20, 336–344. doi: 10.1093/hmg/ddq468

Cavaco, S., Martins Da Silva, A., Santos, E., Coutinho, E., Marinho, A., Moreira, I., et al. (2012). Are cognitive and olfactory dysfunctions in neuropsychiatric lupus erythematosus dependent on anxiety or depression? *J. Rheumatol.* 39, 770–776. doi: 10.3899/jrheum.110574

Chun, J. J., Schatz, D. G., Oettinger, M. A., Jaenisch, R., and Baltimore, D. (1991). The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system. *Cell* 64, 189–200. doi: 10.1016/0092-8674(91)90220-S

Dawson, P. A., Steane, S. E., and Markovich, D. (2005). Impaired memory and olfactory performance in NaSi-1 sulphate transporter deficient mice. *Behav. Brain Res.* 159, 15–20. doi: 10.1016/j.bbr.2004.09.020

Drayton, D. L., Liao, S., Mounzer, R. H., and Ruddle, N. H. (2006). Lymphoid organ development: from ontogeny to neogenesis. *Nat. Immunol.* 7, 344–353. doi: 10.1038/ni1330

Erb, K., Bohner, G., Harms, L., Goektas, O., Fleiner, F., Dommes, E., et al. (2012). Olfactory function in patients with multiple sclerosis: a diffusion tensor imaging study. *J. Neurol. Sci.* 316, 56–60. doi: 10.1016/j.jns.2012.01.031

Feng, B., Bulchand, S., Yaksi, E., Friedrich, R. W., and Jesuthasan, S. (2005). The recombination activation gene 1 (Rag1) is expressed in a subset of zebrafish olfactory neurons but is not essential for axon targeting or amino acid detection. *BMC Neurosci.* 6:46. doi: 10.1186/1471-2202-6-46

Fleming, S. M., Tetreault, N. A., Mulligan, C. K., Hutson, C. B., Masliah, E., and Chesselet, M. F. (2008). Olfactory deficits in mice overexpressing human wildtype alpha-synuclein. *Eur. J. Neurosci.* 28, 247–256. doi: 10.1111/j.1460-9568.2008.06346.x

Forni, P. E., and Wray, S. (2014). GnRH, anosmia and hypogonadotropic hypogonadism - Where are we? *Front. Neuroendocrinol.* 36, 165-77. doi: 10.1016/j.yfrne.2014.09.004

Garcia-Gonzalez, D., Murcia-Belmonte, V., Clemente, D., and De Castro, F. (2013). Olfactory system and demyelination. *Anat. Rec.* 296, 1424–1434. doi: 10.1002/ar.22736

Glatz, R., and Bailey-Hill, K. (2011). Mimicking nature's noses: from receptor deorphaning to olfactory biosensing. *Prog. Neurobiol.* 93, 270–296. doi: 10.1016/j.pneurobio.2010.11.004

Glinka, M. E., Samuels, B. A., Diodato, A., Teillon, J., Feng Mei, D., Shykind, B. M., et al. (2012). Olfactory deficits cause anxiety-like behaviors in mice. *J. Neurosci.* 32, 6718–6725. doi: 10.1523/JNEUROSCI.4287-11.2012

Graham, C. S., Graham, B. G., Bartlett, J. A., Heald, A. E., and Schiffman, S. S. (1995). Taste and smell losses in HIV infected patients. *Physiol. Behav.* 58, 287–293. doi: 10.1016/0031-9384(95)00049-O

Hoover, K. C. (2010). Smell with inspiration: the evolutionary significance of olfaction. *Am. J. Phys. Anthropol.* 143(Suppl. 51), 63–74. doi: 10.1002/ajpa.21441

Huckins, L. M., Logan, D. W., and Sánchez-Andrade, G. (2013). Olfaction and olfactory-mediated behaviour in psychiatric disease models. *Cell Tissue Res.* 354, 69–80. doi: 10.1007/s00441-013-1617-7

Jessen, J. R., Jessen, T. N., Vogel, S. S., and Lin, S. (2001). Concurrent expression of recombination activating genes 1 and 2 in zebrafish olfactory sensory neurons. *Genesis* 29, 156–162. doi: 10.1002/gene.1019

