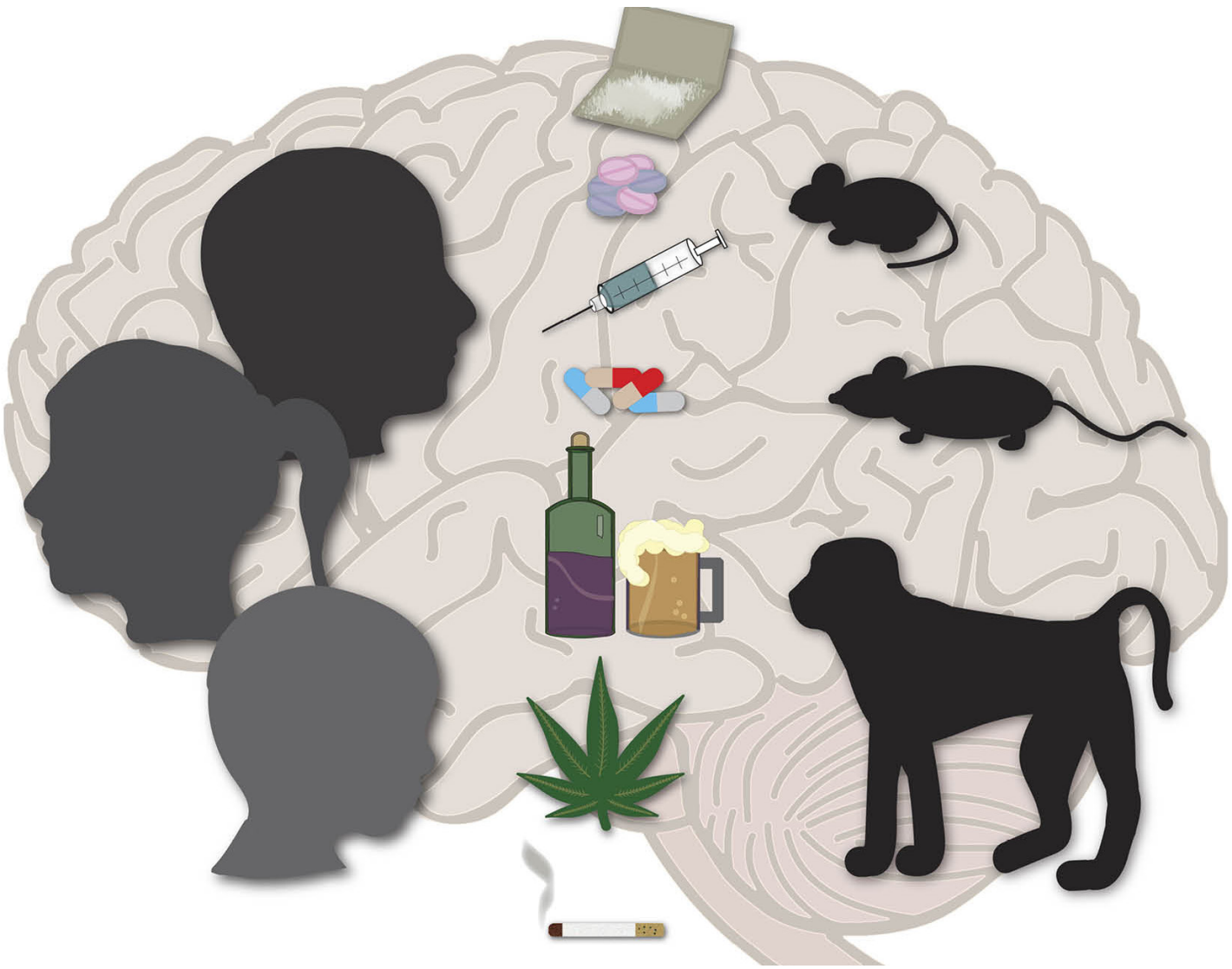
LONG-TERM CONSEQUENCES OF ADOLESCENT DRUG USE: EVIDENCE FROM PRE-CLINICAL AND CLINICAL MODELS

EDITED BY: Mary M. Torregrossa, Jacqueline M. Barker and Shannon L. Gourley
PUBLISHED IN: Frontiers in Behavioral Neuroscience, Frontiers in Pharmacology, Frontiers in Neuroscience and Frontiers in Psychiatry



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-530-0
DOI 10.3389/978-2-88945-530-0

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

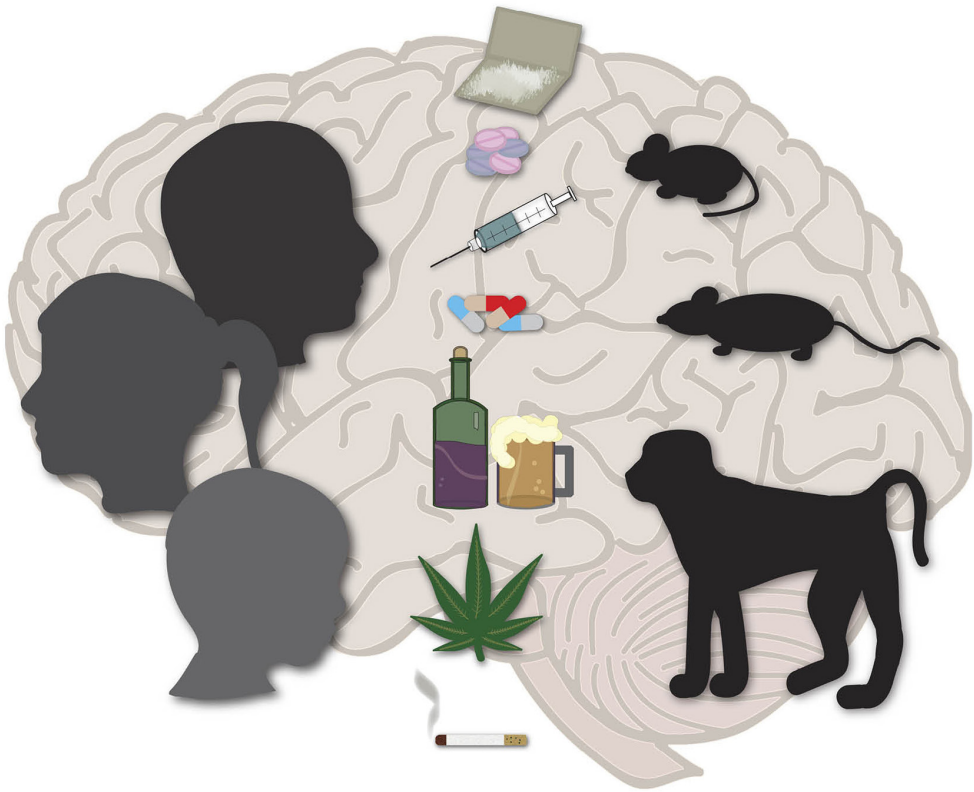
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - 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

LONG-TERM CONSEQUENCES OF ADOLESCENT DRUG USE: EVIDENCE FROM PRE-CLINICAL AND CLINICAL MODELS

Topic Editors:
Mary M. Torregrossa, University of Pittsburgh, United States
Jacqueline M. Barker, Drexel University, United States
Shannon L. Gourley, Emory University, United States



Long-Term Consequences of Adolescent Drug Use: Evidence from Pre-Clinical and Clinical Models. Image: A.G. Allen.

The purpose of this collection is to provide a forum to integrate pre-clinical and clinical investigations regarding the long-term consequences of adolescent exposure to drugs of abuse. Adolescence is characterized by numerous behavioral and biological changes, including substantial neurodevelopment. Behaviorally, adolescents are more likely to engage in risky activities and make impulsive decisions. As such, the majority of substance use begins in adolescence, and an earlier age of onset of use (<15 yr) is strongly associated with the risk for developing a substance use disorder later in life. Furthermore, adolescent drug use may negatively impact ongoing neurological development, which could lead to long-term cognitive and emotional deficits. A large number of clinical studies have investigated both the acute and long-term effects of adolescent drug use on functional outcomes. However,

the clinical literature contains many conflicting findings, and is often hampered by the inability to know if functional differences existed prior to drug use. Moreover, in human populations it is often very difficult to control for the numerous types of drugs, doses, and combinations used, not to mention the many other environmental factors that may influence adult behavior. Therefore, an increase in the number of carefully controlled studies using relevant animal models has the potential to clarify which adolescent experiences, particularly what drugs used when, have long-term negative consequences.

Despite the advantages of animal model systems in clarifying these issues, the majority of pre-clinical addiction research over the past 50+ years has been conducted in adult animals. Moreover, few addiction-related studies have investigated the long-term neurocognitive consequences of drug exposure at any age. In the past 10 years of so, however, the field of adolescent drug abuse research has burgeoned. To date, the majority of this research has focused on adolescent alcohol exposure using a variety of animal models. The results have given the field important insight into why adolescents are more likely to drink alcohol to excess relative to adults, and the danger of adolescent alcohol use (e.g., in leading to a persistence of excessive drinking in adulthood). More recently, research regarding the effects of adolescent exposure to other drugs of abuse, including nicotine, cocaine, and cannabinoids has expanded.

Therefore, we are at unique point in time, when emerging results from carefully controlled pre-clinical studies can inform the sometimes confusing clinical literature. In addition, we expect an influx of prospective clinical studies in response to a cross-institute initiative at NIH, known as the ABCD grant. Several institutes are enrolling children prior to adolescence (and the initiation of drug use), in order to control for pre-existing neurobiological and neurobehavioral differences and to monitor the age of initiation and amount of drug used more carefully than is possible using retrospective designs.

Citation: Torregrossa, M. M., Barker, J. M., Gourley, S. L., eds. (2018). Long-Term Consequences of Adolescent Drug Use: Evidence from Pre-Clinical and Clinical Models. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-530-0

Table of Contents

frontiers in Behavioral Neuroscience

EDITORIAL
published: 03 May 2018
doi: 10.3389/fnbeh.2018.00083

Editorial: Long-Term Consequences of Adolescent Drug Use: Evidence From Pre-clinical and Clinical Models

Alonzo J. Whyte[1], *Mary M. Torregrossa*[2]*, *Jacqueline M. Barker*[3] and *Shannon L. Gourley*[1,4]

[1] Departments of Pediatrics and Psychiatry, Yerkes National Primate Research Center, Emory University, Atlanta, GA, United States, [2] Department of Psychiatry, Translational Neuroscience Program, University of Pittsburgh, Pittsburgh, PA, United States, [3] Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, PA, United States, [4] Graduate Programs in Neuroscience and Molecular and Systems Pharmacology, Emory University, Atlanta, GA, United States

Keywords: addiction, adolescence, prefrontal, decision making, impulsivity

Editorial on the Research Topic

Long-Term Consequences of Adolescent Drug Use: Evidence From Pre-clinical and Clinical Models

Adolescence is characterized by risky behavior and impulsive decision making, which often exposes adolescents to psychoactive substances. Meanwhile, neural circuits refine and mature during adolescence, creating a window of opportunity for environmental insults to impact brain maturation and potentially confer susceptibility to life-long drug addiction. Indeed, an early age of first drug use (before 15 years old) is strongly associated with risk for developing a substance use disorder later in life (Kessler et al., 2001). A large number of clinical studies have investigated both the acute and long-term effects of adolescent substance use on a number of neurobehavioral measures, resulting in a clinical research literature that can at times be conflicting, likely due to difficulties in controlling for environmental factors—including which drugs were ingested, when, and in what combinations. While animal models allow for control over all of these factors, the majority of preclinical addiction research has historically used adult animals and thus failed to address the effects of drug and alcohol exposure during adolescence. We recently organized a Special Issue of *Frontiers in Neuroscience*, meant to collect both clinical and preclinical investigations focused on factors associated with illicit drug vulnerability in adolescents, and the long-term consequences of adolescent drug exposure. This Editorial provides an overview of our Special Issue, organized in 3 themes.

THEME 1: RISKS PREDISPOSING ADOLESCENTS TO SUBSTANCE USE

Although many adolescents engage in risky, experimental substance use without developing later-life addiction, certain non-drug-related, functional differences appear to predispose adolescents to increased risk for addiction. Clark et al. studied a diverse and representative sample of adolescents in the National Consortium on Alcohol and Neurodevelopment in Adolescence (NCANDA) and report that poor daily executive functioning (higher-order cognitive skills such as mental flexibility that optimize responding to maximize reward) significantly relates to increased substance use risk during adolescence. Tervo-Clemmens et al. focused further on the executive function characteristics in adolescents that increase risk for substance use. In a sample of youths from the

OPEN ACCESS

Edited and reviewed by:
Nuno Sousa,
Instituto de Pesquisa em Ciências da Vida e da Saúde (ICVS), Portugal

***Correspondence:**
Mary M. Torregrossa
torregrossam@upmc.edu

Received: *06 April 2018*
Accepted: *16 April 2018*
Published: *03 May 2018*

Citation:
Whyte AJ, Torregrossa MM, Barker JM and Gourley SL (2018) Editorial: Long-Term Consequences of Adolescent Drug Use: Evidence From Pre-clinical and Clinical Models. Front. Behav. Neurosci. 12:83. doi: 10.3389/fnbeh.2018.00083

NCANDA study, Tervo-Clemmens et al. replicated previous findings indicating that poor response inhibition is associated with substance use risk. The nature of this risk was further elucidated using a reward-anti-saccade task in which participants are primed with visual cues during a fixation period. The visual cue provides information as to whether correct performance on the subsequent response trial will be rewarded (monetarily) or not incentivized. During response trials, participants must perform a saccade away from the presented visual stimulus. Although incentivizing accurate responding improved performance in the anti-saccade task, incentives alone were insufficient to modify associations between response inhibition and substance use risk, indicating that deficits in response inhibition and heightened sensitivity to reward are distinct neurocognitive features of adolescent substance use risk.

In addition to the general risk that poor daily executive function confers, early-life events and sex may also impact long-term risk for substance abuse. In their review article, Weil and Karelina summarize clinical and preclinical evidence suggesting that traumatic brain injury during adolescence increases risk for alcohol use disorder later in life. For males, this pattern is particularly pertinent, as young men are more likely to suffer from abuse-related head injury, traumatic brain injury, and also alcohol abuse. In preclinical studies, Barfield and Gourley sought to uncover sex-specific behavioral consequences of experiencing elevated levels of the primary stress hormone corticosterone during adolescence. Using an instrumental reversal learning task, Barfield reports that female, but not male, mice exhibit inflexible, perseverative-like response errors following early-adolescent corticosterone. These findings are significant because pre-existing deficits in behavioral flexibility may accelerate the transition from recreational substance use to addiction (Dalley et al., 2004; Perry and Carrol, 2008; Cervantes et al., 2013). Further, longer periods of corticosterone exposure in adolescence impair reversal performance in male mice in the same task (Shapiro et al., 2017). Related findings from Barker et al. indicate that high dose binge-like ethanol exposure during adolescence may selectively promote habitual behavior in female rats, with adolescent male rats showing no alterations in response strategy selection in adulthood. Together, these preclinical reports suggest that adolescent females are more vulnerable to developing drug- and stressor-related errors in updating action-outcome associations, which may confer vulnerability to drug use and misuse.

THEME 2: EFFECTS OF ADOLESCENT DRUG EXPOSURE ON BRAIN PHYSIOLOGY AND LONG-TERM BEHAVIOR

Across multiple mammalian species, exposure to drugs of abuse during adolescence is associated with deficits in neurodevelopment. Using data from the longitudinal Brain and Alcohol Research in College Students (BARCS) study, Meda et al. report that, compared to the typical decline in gray matter volume associated with adolescent neurodevelopment, young adults who engage in heavy drinking display greater volume loss in regions such as the frontal cortex and hippocampus. In this investigation, participants initially underwent MRI brain scans and self-reported their drinking behavior during young adulthood (average age 18 years old) and were then sampled again 2 years later. Interestingly, Meda et al. also show that many of the participants who self-reported heavy drinking at the beginning of the study already had smaller frontal cortices, potentially suggestive of a pre-disposition toward substance abuse. Also affected by adolescent substance exposure are the white matter tracts that connect gray matter regions. Li et al. find that adolescent cynomolgus monkeys exposed repeatedly to ketamine have reduced white matter integrity in connections between brain regions involved in executive functions and learning and memory such as the frontal cortex, striatum, thalamus, and hippocampus. Neural changes are also detectable at the molecular level: Liu and Crews report that rats exposed to binge-like ethanol treatment during adolescence exhibit less neurogenesis within the dentate gyrus region of the hippocampus, and also the subventricular zone. Further, Vore et al. report that rats exposed to binge-like ethanol treatment during adolescence have reduced cytokine expression in response to stress and immune challenge.

Damage to specific neurocircuits by adolescent drug exposure is also associated with long-term effects on learning and memory and executive functions. Sanchez Marin et al. report that adolescent rats exposed to binge-like ethanol treatment not only have aberrant mRNA levels of genes encoding enzymes and receptors in the endocannabinoid system and neuroinflammation-related factors within the frontal cortex, and higher mRNA levels of stress-related genes within the hippocampus, but in addition, develop deficits in the novel object recognition test of learning and memory. Similarly, Marco et al. find that binge-like ethanol exposure not only reduces cannabinoid receptor type 2 protein levels in the frontal cortex, but also causes significant deficits in novel object recognition. Last, Kruse et al. show that, in rats, deficits in risk-based decision making (overly risky behavior), resultant from adolescent ethanol self-administration, are not caused by impulsivity but rather are due to heightened sensitivity to reward-related conditioning stimuli.

THEME 3: PRECLINICAL STUDIES COULD HELP TO CLARIFY CAUSE-AND-EFFECT IN RISK

The nature of clinical studies often precludes direct cause-and-effect analyses of mechanisms underlying substance use risk. Several studies within this collection use preclinical models to tease out these distinctions, and they report important null and unexpected effects of adolescent drug exposure. For example, in female rats, nicotine exposure during adolescence unexpectedly fails to impair behavioral flexibility or energize ethanol self-administration and reinstatement in adulthood (Madayag et al.). Nor does adolescent ethanol exposure energize later-life self-administration in male rats (Carvajal et al.).

In several psychosis-related behavioral tasks, Jaehne et al. report that, despite the known actions of methamphetamine on serotonergic neurotransmission, mice lacking the 5-HT1A receptor and exposed to methamphetamine during adolescence are indistinguishable from methamphetamine-exposed wildtype control mice. Finally, Kirschmann et al. present findings that challenge the popular belief that adolescent exposure to marijuana causes long-term deficits in executive functions. Using an operant conditioning procedure that allows adolescent rats to self-administer cannabinoids, Krishmann et al. report that adolescent cannabinoid self-administration in female rats has no detectable effects on spatial or working memory performance. Further, experimenter-administered cannabinoids actually *improved* spatial and working memory performance in adulthood.

SUMMARY

Together, these studies provide insight into factors that may alter adolescent brain development and increase, decrease, or have no effect on subsequent risk for addiction in adulthood. Overall, these articles highlight the necessity of clinical studies focused on the real-world risks and consequences of adolescent substance use and abuse, combined with more controlled (and controllable) preclinical studies aimed at elucidating mechanistic factors by which adolescent drug exposure impacts neurocognitive functions, neural structure, and neuronal plasticity during development and into adulthood.

AUTHOR CONTRIBUTIONS

AW wrote the first draft of the manuscript. All other authors edited the manuscript and contributed to the final version of the manuscript. All authors approved the manuscript for publication.

ACKNOWLEDGMENTS

AW is supported by NIH 5K12GM000680-18; MT is supported by NIH DA042029, DA041563, AA025547; JB is supported by NIH AA024499; SG is supported by NIH DA042358 and DA044297. The Yerkes National Primate Research Center is funded in part by NIH ORIP/OD011132.

REFERENCES

Cervantes, M. C., Laughlin, R. E., and Jentsch, J. D. (2013). Cocaine self-administration behavior in inbred mouse lines segregating different capacities for inhibitory control. *Psychopharmacology* 229, 515–525. doi: 10.1007/s00213-013-3135-4

Dalley, J. W., Cardinal, R. N., and Robbins, T. W. (2004). Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. *Neurosci. Biobehav. Rev.* 28, 771–784. doi: 10.1016/j.neubiorev.2004.09.006

Kessler, R. C., Aguilar-Gaxiola, S., Berglund, P. A., Caraveo-Anduaga, J. J., DeWit, D. J., Greenfield, S. F., et al. (2001). Patterns and predictors of treatment seeking after onset of a substance use disorder. *Arch. Gen. Psychiatry* 58, 1065–1071. doi: 10.1001/archpsyc.58.11.1065

Perry, J. L., and Carrol, M. E. (2008). The role of impulsive behavior in drug abuse. *Psychopharmacology* 200, 1–26. doi: 10.1007/s00213-008-1173-0

Shapiro, L. P., Omar, M. H., Koleske, A. J., and Gourley, S. L. (2017). Corticosteroid-induced dendrite loss and behavioral deficiencies can be blocked by activation of Abl2/Arg kinase. *Mol. Cell Neurosci.* 85, 226–234. doi: 10.1016/j.mcn.2017.10.007

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: 13 November 2017
doi: 10.3389/fnbeh.2017.00223

Adolescent Executive Dysfunction in Daily Life: Relationships to Risks, Brain Structure and Substance Use

Duncan B. Clark[1], Tammy Chung[1], Christopher S. Martin[1], Brant P. Hasler[1], Douglas H. Fitzgerald[1], Beatriz Luna[1], Sandra A. Brown[2], Susan F. Tapert[2], Ty Brumback[2], Kevin Cummins[2], Adolf Pfefferbaum[3,4], Edith V. Sullivan[4], Kilian M. Pohl[3], Ian M. Colrain[3], Fiona C. Baker[3], Michael D. De Bellis[5], Kate B. Nooner[6] and Bonnie J. Nagel[7]*

[1] Department of Psychiatry, University of Pittsburgh, Pittsburgh, PA, United States, [2] Department of Psychology and Psychiatry, University of California, San Diego, La Jolla, CA, United States, [3] Center for Health Sciences, SRI International, Menlo Park, CA, United States, [4] Department of Psychiatry and Behavioral Sciences, School of Medicine, Stanford University, Palo Alto, CA, United States, [5] Department of Psychiatry, Duke University, Durham, NC, United States, [6] Department of Psychology, University of North Carolina Wilmington, Wilmington, NC, United States, [7] Department of Psychiatry, Oregon Health and Science University, Portland, OR, United States

During adolescence, problems reflecting cognitive, behavioral and affective dysregulation, such as inattention and emotional dyscontrol, have been observed to be associated with substance use disorder (SUD) risks and outcomes. Prior studies have typically been with small samples, and have typically not included comprehensive measurement of executive dysfunction domains. The relationships of executive dysfunction in daily life with performance based testing of cognitive skills and structural brain characteristics, thought to be the basis for executive functioning, have not been definitively determined. The aims of this study were to determine the relationships between executive dysfunction in daily life, measured by the Behavior Rating Inventory of Executive Function (BRIEF), cognitive skills and structural brain characteristics, and SUD risks, including a global SUD risk indicator, sleep quality, and risky alcohol and cannabis use. In addition to bivariate relationships, multivariate models were tested. The subjects ($n = 817$; ages 12 through 21) were participants in the National Consortium on Alcohol and Neurodevelopment in Adolescence (NCANDA) study. The results indicated that executive dysfunction was significantly related to SUD risks, poor sleep quality, risky alcohol use and cannabis use, and was not significantly related to cognitive skills or structural brain characteristics. In multivariate models, the relationship between poor sleep quality and risky substance use was mediated by executive dysfunction. While these cross-sectional relationships need to be further examined in longitudinal analyses, the results suggest that poor sleep quality and executive dysfunction may be viable preventive intervention targets to reduce adolescent substance use.