Jessen, J. R., Willett, C. E., and Lin, S. (1999). Artificial chromosome transgenesis reveals long-distance negative regulation of rag1 in zebrafish. *Nat. Genet.* 23, 15–16. doi: 10.1038/12609

Krusemark, E. A., Novak, L. R., Gitelman, D. R., and Li, W. (2013). When the sense of smell meets emotion: anxiety-state-dependent olfactory processing and neural circuitry adaptation. *J. Neurosci.* 33, 15324–15332. doi: 10.1523/JNEUROSCI.1835-13.2013

Kudyakova, T. I., Sarycheva, N. Y., and Kamenskii, A. A. (2007). Orientation and exploratory behavior and anxiety of CBA mice with anosmia induced by N-trimethylindole (skatole). *Bull. Exp. Biol. Med.* 143, 1–4. doi: 10.1007/s10517-007-0001-6

Leinwand, S. G., and Chalasani, S. H. (2011). Olfactory networks: from sensation to perception. *Curr. Opin. Genet. Dev.* 21, 806–811. doi: 10.1016/j.gde.2011.07.006

Luo, A. H., Cannon, E. H., Wekesa, K. S., Lyman, R. F., Vandenbergh, J. G., and Anholt, R. R. (2002). Impaired olfactory behavior in mice deficient in the alpha subunit of G(o). *Brain Res.* 941, 62–71. doi: 10.1016/S0006-8993(02)02566-0

Lutterotti, A., Vedovello, M., Reindl, M., Ehling, R., Dipauli, F., Kuenz, B., et al. (2011). Olfactory threshold is impaired in early, active multiple sclerosis. *Mult. Scler.* 17, 964–969. doi: 10.1177/1352458511399798

Maione, F., Paschalidis, N., Iqbal, A. J., Crompton, T., Perretti, M., and D'acquisto, F. (2010). Analysis of the inflammatory response in HY-TCR transgenic mice highlights the pathogenic potential of CD4- CD8- T cells. *Autoimmunity* 43, 672–681. doi: 10.3109/08916931003678296

Maione, F., Paschalidis, N., Mascolo, N., Dufton, N., Perretti, M., and D'acquisto, F. (2009). Interleukin 17 sustains rather than induces inflammation. *Biochem. Pharmacol.* 77, 878–887. doi: 10.1016/j.bcp.2008.11.011

Makino, N., Ookawara, S., Katoh, K., Ohta, Y., Ichikawa, M., and Ichimura, K. (2009). The morphological change of supporting cells in the olfactory epithelium after bulbectomy. *Chem. Senses* 34, 171–179. doi: 10.1093/chemse/bjn074

Mcgowan, P. O., Hope, T. A., Meck, W. H., Kelsoe, G., and Williams, C. L. (2011). Impaired social recognition memory in recombination activating gene 1-deficient mice. *Brain Res.* 1383, 187–195. doi: 10.1016/j.brainres.2011.02.054

Midilli, R., Gode, S., Oder, G., Kabasakal, Y., and Karci, B. (2013). Nasal and paranasal involvement in primary Sjogren's syndrome. *Rhinology* 51, 265–267. doi: 10.4193/Rhino13.001

Mombaerts, P. (2004). Genes and ligands for odorant, vomeronasal and taste receptors. *Nat. Rev. Neurosci.* 5, 263–278. doi: 10.1038/nrn1365

Mueller, C., Temmel, A. F., Quint, C., Rieger, A., and Hummel, T. (2002). Olfactory function in HIV-positive subjects. *Acta Otolaryngol.* 122, 67–71. doi: 10.1080/00016480252775760

Munger, S. D. (2009). Olfaction: noses within noses. *Nature* 459, 521–522. doi: 10.1038/459521a

Murthy, V. N. (2011). Olfactory maps in the brain. *Annu. Rev. Neurosci.* 34, 233–258. doi: 10.1146/annurev-neuro-061010-113738

Paschalidis, N., Iqbal, A. J., Maione, F., Wood, E. G., Perretti, M., Flower, R. J., et al. (2009). Modulation of experimental autoimmune encephalomyelitis by endogenous annexin A1. *J. Neuroinflamm.* 6:33. doi: 10.1186/1742-2094-6-33