Keywords: adolescents, executive functioning, neurocognitive testing, neuroimaging, alcohol

OPEN ACCESS

Edited by:
Shannon Leigh Gourley, Emory University School of Medicine, United States

Reviewed by:
Frauke Nees, Zentralinstitut für Seelische Gesundheit, Germany
Christos Frantzidis, Aristotle University of Thessaloniki, Greece

****Correspondence:***
Duncan B. Clark
clarkdb@upmc.edu

Received: *27 April 2017*
Accepted: *24 October 2017*
Published: *13 November 2017*

Citation:
Clark DB, Chung T, Martin CS, Hasler BP, Fitzgerald DH, Luna B, Brown SA, Tapert SF, Brumback T, Cummins K, Pfefferbaum A, Sullivan EV, Pohl KM, Colrain IM, Baker FC, De Bellis MD, Nooner KB and Nagel BJ (2017) Adolescent Executive Dysfunction in Daily Life: Relationships to Risks, Brain Structure and Substance Use. Front. Behav. Neurosci. 11:223. doi: 10.3389/fnbeh.2017.00223

INTRODUCTION

Executive functioning is a broad construct comprised of behavioral competencies and cognitive skills, including attention, inhibition, mental flexibility, working memory, self-monitoring, planning, and emotional control (Chan et al., 2008). These self-control capabilities and higher order cognitive skills support the optimization of responding to environmental and personal

challenges so as to maximize reward opportunities and achieve long-term goals (Best and Miller, 2010; Diamond and Lee, 2011). Problems in executive functioning in daily life (i.e., executive dysfunction) and cognitive skills assessed by performance-based testing may, in fact, reflect different constructs.

Characteristics reflecting executive dysfunction has been found to be associated with substance use disorder (SUD) risks and outcomes (Clark and Winters, 2002; Tarter et al., 2003; Clark et al., 2008). Pertinent indicators include dysregulation in cognitive (e.g., attention deficits), behavioral (e.g., impulsivity), and affective (e.g., affective lability) domains (Clark and Winters, 2002; Vanyukov et al., 2009; Clark et al., 2012). Most studies on these relationships have not explicitly and comprehensively measured executive dysfunction dimensions, however, leaving remaining questions on the extent to which SUD risks and outcomes may relate to specific dimensions or global executive dysfunction indicators.

Several studies have examined executive dysfunction in relationship to SUD risks and outcomes using a comprehensive multidimensional executive dysfunction measure, the Behavior Rating Inventory of Executive Function (BRIEF-SR: Gioia et al., 2002a,b; Roth et al., 2015). Expressly developed to measure eight executive dysfunction dimensions, the BRIEF has been demonstrated to be a valid and practical tool in school and clinical settings as well as in research (Egeland and Fallmyr, 2010; Roth et al., 2015). The BRIEF has been utilized in adolescent research to study executive dysfunction in relationship to SUD risks and outcomes, including disruptive behavior disorders (Wang et al., 2012; Long et al., 2015), sleep problems (Anderson et al., 2009; Caruso et al., 2014), academic achievement (Langberg et al., 2013; Samuels et al., 2016), performance-based cognitive tests (Shimoni et al., 2012; Long et al., 2015), brain structure and function (Clark et al., 2012; Ziegler et al., 2013; Zhai et al., 2015), and substance use (Clark et al., 2012). While comprehensively measuring executive dysfunction dimensions, most of these prior studies have had small sample sizes. Compared with prior studies, the present study includes a broader array of assessment domains with a considerably larger sample.

Among adolescents, poor sleep has been found to be associated with difficulties that may reflect executive dysfunction, such as inattention and depression (Millman, 2005). Limited research has specifically addressed the relationship between sleep quality and expressly measured executive dysfunction among adolescents. In 236 healthy adolescents (age 13–16 years: Anderson et al., 2009), increased sleepiness was significantly associated with more problems on dimensions including Working Memory, Task Planning, Orderliness, and Task Completion (i.e., BRIEF Metacognition) and not associated with Inhibitory Control, Flexibility, Emotional Control and Monitoring (i.e., BRIEF Behavioral Regulation). Sleep problems and circadian misalignment have been found to predict adolescent alcohol and other substance use (Hasler and Clark, 2013; Hasler et al., 2014, 2016, 2017). A study with a larger sample is needed to determine whether the relationships observed between executive dysfunction and poor sleep (Anderson et al., 2009) can be confirmed. Since poor sleep may influence executive dysfunction and executive dysfunction may, in turn, influence substance use, an examination of the relationships among these characteristics may suggest mechanisms, preventive intervention targets, and directions for future research.

The construct of executive functioning refers to behaviors in daily life as well as specific cognitive skills. A hypothesis about executive functioning in daily life is that variations in specific cognitive skills reflect the capabilities needed for effective functioning (Willcutt et al., 2005). To the extent that variations in objectively assessed cognitive skills correlate with executive dysfunction, such testing may be useful in depicting a mechanism for functional deficits. Relevant constructs assessed by performance-based cognitive testing include attention (e.g., Continuous Performance Test: Kurtz et al., 2001) and working memory (e.g., N-Back Test: Ragland et al., 2002). Several studies with small adolescent samples have noted, however, that performance-based cognitive tests and executive dysfunction ratings have little or no correspondence (Mahone et al., 2009; Cyders and Coskunpinar, 2012; Boschloo et al., 2014; Long et al., 2015; see Toplak et al., 2013 for review), with some exceptions (e.g., BRIEF and Working Memory by cognitive testing: Faridi et al., 2015). These studies suggest that cognitive testing of these constructs may not be particularly informative in understanding executive dysfunction in daily life. The present study examined these relationships in a large sample.

Executive dysfunction may reflect delays or deficits in neuromaturation. To the extent that variations in structural brain characteristics are found to correlate with executive dysfunction, such findings would support a hypothesis that specific observable brain characteristics reflect the neurobiological foundation for executive functioning. Prefrontal cortex (PFC) development has been hypothesized to be the neurobiological foundation for maturing executive functioning during adolescence, with the interaction of PFC with other functionally specialized brain regions critical for integrative executive functions (Spear, 2000). During adolescence, the maturation of executive cognitive skills occurs in parallel with increasing organization or integrity of white matter tracts projecting to PFC (Klingberg et al., 1999; Chambers et al., 2003; Takahashi et al., 2004; Lenroot and Giedd, 2006; Ashtari et al., 2007; Paus, 2010). The frontoparietal network (Fassbender et al., 2006), including tracts such as the superior longitudinal fasciculus (SLF) that connect the frontal and parietal cortex. The delay or disruption in the maturation of white matter integrity has been implicated as a neurobiological substrate for executive dysfunction (Lipton et al., 2009; Skranes et al., 2009; Clark et al., 2012). A few studies with adolescents have examined executive dysfunction, measured by BRIEF, in relationship to structural brain characteristics. Among 35 adolescents with SUD and 20 controls, worse executive dysfunction was significantly correlated with less PFC and parietal white matter integrity by diffusion tensor imaging fractional anisotropy (FA: Clark et al., 2012). Among 35 children and adolescents (Mahone et al., 2009), problems indicated on the BRIEF Working Memory scale were significantly correlated with smaller frontal gray matter volume, but not with temporal, parietal or occipital gray matter volume. In the NIH MRI study (Faridi et al., 2015), BRIEF Inhibitory Control was not significantly correlated with cortical gray thickness, while BRIEF Working Memory and Emotional

Control showed greater cortical thickness associated with fewer problems only in the parahippocampal gyri. These studies do not clearly establish consistent relationships between adolescent executive dysfunction and structural brain characteristics, and the present larger study will clarifiy these relationships.

To the extent that executive dysfunction is found to correlate with the early development of risky substance use patterns, such findings would support the hypothesis that deficits in self-control reflected in executive dysfunction may contribute to SUD. In a few adolescent samples, executive dysfunction, indicated by BRIEF, have been found to be associated with substance involvement. BRIEF Global Composite (GEC) has been found to be significantly different among adolescents with SUD ($n = 35$) and control adolescents ($n = 20$), with 29% of the SUD group showing scores considered to indicate clinical problems (i.e., $\geq$70; Clark et al., 2012). In this sample, BRIEF GEC mediated the relationship between disruption of frontoparietal white matter integrity and cannabis symptoms. More everyday executive functioning problems indicated by BRIEF scores have also been observed in young adults with substance use (Hadjiefthyvoulou et al., 2012). More extensive study of these relationships is needed, particularly the examination of the relationship between executive dysfunction and the adolescent onset of risky substance use patterns.

This study examined these relationships at the initial assessment among subjects in National Consortium on Alcohol and Neurodevelopment in Adolescents (NCANDA), providing the opportunity to examine executive dysfunction in a relatively large, representative sample with a broad array of hypothetically related psychosocial, neurocognitive and brain measures. The aims of this study were to determine the relationships between executive dysfunction in daily life, measured by the Behavior Rating Inventory of Executive Function (BRIEF), cognitive skills and structural brain characteristics, and SUD risks, including a global SUD risk indicator, sleep quality, and risky alcohol and cannabis use. In addition to bivariate relationships, multivariate models were tested.

METHODS

Participants

NCANDA participants were 831 youth ranging in age from 12 to 21 years. NCANDA examines adolescent neurodevelopmental risks for and outcomes of alcohol use in a large, multisite, accelerated longitudinal design (see Brown et al., 2015 for additional details). NCANDA subjects were recruited from five sites: Duke University, University of Pittsburgh, Oregon Health and Science University, University of California, San Diego, and SRI International. For this analysis, 10 subjects were missing BRIEF, and 4 were excluded for invalid results (see below). The demographic characteristics were as follows: age: mean: 16.2 years, s.d. 2.5, range: 12.0–21.9; female: $n = 417$, male: $n = 400$; race: white: $n = 586$ (71.7%), African American: 95 (11.6%), Asian: $n = 62$ (7.6%); Native American: $n = 3$ (<1%); Pacific Islander: $n = 4$ (<1%); multiple: $n = 67$ (8.2%); ethnicity: Hispanic: $n = 96$ (11.8%), non-Hispanic: $n = 721$ (88.2%). These race and ethnicity proportions are similar to the U.S. population (Brown et al., 2015). Most subjects ($n = 692$, 83%) had little or no alcohol use history, and a subsample ($n = 139$, 17%) had a history of risky alcohol use based on exceeding established alcohol use thresholds (National Institute on Alcohol Abuse and Alcoholism, 2011). Youth at increased risk for problematic alcohol use, based on one or more risk factors including early alcohol use, family SUD history, disruptive behavior disorder symptoms, or two or more anxiety or depression symptoms, comprised approximately 50% of the sample. The institutional review board at each site approved the study. Adult participants consented to participate, and minors provided assent with parental or legal guardian consent.

Design

NCANDA uses an accelerated longitudinal design (Duncan et al., 1996, 2006), sampling subjects from a broad age span with subsequent follow-up assessments to characterize development across an expansive period. At the initial assessment, youth completed a comprehensive assessment of substance use, psychiatric symptoms and diagnoses, personality factors, and functioning in major life domains (Brown et al., 2015); a neurocognitive battery (Sullivan et al., 2016), and a neuroimaging assessment with structural and diffusion tensor imaging (Pfefferbaum et al., 2016; Pohl et al., 2016). One parent of each youth also completed an assessment. These analyses utilized the initial assessment data.

Measures

BRIEF

Behavior Rating Inventory of Executive Function-Self-Report Version (BRIEF-SR: Baron, 2000; Gioia et al., 2002a,b; Guy et al., 2004; Roth et al., 2015): The BRIEF-SR is an 80-item adolescent self-report behavior rating scale of purposeful, goal-directed, problem-solving behavior. For each item, the subject is asked: "Over the past 6 months, how often has each of the following behaviors been a problem?" The response choices are "Often" (3 points), "Sometimes" (2 points) and "Never" (1 point). The instrument yields a summary score, the Global Executive Composite (GEC), two composite indices, and eight scales: (1) The ***Inhibitory Control*** scale (13 items) assesses inhibitory control and impulsivity (e.g., *"I have problems waiting my turn," "I don't think of consequences before acting," "I get out of control more than my friends"*). (2) The ***Flexibility*** scale (10 items), including behavioral and cognitive characteristics, assesses the ability to make transitions and flexibly solve problems (e.g., *"I have trouble changing from one activity to another"*). (3) The ***Emotional Control*** scale (10 items) assesses the ability to modulate emotional responses in response to situational demands (e.g., *"I overreact to small problems," "I get upset easily"*). (4) The ***Monitoring*** scale (5 items) assesses self-awareness of interpersonal strengths and weaknesses (e.g., *"I don't know when my actions bother others"*). (5) The ***Working Memory*** scale (12 items) assesses holding information in mind for the purpose of completing a task (e.g., *"I forget instructions easily," "I have trouble with jobs or tasks that have more than one step"*). (6) The Task ***Planning*** scale (Plan: 13 items) assesses planning steps to complete a task and the anticipation of future consequences (e.g.,

"I don't plan ahead for school assignments," "I don't think ahead about possible problems" "I have trouble carrying out the things that are needed to reach a goal, such as saving money for special needs, studying to get good grades, etc."). (7) The ***Organization*** scale (Organize: 7 items) assesses the ability to keep work and school materials organized (e.g., *"My backpack/schoolbag is disorganized"*). (8) The ***Task Completion*** assesses the ability to complete school or work in a timely fashion by 10 items (e.g., *"I have difficulty finishing a task on my own"*). The ***Behavioral Regulation Index (BRI)*** combines Inhibit, Shift, Emotional Control, and Monitor scales (internal consistency: 0.96). The ***Metacognition Index (MCI)*** is comprised of Initiate, Working Memory, Plan and Organize, Task Monitor, and Organization of Materials subscales (internal consistency: 0.72). In addition, validity scales include the ***Inconsistency*** scale, which determines whether the subject responded in a consistent manner by comparing 10 pairs of similar items, and the ***Negativity*** scale (10 items), which determines whether the subject responds to selected items in an unusually negative manner. Raw scores are converted to age indexed t-scores, with higher scores indicating more problems. Subjects with missing ($n = 10$) or invalid ($n = 4$) BRIEF scores were excluded from all analyses.

Risk Factors for Alcohol Use Disorder (AUD)

The NCANDA sample (Brown et al., 2015) was configured to include approximately 50% of the sample at higher risk for problematic alcohol use based on screening evidence for one of the following: (1) early alcohol use (i.e., first standard drink before age 15 years old); (2) a family history of alcohol or other substance problems; (3) endorsement of one or more conduct disorder or antisocial personality disorder symptoms or t score ≥ 60 on the externalizing score of the Achenbach system of Empirically Based Assessments (ASEBA: Achenbach and Rescorla, 2001a,b); (4) endorsement of two or more internalizing symptoms or $t \geq 60$ on ASEBA internalizing. ***Risk Density*** was calculated as the sum of the presence (1) or absence (0) of each of these characteristics (range: 0–4).

Subjective Sleep Quality

The Pittsburgh Sleep Quality Index (PSQI: Buysse et al., 1989) has been shown to be comprised of two factors, sleep efficiency and perceived sleep quality (Mollayeva et al., 2016). The PSQI has been validated in adolescent samples (de la Vega et al., 2015) and has been commonly used in adolescent research (e.g., Noone et al., 2014). The perceived sleep quality factor includes a subjective sleep quality item which correlates highly with the factor score and loads more strongly on this factor than do other items (Mollayeva et al., 2016). The subjective sleep quality item asks "During the past month, how would you rate your sleep quality overall?" with response options "very good," "fairly good," "fairly bad," and "very bad."

Performance Based Cognitive Tests

The NCANDA performance based cognitive testing protocol was designed to assess functional domains relevant for alcohol involvement risks and outcomes (see Sullivan et al., 2016 for details). The NCANDA cognitive and motor battery assessed eight domains: general ability, attention, abstraction, emotion, working memory, balance, and motor speed (Sullivan et al., 2016, 2017). Most of these domains were assessed with computer-administered WebCNP tests (Gur et al., 2010). For examining cognitive skills that may be pertinent to executive dysfunction here, the domains of attention, emotion and working memory are particularly pertinent, and the examination of general ability may provide information on the global effects of problems in this arena. The functional domains and tests included here were: (1) Attention: Continuous Performance Test (Kurtz et al., 2001); (2) Emotion: Emotion Recognition Test (Gur et al., 2002), Measured Emotion Differentiation (Fossati, 2012); (3) Working Memory: Short Fractal N-Back Test (Ragland et al., 2002); and (4) General Ability: Vocabulary Test (Lee et al., 2014), WRAT-4 Math Calculations and Word Reading (Wilkinson and Robertson, 2006). These computer administered WebCNP tests were used to generate domain specific accuracy and speed (response time) z scores. In prior analyses, older age was associated with better scores on attention, emotion, and general ability (Sullivan et al., 2016), so age was used as a covariate in these analyses.

MRI Acquisition and Analysis

The NCANDA neuroimaging battery includes structural indices (Pfefferbaum et al., 2016) and diffusion tensor imaging (DTI: Pohl et al., 2016), and more detailed descriptions of the protocols may be found in those publications. Briefly, T1-weighted, 3D images were collected in the sagittal plane on systems from two manufacturers: 3T General Electric (GE) Discovery MR750 at three sites and 3T Siemens TIM TRIO scanners at two sites. The GE sites used an Array Spatial Sensitivity Encoding Technique (ASSET) for parallel and accelerated imaging with an 8-channel head coil and acquired an Inversion Recovery-Spoiled Gradient Recalled (IR-SPGR) echo sequence. The Siemens sites used a 12-channel head coil and parallel imaging and temporal acceleration with iPAT and acquired an MPRAGE sequence. Each site scanned the ADNI phantom on each day that participants were scanned. Analysis proceeded via the NCANDA NeuroInformatics Platform (Rohlfing et al., 2014) and involved skull stripping applied to the extracted maps. The SRI24 atlas-based analysis pipeline was used to identify intracranial volume (ICV), supratentorial volume (svol), and pons, corpus callosum, subcortical white matter (including the centrum semiovale), and lateral ventricular volumes. FreeSurfer (Dale et al., 1999) was used on skull-stripped data to create bilateral surface area, volume, and thickness of frontal, temporal, parietal, occipital, cingulate cortices derived from the Desikan-Killiany regions-of-interest (ROI) scheme (Desikan et al., 2006) plus the insular cortex. Volume was expressed in cc, surface area in cm^2, and thickness in mm. All ROIs were adjusted for linear scaling factors from the ADNI phantom (Clarkson et al., 2009). To acquire Diffusion-Weighted Images (DWI, a.k.a. diffusion tensor imaging or DTI), GE and Siemens sites applied a 2D Axial Spin Echo, Echo-Planar protocol, as well as a reverse phase acquisition of the 2D Axial Spin Echo-Planar protocol for B0-field inhomogeneity spatial distortion correction. To achieve common anatomical coordinates across subjects, each subject's

fractional anisotropy (FA) data set was registered to the FA channel of the SRI24 atlas. Cortical volume and thickness were smaller with increasing age, and cortical volumes greater in males than females (Pfefferbaum et al., 2016). For DTI indices, increasing age was associated with higher FA and lower diffusivity measures (Pohl et al., 2016). Consequently, statistical analyses on structural brain characteristics in the present study were controlled for age and sex.

Substance Use

Past and recent alcohol and other substance use were determined by the Customary Drinking and Drug Use Record (CDDR: Brown et al., 1998). The measure includes items on alcohol and marijuana use, including use frequency in the past year, and the maximum number of drinks in a drinking episode during the past year. At **Risk Alcohol Use:** Alcohol use frequency in the past year has been found to useful for identifying use patterns associated with alcohol use disorder (AUD) among adolescents (e.g., Chung et al., 2012; Clark et al., 2016). The National Institute on Alcohol Abuse and Alcoholism (NIAAA) Alcohol Screening and Brief Intervention for Youth: A Practitioner's Guide (NIAAA Youth Guide) recommends age specific stratified past year alcohol use frequency thresholds to define low, moderate and high risk for AUD. Moderate Risk is defined at age 12–15 as: ≥1 days; ages 16–17: ≥3 days; ages 18: ≥12 days. High Risk is defined at age 12–15 as: ≥3 days; age 16: ≥12 days; age 17: ≥24 days; age 18: ≥52 days. Subjects below these thresholds were classified as Low Risk. **Binge Alcohol Use:** While the traditional definition of a drinking binge has typically been applied across development, binge definitions appropriate for younger adolescents have been developed to account for smaller body size among youth (Donovan, 2009). Using the "lifetime greatest number of drinks" response, age-specific binge thresholds were calculated as follows: ages 9 to 13 years: ≥3 drinks; 14 or 15 years: ≥4 for males, ≥3 for females; 16 or 17 years: ≥5 for males, ≥3 for females.

Statistical Analyses

Overall NCANDA data management has been described (Rohlfing et al., 2014). (NCANDA Data releases used in these analyses: 000001_V1; 00010_V3; 00011; 00012_V2.) The analyses presented to test hypotheses were by analysis of variance (ANOVA) for categorical variables or Pearson correlations for continuous variables. Covariates for all analyses were age, sex and SES. A sequential procedure (Ludbrook, 1998) was used to reduce Type 1 error or family wise error rate, first testing the global hypothesis represented by the BRIEF Global Composite (i.e., GEC), followed by tests examining the BRIEF indices and scales with Šidák corrections (Šidák, 1967; Ludbrook, 1998). For analyses examining BRIEF composite, indices and scales, BRIEF GEC was examined first ($p < 0.05$) and, where the relationship between GEC and the dependent measure were significant, relationships with Behavioral Regulation Index (BRI) and Metacognition Index (MCI), then the eight scales, were interpreted. Corrections for multiple tests were implemented as follows: Behavioral Regulation Index or BRI and Metacognition Index or MCI: $\alpha = 0.05$ for 2 tests: $p < 0.025$; 8 scales: $\alpha = 0.05$ for 8 tests: $p < 0.0064$). To reduce type 1 error in sets of analyses examining correlations between multiple continuous variables (i.e., cognitive test scores and brain structure variables) and BRIEF GEC, the Šidák procedure was applied, in which the overall number of comparisons was taken into consideration to determine the significance threshold (Cognitive skill domains, with scores for accuracy and speed: $\alpha = 0.05/2$ or $p < 0.025$; MR Structural Gray Indices: $\alpha = 0.05/18$ or $p < 0.0028$; DTI: $\alpha = 0.05/32$ or $p < 0.0016$). For variables with significant relationships with BRIEF GEC, multivariate models were constructed to test mediation hypotheses (Baron and Kenny, 1986).

RESULTS

BRIEF Description

For subjects with valid BRIEF data ($n = 817$), mean scale t-scores ranged from 43.5 ± 8.6 (BRIEF Inhibitory Control) to 46.1 ± 9.9 (BRIEF Task Completion), with composite t-scores also in this range (BRI: 43.6 ± 9.4; MCI: 44.6 ± 10.6; GEC: 43.9 ± 10.4).

Demographic Characteristics

BRIEF composite scores did not significantly differ by gender (e.g., GEC: F: 44.0 ± 11.1; M: 43.8 ± 9.6; $t = 0.2$, d.f. 815, $p = 0.8$). BRIEF GEC was not significantly correlated with age ($r = 0.06$, $n = 817$, $p = 0.10$). BRIEF GEC was significantly correlated with SES ($r = -0.15$, $n = 731$, $p < 0.001$). Race (White: $n = 586$, 43.7 ± 10.1; African American: $n = 95$, 43.3 ± 11.7; Asian: $n = 62$, 43.9 ± 8.9; Other: $n = 67$, 46.6 ± 12.0) was not significantly associated with BRIEF GEC ($F = 1.7$, d.f. 3,806, $p = 0.16$). Ethnicity (Non-Hispanic: $n = 721$, 43.2 ± 11.9; Hispanic: 49.1 ± 11.9) was significantly associated with BRIEF GEC ($F = 28.4$, 1,815, $p < 0.001$). To a substantial extent, this relationship was accounted for by SES (multivariate model: $n = 725$; SES: $F = 13.1$, $p < 0.001$; Race: $F = 1.6$, $p = 0.6$; Ethnicity: $F = 4.9$, $p = 0.03$).