Perricone, C., Shoenfeld, N., Agmon-Levin, N., De Carolis, C., Perricone, R., and Shoenfeld, Y. (2013). Smell and autoimmunity: a comprehensive review. *Clin. Rev. Allergy. Immunol.* 45, 87–96. doi: 10.1007/s12016-012-8343-x

Rattazzi, L., Piras, G., Ono, M., Deacon, R., Pariante, C. M., and D'acquisto, F. (2013). CD4(+) but not CD8(+) T cells revert the impaired emotional behavior of immunocompromised RAG-1-deficient mice. *Transl. Psychiatry* 3, e280. doi: 10.1038/tp.2013.54

Ruddle, N. H., and Akirav, E. M. (2009). Secondary lymphoid organs: responding to genetic and environmental cues in ontogeny and the immune response. *J. Immunol.* 183, 2205–2212. doi: 10.4049/jimmunol.0804324

Sakano, H. (2010). Neural map formation in the mouse olfactory system. *Neuron* 67, 530–542. doi: 10.1016/j.neuron.2010.07.003

Schwob, J. E. (2002). Neural regeneration and the peripheral olfactory system. *Anat. Rec.* 269, 33–49. doi: 10.1002/ar.10047

Shoenfeld, N., Agmon-Levin, N., Flitman-Katzevman, I., Paran, D., Katz, B. S., Kivity, S., et al. (2009). The sense of smell in systemic lupus erythematosus. *Arthritis Rheum.* 60, 1484–1487. doi: 10.1002/art.24491

Shoenfeld, Y. (2007). To smell autoimmunity: anti-P-ribosomal autoantibodies, depression, and the olfactory system. *J. Autoimmun.* 28, 165–169. doi: 10.1016/j.jaut.2007.02.012

Suzuki, Y., Takeda, M., Obara, N., and Suzuki, N. (1998). Bulbectomy of neonatal mice induces migration of basal cells from the olfactory epithelium. *Brain Res. Dev. Brain Res.* 108, 295–298. doi: 10.1016/S0165-3806(98)00044-3

Takeuchi, H., and Sakano, H. (2014). Neural map formation in the mouse olfactory system. *Cell. Mol. Life Sci.* 71, 3049–3057. doi: 10.1007/s00018-014-1597-0

Vance, D., and Burrage, J. (2006). Chemosensory declines in older adults with HIV: identifying interventions. *J. Gerontol. Nurs.* 32, 42–48. doi: 10.1080/J148v24n02_03

Vance, D. E. (2004). Chemosensory loss in adults aging with HIV. *Percept. Mot. Skills* 99, 1087–1090. doi: 10.2466/pms.99.7.1087-1090

Vance, D. E. (2007). Olfactory and psychomotor symptoms in HIV and aging: potential precursors to cognitive loss. *Med. Sci. Monit.* 13, SC1–SC3.

Wersinger, S. R., Caldwell, H. K., Martinez, L., Gold, P., Hu, S. B., and Young, W. S. III. (2007). Vasopressin 1a receptor knockout mice have a subtle olfactory deficit but normal aggression. *Genes Brain Behav.* 6, 540–551. doi: 10.1111/j.1601-183X.2006.00281.x

Woodley, S. K., and Baum, M. J. (2003). Effects of sex hormones and gender on attraction thresholds for volatile anal scent gland odors in ferrets. *Horm. Behav.* 44, 110–118. doi: 10.1016/S0018-506X(03)00126-0

Wray, S. (2010). From nose to brain: development of gonadotrophin-releasing hormone-1 neurones. *J. Neuroendocrinol.* 22, 743–753. doi: 10.1111/j.1365-2826.2010.02034.x

Wrenn, C. C., Harris, A. P., Saavedra, M. C., and Crawley, J. N. (2003). Social transmission of food preference in mice: methodology and application to galanin-overexpressing transgenic mice. *Behav. Neurosci.* 117, 21–31. doi: 10.1037/0735-7044.117.1.21

Yang, M., and Crawley, J. N. (2009). Simple behavioral assessment of mouse olfaction. *Curr. Protoc. Neurosci.* 48, 8.24.1–8.24.12. doi: 10.1002/0471142301.ns0824s48

Zou, D. J., Chesler, A., and Firestein, S. (2009). How the olfactory bulb got its glomeruli: a just so story? *Nat. Rev. Neurosci.* 10, 611–618. doi: 10.1038/nrn2666

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on