NCANDA Risk Density

Risk Density (**Table 1**) was determined by summing the number of risks present (range: 0–4; 0: $n = 381$, 46.6%; 1: 276, 33.8%; 2: 119, 14.6%; 3: 37, 4.5%; 4: 4, 0.5%). Due to few participants with 4 risk characteristics, participants with 3 or 4 risk factors were combined for analyses. Risk Density was significantly associated with BRIEF GEC, with higher Risk Density associated with greater dysfunction (**Table 1**; **Figure 1**). All BRIEF indices and scales were similarly significantly related to Risk Density. Abnormal BRIEF GEC scores (≥70), present in 2% of the sample, were associated with Risks (0 Risks, 0%; 1 risk, 2%, 2 risks, 4%, 3 or 4 Risks, 20%: Likelihood Ratio $\chi^2 = 68.0$, d.f. = 3, $p < 0.001$). Risk Density was significantly associated with At Risk Alcohol Use ($F = 40.5$, $p < 0.001$), Binge Alcohol Episodes ($F = 7.4$, $p < 0.01$), and Marijuana Use ($F = 10.3$, $p < 0.01$) (**Table 8**).

Sleep Quality

Sleep Quality was significantly associated with BRIEF GEC, with poorer slep quality associated with greater dysfunction (**Table 2**; **Figure 2**). All BRIEF indices and scales were similarly significantly related to Sleep Quality. Sleep Quality was significantly associated with At Risk Alcohol Use ($F = 5.6$,

TABLE 1 | BRIEF global composite, indexes and scales by NCANDA risk density categories.

	0	1	2	3–4	*F*
	***n* = 381**	***n* = 276**	***n* = 119**	***n* = 41**	
Inhibitory control	40.8 ± 7.2	44.5 ± 8.2	46.9 ± 9.0	54.0 ±12.0	37.3[c]
Flexibility	42.7 ± 9.3	46.9 ± 10.1	51.1 ± 12.4	55.4 ± 11.1	28.9[c]
Emotional control	42.4 ± 7.3	46.2 ± 8.5	50.9 ± 10.6	53.3 ± 11.5	36.6[c]
Monitoring	42.5 ± 7.6	45.3 ± 8.6	50.2 ± 9.2	54.1 ± 10.1	33.5[c]
Behavioral regulation	40.1 ± 7.7	44.7 ± 8.7	49.5 ± 10.2	55.0 ± 11.0	51.3[b]
Working memory	41.9 ± 8.4	46.9 ± 10.0	50.1 ± 10.8	56.9 ± 12.0	40.0[c]
Task plan	41.9 ± 8.7	46.7 ± 9.9	50.6 ± 1.5	56.7 ± 10.8	39.9[c]
Orderly	43.0 ± 8.7	46.9 ± 9.5	49.2 ± 10.0	54.4 ± 11.2	24.4[c]
Completion	42.3 ± 7.3	47.5 ± 9.8	51.4 ± 11.0	57.9 ± 12.7	47.6[c]
Metacognition	40.9 ± 8.5	46.6 ±10.2	50.4 ± 11.2	57.7 ± 11.9	50.1[b]
Global composite	39.9 ± 8.2	45.0 ± 9.6	50.0 ± 10.9	56.5 ± 11.7	55.8[a]

d.f. 3, 724; covariates: sex, age, SES.
[a]*p < 0.05 for test of GEC.*
[b]*p < 0.025 for 2 tests of Indexes.*
[c]*p < 0.0064 for 8 tests of Scales.*

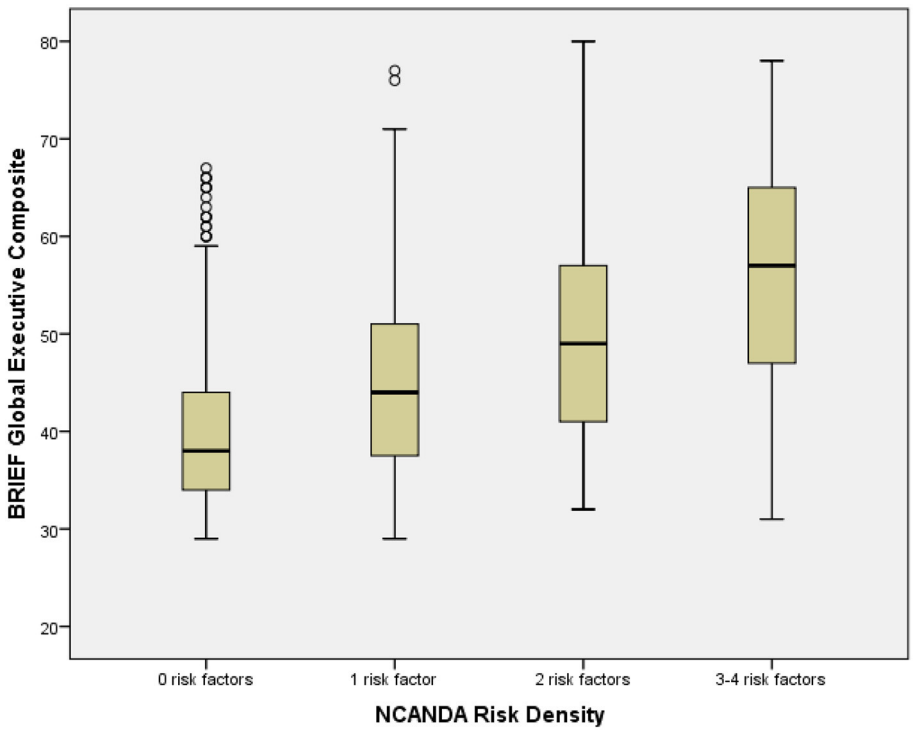


FIGURE 1 | Box plot of BRIEF global composite by NCANDA risk density categories. The figure shows box plots for NCANDA Risk Density categories. The box plot represents the interquartile range (IQR), with the shaded box representing the second and third quartiles and center demarcations indicating the median. The whiskers represent the maximum and minimum values up to 1.5 × IQR, and the circles represent values beyond 1.5 × IQR.

$p < 0.05$), Binge Alcohol Episodes ($F = 3.9$, $p < 0.05$), and Marijuana Use ($F = 5.8$, $p < 0.05$) (**Table 8**).

Cognitive Skill Domain Scores

Cognitive Skill Domain Scores were not significantly correlated with BRIEF GEC (**Table 3**). Note that, while a correction for two tests per domain (i.e., accuracy and speed) was thought applicable, a less stringent threshold of $p < 0.05$ did not change the interpretation of the results. Since there may be some interest in the observed correlations for specific variable pairs (e.g., Working Memory by BRIEF scale by Cognitive Skill Domain), the full table of analyses is presented.

TABLE 2 | BRIEF global composite, indexes and scales by sleep quality categories.

	Very good	Fairly good	Fairly bad	Very bad	*F*
	***n* = 218**	***n* = 430**	***n* = 71**	***n* = 12**	
Inhibitory control	41.0 ± 7.1	44.0 ± 8.7	48.3 ± 10.7	47.2 ± 9.8	14.5[c]
Flexibility	42.7 ± 9.6	46.5 ± 10.7	51.5 ± 10.6	53.8 ± 15.4	15.4[c]
Emotional control	42.9 ± 8.1	46.2 ± 9.3	48.2 ± 9.5	49.5 ± 9.8	9.6[c]
Monitoring	42.3 ± 7.7	45.9 ± 8.9	48.6 ± 9.3	48.5 13.2	12.8[c]
Behavioral regulation	40.4 ± 8.2	44.4 ± 9.4	48.9 ± 10.3	49.7 ± 11.8	18.7[b]
Working memory	42.0 ± 9.3	46.0 ± 9.8	52.3 ± 11.9	54.9 ± 11.9	24.2[c]
Task plan	41.3 ± 9.3	46.0 ± 9.8	53.3 ± 11.9	53.5 ± 11.3	28.5[c]
Orderly	42.2 ± 7.9	46.5 ± 9.7	50.6 ± 11.3	55.4 ± 9.6	20.4[c]
Task completion	42.9 ± 8.3	46.2 ± 9.6	54.4 ± 11.8	54.3 ± 12.5	27.1[c]
Metacognition	40.9 ± 9.2	45.5 ± 10.0	53.3 ± 12.2	55.3 ± 12.6	31.8[b]
Global composite	39.9 ± 9.0	44.7 ± 10.3	51.4 ± 11.8	52.9 ± 12.4	28.3[a]

d.f. 2, 723; covariates: sex, age, SES.
[a]*p < 0.05 for test of BRIEF GEC.*
[b]*p < 0.025 for 2 tests of Indexes.*
[c]*p < 0.0064 for 8 tests of Scales.*

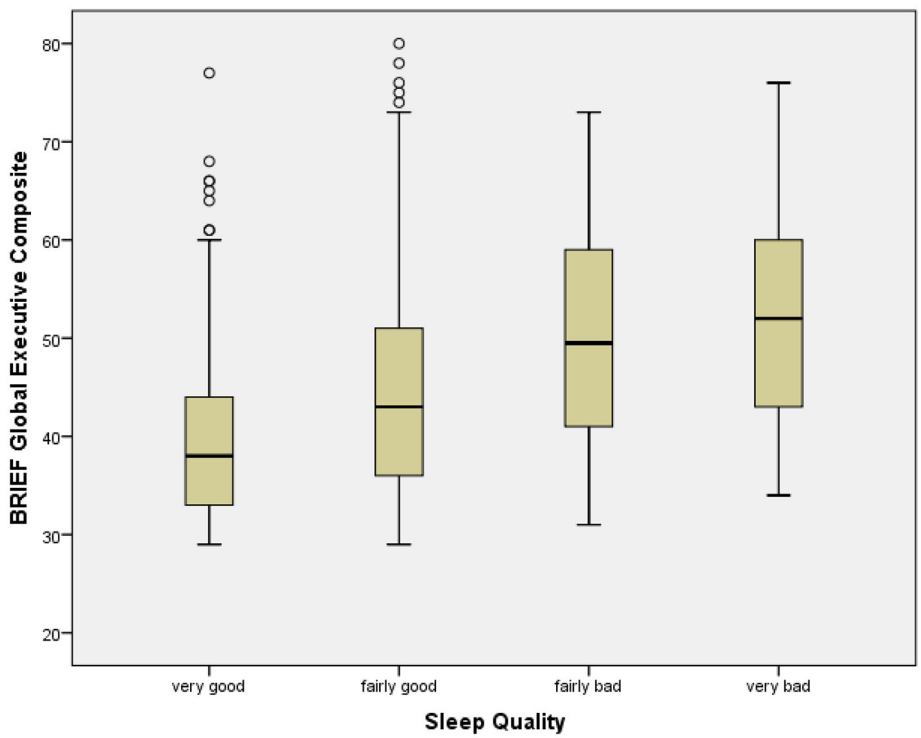


FIGURE 2 | Box plot of BRIEF global executive composite by sleep quality categories. The figure shows box plots for Sleep Quality categories. The box plot represents the interquartile range (IQR), with the shaded box representing the second and third quartiles and center demarcations indicating the median. The whiskers represent the maximum and minimum values up to 1.5 × IQR, and the circles represent values beyond 1.5 × IQR.

Cortical Structure

Using a correction for multiple comparisons for tests across the BRIEF GEC by structural variables matrix (i.e., 18 tests), BRIEF GEC was not significantly correlated with gray matter volume, cortical thickness or cortical surface area in frontal temporal, parietal, occipital, cingulate, or insula regions (**Table 4**).

TABLE 3 | BRIEF global composite, indexes and scales by cognitive skill domain scores.

	Attention		Emotion recognition		Working memory		General abiliity	
	a	s	a	s	a	s	a	s
Inhibitory control	−0.08	−0.01	−0.01	0.13	0.01	0.00	−0.04	0.08
Flexibility	−0.02	−0.04	−0.02	0.03	0.02	−0.02	−0.01	0.02
Emotional control	−0.03	−0.01	0.03	0.05	0.04	0.02	−0.04	0.01
Monitoring	−0.03	−0.05	−0.03	0.07	−0.02	−0.02	−0.04	0.04
Behavioral regulation	−0.05	−0.03	−0.01	0.08	0.02	0.00	−0.04	0.04
Working memory	−0.05	−0.03	0.03	0.06	0.00	0.00	−0.04	0.04
Task planning	−0.04	−0.03	0.04	0.09	0.04	−0.02	0.01	0.07
Organization	0.01	−0.03	0.06	0.06	0.03	−0.02	0.01	0.07
Task completion	−0.05	−0.06	0.01	0.05	0.00	−0.05	−0.13	−0.02
Metacognition	−0.04	−0.03	0.04	0.07	0.02	−0.03	−0.04	0.05
Global composite	−0.05	−0.03	0.02	0.08	0.02	−0.01	−0.04	0.05

A, accuracy; s, speed; Pearson correlations [r]; d.f. 707; covariates: sex, age, SES.
With corrections for multiple comparisons, none of the above statistical test reached statistical significance.

TABLE 4 | BRIEF global composite by MR structural gray segment volume, thickness and surface area.

	Volume	Thickness	Surface area
Frontal	−0.01	−0.04	0.02
Temporal	0.02	0.00	0.04
Parietal	0.01	0.00	0.03
Occipital	−0.02	−0.01	0.00
Cingulate	0.00	−0.02	0.02
Insula	−0.03	−0.05	−0.01

Pearson correlations [r]; d.f. 707; covariates: sex, age, SES.
With corrections for multiple comparisons, none of the above statistical test reached statistical significance.

TABLE 5 | BRIEF global composite by DTI indices for fasciculi and tracts.

	FA	MD	L1	LT
FASCICULI				
Superior longitudinal	−0.07	0.08	0.03	0.04
Superior frontal-occipital	−0.07	0.00	−0.06	0.02
Sagittal stratum	0.01	0.01	0.04	−0.02
Uncinate	−0.09	0.08	0.00	0.08
LIMBIC TRACTS				
Fornix	0.01	0.01	0.02	0.00
Striatia terminalis	0.01	0.06	0.09	−0.01
Anterior middle cingulum	−0.03	0.11	0.09	0.03
Inferior cingulum	0.01	0.00	0.01	−0.01

FA, fractional anisotrophy; MD, mean diffusivity; L1, axial diffusivity; LT, radial diffusivity. Pearson correlations [r]; d.f. 703; covariates: sex, age, SES. With corrections for multiple comparisons, none of the above statistical test reached statistical significance.

DTI

Using a correction across the presented BRIEF GEC by structural variables (i.e., 32 tests), BRIEF GEC was not significantly correlated with DTI indices for fasciculi and tracts by region or DTI variable (**Table 5**).

Alcohol and Cannabis Use

At Risk Alcohol Use Category was determined by the past year alcohol use days frequency thresholds described in the National Institute on Alcohol Abuse and Alcoholism Youth Guide (2011). Overall (**Table 6**), 83% ($n = 676$) were Low Risk, 12% ($n = 95$) were Moderate Risk, and 5% ($n = 41$) were High Risk. At Risk Alcohol Use Category was significantly related to BRIEF GEC ($F = 7.0$, d.f. 2,723, $p = 0.001$), with higher risk associated with more problems. At Risk Alcohol Use Category was significantly associated with BRIEF BRI and MCI, and scales including Inhibitory Control, Emotional Control, and Task Planning.

Age Defined Binge Alcohol Use

Past year age defined binge alcohol use (**Table 7**) was present in 129 subjects (15.9%). Including sex, age and SES as covariates, Age Defined Binge Alcohol Use was significantly related to BRIEF GEC ($F = 11.1$, d.f. 1,721, $p = 0.001$), with the presence of at least one binge in the past year associated with more problems. Age Defined Binge Alcohol Use was associated with more problems on BRIEF BRI and MCI, as well as on the including Inhibitory Control scale.

Cannabis Use

Past year cannabis use was present in 123 subjects (**Table 7**: 15.2%). Cannabis Use was significantly related to BRIEF GEC ($F = 9.1$, d.f. 1,720, $p = 0.003$), with the presence of cannabis use in the past year associated with more problems. Cannabis Use was associated with more problems on BRIEF BRI and MCI, as well as scales including Inhibitory Control, Emotional Control, and Task Planning.

Multivariate Models

For variables shown to be significantly related to BRIEF GEC (i.e., NCANDA Risk Density and Sleep Quality), multivariate

TABLE 6 | At Risk Alcohol Use Frequency x BRIEF scales.

	Low risk	Moderate risk	High risk	*F*
	n = 616	*n* = 82	*n* = 31	
Inhibitory control	43.1 ± 8.4	46.3 ± 10.7	47.3 ± 9.0	8.1[c]
Flexibility	45.7 ± 10.8	48.0 ± 11.4	48.1 ± 11.7	2.3
Emotional control	45.1 ± 9.1	48.9 ± 9.2	47.1 ± 10.6	7.3[c]
Monitoring	44.8 ± 8.8	47.3 ± 10.0	47.6 ± 8.8	3.7
Behavioral regulation	43.3 ± 9.5	47.1 ± 10.5	46.7 ± 10.3	7.4[b]
Working memory	45.1 ± 10.3	48.6 ± 11.7	48.5 ± 9.4	5.4
Task plan	45.0 ± 10.4	47.9 ± 11.0	51.8 ± 11.0	8.0[c]
Orderly	45.5 ± 9.8	47.5 ± 10.1	48.9 ± 9.0	2.4
Task completion	45.7 ± 10.0	49.2 ± 11.0	48.1 ± 11.4	3.9
Metacognition	44.5 ± 10.7	48.0 ± 11.6	49.3 ± 10.5	5.9[b]
Global composite	43.5 ± 10.5	47.5 ± 11.2	48.1 ± 10.6	7.0[a]

d.f. 2, 723; covariates: sex, age, SES.
[a]*p < 0.05 for test of BRIEF GEC.*
[b]*p < 0.025 for 2 tests of Indices.*
[c]*p < 0.0064 for 8 tests of Scales.*

models were tested (**Table 8**). NCANDA Risk Density was significantly related to Past Year At Risk Alcohol Use Frequency, Age Defined Binge Alcohol Use and Cannabis Use (i.e., substance use). In multivariate models including BRIEF GEC, the relationship between Risk Density and substance use variables diminished but remained significant, suggesting that mediation was not demonstrated. Sleep Quality was significantly related to substance use variables. In multivariate models including BRIEF GEC, the relationship between Sleep Quality and substance use variables was not statistically significant, suggesting mediation. In a model with NCANDA Risk Density, Sleep Quality and BRIEF GEC, the relationship between NCANDA Risk Density and substance use remained significance while Sleep Quality was not significantly related to substance use.

DISCUSSION

In NCANDA, BRIEF (Guy et al., 2004) was utilized as an ecologically valid complement to performance based cognitive testing to measure executive functioning constructs. While BRIEF scores were noted to be, on average, somewhat lower than might be expected from the scale construction samples, subsequent studies have similarly noted that normative samples have mean calculated *t*-scores similar to the NCANDA sample (Roth et al., 2015). BRIEF scores were not related to age, sex, or race, and showed a significant relationship with SES. Prior studies have shown relationships between SES and executive functioning, with attributions of this correlation interpreted as related to parent education and other home and family environment characteristics (Hackman et al., 2015).

As expected, higher BRIEF scores were systematically correlated with NCANDA Risk Density. Previous studies have indicated that characteristics related to executive functioning are associated with SUD risk indicators, including externalizing characteristics (Familiar et al., 2015; Long et al., 2015), internalizing characteristics (Clark et al., 1997), family SUD history (Tapert and Brown, 2000), and the early adolescent onset of alcohol use (Tarter et al., 2003). However, the multivariate models indicated that the inclusion of BRIEF was accompanied by only a modest reduction in the relationship between NCANDA Risk Density and substance use, suggesting that the mediation hypothesis was not supported. These analyses of cross-sectionally collected data were not ideal for determining directional influences, and subsequent NCANDA analyses will be able to further examine the extent to which the relationship between risk factors and later substance use may be mediated by executive dysfunction.

Poor sleep quality was significantly and consistently correlated with all executive dysfunction dimensions. While prior research has suggested that sleep problems are associated with executive dysfunction in daily life, the robust correlations observed here indicates a more extensive relationship than has been previously reported. Since prior research utilizing BRIEF in relationship to sleep has involved smaller samples, the present finding may benefit from greater statistical power. The multivariate models indicated that the inclusion of BRIEF was accompanied by a reduction in the relationship between Sleep Quality and substance use, indicating that the mediation hypothesis was supported. Since the present findings are based on cross-sectional data, however, analyses informed by changes in these relationships over time will be undertaken in the course of the NCANDA study. In addition, polysomnography being examined in an NCANDA subset will complement subjective sleep assessments (Baker et al., 2016). Sleep difficulties have been shown to predict problematic alcohol use (Hasler and Clark, 2013; Hasler et al., 2014), and subsequent NCANDA analyses will be able to examine the extent to which executive dysfunction mediates this relationship over the course of adolescent development.

The self-report of executive dysfunction in daily life assessed by BRIEF were not significantly correlated with performance based cognitive test results. While one might expect there to be some correspondence between these indicators, our results are consistent with several prior studies. A recent review of 20 studies (Toplak et al., 2013) on the relationship between self-report and performance-based executive functioning assessments found that, of 286 relevant correlations, only 68 (24%) were statistically significant, and the median correlation was only 0.19. The authors concluded that these approaches generally assess different underlying constructs. The results here were consistent with these observations. Broadly conceived, performance-based cognitive tests and BRIEF scores assess executive functioning dimensions. However, our results, consistent with prior studies (Toplak et al., 2013), indicate that executive dysfunction in daily life, measured by BRIEF, and performance based cognitive testing measure distinct constructs.

BRIEF Global Composite scores were not significantly correlated with cortical structure or DTI indicators. These findings were consistent with some prior observations (e.g., Faridi et al., 2015). The current study had a substantially larger and more representative sample than some other pertinent prior studies with positive findings (Mahone et al., 2009;

TABLE 7 | Past year age defined binge and marijuana use × BRIEF scales.

	Age defined binges			Marijuana use		
	No $n = 626$	**Yes $n = 100$**	***F***	**No $n = 621$**	**Yes $n = 104$**	***F***
Inhibitory control	43.2 ± 8.6	46.1 ± 9.6	11.1[c]	43.1 ± 8.5	46.5 ± 9.9	12.2[c]
Flexibility	45.7 ± 10.7	48.0 ± 11.4	4.4	45.6 ± 10.6	48.3 ± 11.5	4.9
Emotional control	45.3 ± 9.5	46.9 ± 9.5	3.4	45.1 ± 9.1	48.1 ± 9.3	8.4[c]
Monitoring	44.8 ± 8.8	47.3 ± 9.8	6.5	44.8 ± 8.8	47.4 ± 9.3	5.5
Behavioral regulation	43.4 ± 9.4	46.3 ± 9.9	8.7[b]	43.2 ± 9.4	47.0 ± 10.2	11.0[b]
Working memory	45.3 ± 10.5	47.4 ± 9.5	4.4	45.1 ± 10.4	48.0 ± 10.1	5.3
Task plan	45.1 ± 10.4	48.1 ± 10.7	5.5	45.0 ± 10.5	48.6 ± 10.1	7.5[c]
Orderly	45.4 ± 9.7	48.1 ± 9.6	3.7	45.3 ± 9.8	48.2 ± 9.3	4.5
Task completion	45.8 ± 9.8	48.2 ± 11.1	3.7	45.8 ± 10.0	48.3 ± 10.4	2.7
Metacognition	44.7 ± 10.6	47.6 ± 10.8	5.6[b]	44.6 ± 10.7	48.8 ± 10.3	6.2[b]
Global composite	43.6 ± 10.4	46.8 ± 10.6	7.4[a]	43.5 ± 10.4	47.4 ± 10.5	9.1[a]

Binge: d.f. 1, 721; covariates: sex, age, SES; Marijuana: d.f. 1, 720.
[a]*p < 0.05 for test of Composite.*
[b]*p < 0.025 for 2 tests of Indexes.*
[c]*p < 0.0064 for 8 tests of Scales.*

TABLE 8 | Contributions to ANOVA models of substance use outcomes.

	At Risk Alcohol		Binge alcohol		Cannabis use	
Tested covariates	***F***	**(R^2)**	***F***	**(R^2)**	***F***	**(R^2)**
BRIEF alone	13.7***	(0.070)	7.4**	(0.126)	10.3**	(0.141)
Risk alone	40.5 ***	(0.102)	14.1***	(0.134)	28.6***	(0.162)
Risk with BRIEF	27.6***	(0.103)	8.2**	(0.134)	19.1***	(0.162)
BRIEF with risk	1.3		1.5		1.1	
Sleep alone	5.6*	(0.059)	3.9*	(0.122)	5.8*	(0.136)
Sleep with BRIEF	1.6	(0.070)	1.4	(0.126)	2.1	(0.143)
BRIEF with sleep	9.7**		4.8*		6.6**	
BRIEF with risk and sleep	0.7	(0.103)	0.8	(0.135)	0.4	(0.163)
Risk with BRIEF and sleep	27.0***		7.8**		18.5***	
Sleep with BRIEF and risk	1.0		1.1		1.6	

*Adjusted R^2 for the model; F-statistic with d.f. 1, 701; covariates: sex, age, SES; *p < 0.05, **p < 0.01, ***p < 0.001; BRIEF, BRIEF Global Composite; Risk, NCANDA Risk Density; Sleep,Sleep Quality; At Risk Alcohol, At Risk Alcohol Frequency (Past Year); Binge Alcohol, Age Defined Binge Alcohol Use (Past Year); Marijuana Use, Marijuana Use (Past Year).*

Clark et al., 2012). Alternative statistical approaches, such as latent variable modeling, may provide additional insights. For example, among 286 healthy children and adolescents, a latent variable modeling approach revealed a systematic pattern of relationships between cortical networks and BRIEF scores (Ziegler et al., 2013). The examination of network based brain activation patterns, using techniques such as resting state MRI, may show relationships between functional brain development and executive functioning difficulties (Fair et al., 2009; Casey et al., 2016). The neurobiological foundations of executive dysfunction may be more reflected in functional MRI indicators of activation during tasks that require cognitive and behavioral skills in this arena. For example, among 59 young adolescents (ages 12–15), BRIEF cognitive flexibility was associated with orbitofrontal cortex activation during a behavioral inhibition task (Zhai et al., 2015).

Consistent with prior studies (Clark et al., 2012; Hadjiefthyvoulou et al., 2012), BRIEF scores were associated with indicators of risky substance use, including a frequency of alcohol use indicating risk for AUD, age defined binge drinking, and marijuana use. Difficulties with executive functioning and risky substance use may reflect both preexisting psychological dysregulation and substance use resulting in disruption of executive functioning (Clark et al., 2012). While the cross-sectional structure of these NCANDA data from the initial assessment do not support interpretations on the direction of the observed associations, multivariate models were presented that may suggest directions for future studies. The age range in this study was broad, a feature which limits the size of subsamples representing each adolescent stage. For this examination of the initial assessment data, the sample was insufficient to subgroup subjects by age. When data collection for NCANDA has been completed, the acquisition of additional follow-up data in this accelerated longitudinal design and the application of statistical modeling techniques developed for such studies will support the examination of changes in these relationships over the course of adolescence. In addition, analyses with the longitudinal data will clarify the extent to which alternative hypotheses are reflected in observed relationships. While this sample size is larger than many other similar studies, the recruitment strategy and sample characteristics were designed to optimally provide data for later analyses taking advantage of the accelerated longitudinal design, and were not ideal for some of the analyses presented here.

In summary, these analyses found significant and systematic relationships between executive dysfunction in daily life and SUD risk indicators, including a composite risk indicator (i.e., NCANDA Risk Density), poor sleep quality, and risky alcohol and marijuana use. In addition, the study contributed to the available literature on the relationship between BRIEF scores

and performance based cognitive testing, indicating that these methods assess complimentary but distinct constructs. Everyday executive functioning was not systematically related to cortical structure or white matter integrity by DTI. With the on-going collection and analysis of additional follow-up assessments with the measures described here, and the examination of functional brain characteristics and other variables, NCANDA will provide insights on the relationships among difficulties with everyday executive functioning, performance-based cognitive testing of skills reflecting executive functions, structural and functional brain development, and alcohol and other substance use effects on these neurodevelopmental trajectories.

These results suggest that executive dysfunction in daily life is not accounted for by deficits in the cognitive skills measured in the NCANDA protocol. This observation has clinical implications, in that the results here imply that such testing may not provide the expected insights into the origins of executive dysfunction. Also, executive dysfunction was not accounted for by cortical structural or DTI characteristics. While some support has been presented that cognitive tests relate to some related mental disorders (e.g., ADHD: Willcutt et al., 2005), these results are consistent with other negative studies in suggesting that cognitive testing and MRI studies may not provide clinical insights explaining executive dysfunction with typical adolescents.

Consistent with several prior studies (Hasler and Clark, 2013; Hasler et al., 2014, 2016, 2017), poor sleep quality was related to both executive dysfunction and risky substance use. These findings suggest interventions to improve sleep may benefit executive functioning as well as risky substance use. Individually applicable clinical interventions to improve sleep quality have demonstrated effectiveness in adolescents (Gradisar et al., 2014). On a more general level, early school times may be detrimental to sleep quality, later sleep times warrant consideration (Hasler et al., 2014; Minges and Redeker, 2016).

Interventions to improve executive functioning, in addition to having inherent value, may also be be a viable preventive intervention target. Interventions that improve inhibitory control in childhood, for example, hold promise for preventing SUD in adolescence (Riggs and Greenberg, 2009; Diamond and Lee, 2011).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Human Subjects Institutional Review Boards as noted below with written informed consent from all subjects. Institutional review boards approved the study for each site. University of California, San Diego (UCSD) Human Subjects Institutional Review Boards approved the study at each site: University of California, San Diego; Oregon Health & Science University; SRI International; Duke University; University of Pittsburgh. The protocol was approved by these committees. All subjects gave written informed consent in accordance with the Declaration of Helsinki. For participants under 18 years old, a parent provided written informed consent and the under 18 participant provided assent. Participants 18 years old or older provided written informed consent.

AUTHOR CONTRIBUTIONS

All authors (DC, TC, CM, BH, DF, BL, SB, ST, TB, KC, AP, ES, KP, IC, FB, MD, KN, and BN) were involved in study conception, data collection, and manuscript editing.

FUNDING

This work was supported by the U.S. National Institute on Alcohol Abuse and Alcoholism with co-funding from the National Institute on Drug Abuse, the National Institute of Mental Health, and the National Institute of Child Health and Human Development [NCANDA grant numbers: AA021695 (ST), AA021692 (SB+ST), AA021697 (AP+KP), AA021696 (IC+FB), AA021681 (MD), AA021690 (DC), AA021691 (BN)], and a fellowship from the Veteran's Affairs Office of Academic Affiliation (TB).

REFERENCES

Achenbach, T. M., and Rescorla, L. A. (2001a). *Manual for the ASEBA School-Age Forms and Profiles*. Burlington, VT: University of Vermont, Research Center for Children, Youth, and Families.

Achenbach, T. M., and Rescorla, L. A. (2001b). *Manual for the ASEBA Adult Forms and Profiles*. Burlington, VT: University of Vermont, Research Center for Children, Youth, and Families.

Anderson, B., Storfer-Isser, A., Taylor, H. G., Rosen, C. L., and Redline, S. (2009). Associations of executive function with sleepiness and sleep duration in adolescents. *Pediatrics* 123, e701–e707. doi: 10.1542/peds.2008-1182

Ashtari, M., Cervellione, K. L., Hasan, K. M., Wu, J., McIlree, C., Kester, H., et al. (2007). White matter development during late adolescence in healthy males: a cross-sectional diffusion tensor imaging study. *Neuroimage* 35, 501–510. doi: 10.1016/j.neuroimage.2006.10.047

Baker, F. C., Willoughby, A. R., de Zambotti, M., Franzen, P. L., Prouty, D., Javitz, H., et al. (2016). Age-related differences in sleep architecture and electroencephalogram in adolescents in the NCANDA sample. *Sleep* 39, 1429–1439. doi: 10.5665/sleep.5978

Baron, I. S. (2000). Behavior Rating Inventory of Executive Function. *Child Neuropsychol.* 6, 235–238. doi: 10.1076/chin.6.3.235.3152

Baron, R. M., and Kenny, D. A. (1986). The moderator-mediator variable distinction in social psychology research: conceptual, strategic, and statistical considerations. *J. Pers. Soc. Psychol.* 51, 1173–1182.

Best, J. R., and Miller, P. H. (2010). A developmental perspective on executive function. *Child Dev.* 81, 1641–1660. doi: 10.1111/j.1467-8624.2010.01499.x

Boschloo, A., Krabbendam, L., Aben, A., de Groot, R., and Jolles, J (2014). Sorting test, tower test, and BRIEF-SR do not predict school performance of healthy adolescents in preuniversity education. *Front. Psychol.* 5:287. doi: 10.3389/fpsyg.2014.00287

Brown, S. A., Brumback, T., Tomlinson, K., Cummins, K., Thompson, W. K., Nagel, B. J., et al. (2015). The National Consortium on Alcohol and Neurodevelopment in Adolescence (NCANDA): a multi-site study of adolescent development and substance use. *J. Stud. Alcohol Drugs* 76, 895–908. doi: 10.15288/jsad.2015.76.895

Brown, S. A., Myers, M. G., Lippke, L., Tapert, S. F., Stewart, D. G., and Vik, P. W. (1998). Psychometric evaluation of the customary drinking and drug use

record (CDDR): a measure of adolescent alcohol and drug involvement. *J. Stud. Alcohol* 59, 427–438.

Buysse, D., Reynolds, C., Monk, T., Berman, S., and Kupfer, D (1989). The pittsburgh sleep quality index: a new instrument for psychiatric practice and research. *Psychiatry Res.* 28, 193–213

Caruso, N. C., Radovanovic, B., Kennedy, J. D., Couper, J., Kohler, M., Kavanagh, P. S., et al. (2014). Sleep, executive functioning and behavior in children and adolescents with type 1 diabetes. *Sleep Med.* 15, 1490–1499. doi: 10.1016/j.sleep.2014.08.011

Casey, B. J., Galvan, A., and Somerville, L. H. (2016). Beyond simple models of adolescence to an integrated circuit-based account: a commentary. *Dev. Cogn. Neurosci.* 17, 128–130. doi: 10.1016/j.dcn.2015.12.006

Chambers, R. A., Taylor, J. R., and Potenza, M. N. (2003). Developmental neurocircuitry of motivation in adolescence: a critical period of addiction vulnerability. *Am. J. Psychiatry* 160, 1041–1052. doi: 10.1176/appi.ajp.160.6.1041

Chan, R. C. K., Shum, D., Toulopoulou, T., and Chen, E. Y. H. (2008). Assessment of executive functions: review of instruments and identification of critical issues. *Arch. Clin. Neuropsychol.* 23, 201–216. doi: 10.1016/j.acn.2007.08.010

Chung, T., Smith, G. T., Donovan, J. E., Windle, M., Faden, V. B., Chen, C. M., et al. (2012). Drinking frequency as a brief screen for adolescent alcohol problems. *Pediatrics* 129, 1–8. doi: 10.1542/peds.2011-1828

Clark, D. B., Chung, T., Thatcher, D. L., Pajtek, S., and Long, E. C. (2012). Psychological dysregulation, white matter disorganization and substance use disorders in adolescence. *Addiction* 107, 206–214. doi: 10.1111/j.1360-0443.2011.03566.x

Clark, D. B., Martin, C. S., Chung, T., Gordon, A. J., Fiorentino, L., Tootell, M., et al. (2016). Screening for underage drinking and DSM-5 alcohol use disorder in rural primary care practice. *J. Pediatr.* 173, 214–220. doi: 10.1016/j.jpeds.2016.02.047

Clark, D. B., Pollock, N. A., Bromberger, J. T., Bukstein, O. G., Mezzich, A. C., and Donovan, J. E. (1997). Gender and comorbid psychopathology in adolescents with alcohol use disorders. *J. Am. Acad. Child Adolesc. Psychiatry* 36, 1195–1203.

Clark, D. B., Thatcher, D. L., and Tapert, S. (2008). Alcohol, psychological dysregulation and adolescent brain development. *Alcohol. Clin. Exp. Res.* 32, 375–385. doi: 10.1111/j.1530-0277.2007.00601.x

Clark, D. B., and Winters, K. C. (2002). Measuring risks and outcomes in substance use disorders prevention research. *J. Consult. Clin. Psychol.* 70, 1207–1223. doi: 10.1037/0022-006X.70.6.1207

Clarkson, M. J., Ourselin, S., Nielsen, C., Leung, K. K., Barnes, J., Whitwell, J. L., et al. (2009). Comparison of phantom and registration scaling corrections using the ADNI cohort. *Neuroimage* 47, 1506–1513. doi: 10.1016/j.neuroimage.2009.05.045

Cyders, M. A., and Coskunpinar, A (2012). The relationship between self-report and lab task conceptualizations of impulsivity. *J. Res. Pers.* 46, 121–124. doi: 10.1016/j.jrp.2011.11.005

Dale, A. M., Fischl, B., and Sereno, M. I. (1999). cortical surface-based analysis. I. segmentations and surface reconstruction. *Neuroimage* 9, 179–194.

de la Vega, R., Tome-Pires, C., Sole, E., Racine, M., Castarlenas, E., Jensen, M. P., et al. (2015). The Pittsburgh sleep quality index: validity and factor structure in young people. *Psychol. Assess.* 27, e22–e27. doi: 10.1037/pas0000128

Desikan, R. S., Segonne, F., Fischl, B., Quinn, B. T., Dickerson, B. C., Blacker, D., et al. (2006). An automated labeling system for subdividing the human cerebral cortex on MRI scans into gyral based regions of interest. *Neuroimage* 31, 968–980. doi: 10.1016/j.neuroimage.2006.01.021

Diamond, A., and Lee, K (2011). Interventions shown to aid executive function development in children 4 to 12 years old. *Science* 333, 959–964. doi: 10.1126/science.1204529

Donovan, J. E. (2009). estimated blood alcohol concentrations for child and adolescent drinking and their implications for screening instruments. *Pediatrics* 123, e975–e981. doi: 10.1542/peds.2008-0027

Duncan, S. C., Duncan, T. E., and Hops, H. (1996). Analysis of longitudinal data within accelerated longitudinal designs. *Psychol. Methods* 1, 236–248. doi: 10.1037/1082-989X.1.3.236

Duncan, S. C., Duncan, T. E., and Strycker, LA (2006). Alcohol use from ages 9 to 16: a cohort-sequential latent growth model. *Drug Alcohol Depend.* 81, 71–81. doi: 10.1016/j.drugalcdep.2005.06.001

Egeland, J., and Fallmyr, O. (2010). Confirmatory factor analysis of the Behavior Rating Inventory of Executive Function (BRIEF): support for a distinction between emotional and behavioral regulation. *Child Neuropsychol.* 164, 326–337. doi: 10.1080/09297041003601462

Fair, D. A., Cohen, A. L., Power, J. D., Dosenbach, N. U., Church, J. A., Miezin, F, et al. (2009). Functional brain networks develop from "local to distributed" organization. *PLoS Comput. Biol.* 5, 1–14. doi: 10.1371/journal.pcbi.1000381

Familiar, I., Ruisenor-Escudero, H., Giordani, B., Bangirana, P., Nakasujja, N., Opoka, R., et al. (2015). Use of the Behavior Rating Inventory of Executive Function and child behavior checklist in Ugandan children with HIV or a history of severe malaria. *J. Dev. Behav. Pediatr.* 36, 277–284. doi: 10.1097/DBP.0000000000000149

Faridi, N., Karama, S., Burgaleta, M., White, M. T., Evans, A. C., Fonov, V., et al. (2015). Neuroanatomical correlates of behavioral rating versus performance measures of working memory in typically developing children and adolescents. *Neuropsychology* 29, 82–91. doi: 10.1037/neu0000079

Fassbender, C., Simoes-Franklin, C., Murphy, K., Hester, R., Meaney, J., Robertson, I. H., et al. (2006). The role of a right fronto-parietal network in cognitive control - common activations for "cues-to-attend" and response inhibition. *J. Psychophysiol.* 20, 286–296. doi: 10.1027/0269-8803.20.4.286

Fossati, P. (2012). Neural correlates of emotion processing: from emotional to social brain. *Eur. Neuropsychopharmacol.* 22, 487–491. doi: 10.1016/j.euroneuro.2012.07.008

Gioia, G. A., Isquith, P. K., Guy, S. C., and Kenworthy, L. (2002a). *Behavior Rating Inventory of Executive Function (BRIEF)*. Lutz, FL: Psychological Assessment Resources.

Gioia, G. A., Isquith, P. K., Retzlaff, P. D., and Espy, K. A. (2002b). Confirmatory factor analysis of the Behavior Rating Inventory of Executive Function (BRIEF) in a clinical sample. *Child Neuropsychol.* 8, 249–257. doi: 10.1076/chin.8.4.249.13513

Gradisar, M., Smits, M. G., and Bjorvatn, B (2014). Assessment and treatment of delayed sleep phase disorder in adolescents: recent innovations and cautions. *Sleep Med. Clin.* 9, 199–210. doi: 10.1016/j.jsmc.2014.02.005

Gur, R. C., Richard, J., Hughett, P., Calkins, M. E., Macy, L., Bilker, W. B., et al. (2010). A cognitive neuroscience-based computerized battery for efficient measurement of individual differences: standardization and initial construct validation. *J. Neurosci. Methods* 187, 254–262. doi: 10.1016/j.jneumeth.2009.11.017

Gur, R. C., Sara, R., Hagendoorn, M., Marom, O., Hughett, P., Macy, L., et al. (2002). A method for obtaining 3-dimensional facial expressions and its standardization for use in neurocognitive studies. *J. Neurosci. Methods* 15, 137–143. doi: 10.1016/S0165-0270(02)00006-7

Guy, S. C., Isquith, P. K., and Gioia, G. A. (2004). *Behavior Rating Inventory of Executive Function – Self-Report Version.* Lutz, FL: Psychological Assessment Resources.

Hackman, D. A., Gallop, R., Evans, G. W., and Farah, M. J. (2015). Socioeconomic status and executive function: developmental trajectories and mediation. *Dev. Sci.* 18, 686–702. doi: 10.1111/desc.12246

Hadjiefthyvoulou, F., Fisk, J. E., Montgomery, C., and Bridges, N. (2012). Self-reports of executive dysfunction in current ecstasy/polydrug users. *Cogn. Behav. Neurol.* 25, 128–138. doi: 10.1097/WNN.0b013e318261459c

Hasler, B., and Clark, D. B. (2013). Circadian misalignment, reward-related brain function, and adolescent alcohol involvement. *Alcohol. Clin. Exp. Res.* 37, 558–565. doi: 10.1111/acer.12003

Hasler, B. P., Franzen, P., de Zambotti, M., Prouty, D., Brown, S., Tapert, S., et al. (2017). Eveningness and later sleep timing are associated with greater risk for alcohol and marijuana use in adolescence: initial findings from the NCANDA study. *Alcohol. Clin. Exp. Res.* 41, 1154–1165. doi: 10.1111/acer.13401

Hasler, B. P., Kirisci, L., and Clark, D. B. (2016). Restless sleep and variable sleep timing during late childhood accelerate the onset of alcohol and drug

involvement. *J. Stud. Alcohol Drugs* 77, 649–655. doi: 10.15288/jsad.2016.77.649

Hasler, B. P., Martin, C. S., Wood, D. S., Rosario, B., and Clark, D. B. (2014). A longitudinal study of insomnia and other sleep complaints in adolescents with and without alcohol use disorders. *Alcohol. Clin. Exp. Res.* 38, 2225–2233. doi: 10.1111/acer.12474

Klingberg, T., Vaidya, C. J., Gabrieli, J. D., Moseley, M. E., and Hedehus, M. (1999). Myelination and organization of the frontal white matter in children: a diffusion tensor MRI study. *Neuroreport* 10, 2817–2921. doi: 10.1097/00001756-199909090-00022

Kurtz, M. M., Ragland, J. D., Bilker, W., Gur, R. C., and Gur, R. E. (2001). Comparison of the continuous performance test with and without working memory demands in healthy controls and patients with schizophrenia. *Schizophr. Res.* 48, 307–316. doi: 10.1016/S0920-9964(00)00060-8

Langberg, J. M., Dvorsky, M. R., and Evans, S. W. (2013). What specific facets of executive function are associated with academic functioning in youth with attention-deficit/hyperactivity disorder? *J. Abnorm. Child Psychol.* 41, 1145–1159. doi: 10.1007/s10802-013-9750-z

Lee, N. R., Raznahan, A., Wallance, G. L., Alexander-Bloch, A., Clasen, L. S., Lerch, J. P., et al. (2014). anatomical coupling among distributed cortical regions in youth varies as a function of individual differences in vocabulary abilities. *Hum. Brain Mapp.* 35, 1885–1895. doi: 10.1002/hbm.22299

Lenroot, R. K., and Giedd, J. N. (2006). Brain development in children and adolescents: insights from anatomical magnetic resonance imaging. *Neurosci. Biobehav. Rev.* 30, 718–729. doi: 10.1016/j.neubiorev.2006.06.001

Lipton, M. L., Gulko, E., Zimmerman, M. E., Friedman, B. W., Kim, M., Gellella, E., et al. (2009). Diffusion-tensor imaging implicates prefrontal axonal injury in executive function impairment following very mild traumatic brain injury. *Radiology* 252, 816–824. doi: 10.1148/radiol.2523081584

Long, E., Luna, B., Verhulst, B., and Clark, D. B. (2015). Disruptive behavior disorders and indicators of disinhibition in adolescents: the BRIEF-SR, anti-saccade task, and D-KEFS color-word interference test. *J. Adolesc.* 44, 182–190. doi: 10.1016/j.adolescence.2015.07.003

Ludbrook, J. (1998). Multiple comparison procedures updated. *Clin. Exp. Pharmacol. Physiol.* 25, 1032–1037. doi: 10.1111/j.1440-1681.1998.tb02179.x

Mahone, E. M., Martin, R., Kates, W. R., Hay, T., and Horska, A. (2009). Neuroimaging correlates of parent ratings of working memory in typically developing children. *J. Int. Neuropsychol. Soc.* 15, 31–41. doi: 10.1017/S1355617708090164

Millman, R. P. (2005). Excessive sleepiness in adolescents and young adults: causes, consequences, and treatment strategies. *Pediatrics* 115, 1774–1786. doi: 10.1542/peds.2005-0772

Minges, K. E., and Redeker, N. S. (2016). Delayed school start times and adolescent sleep: a systematic review of the experimental evidence. *Sleep Med. Rev.* 28, 82–91. doi: 10.1016/j.smrv.2015.06.002

Mollayeva, T., Thurairajah, P., Burton, K., Mollayeva, S., Shapiro, C. M., and Colantonio, A. (2016). The Pittsburgh Sleep Quality Index as a screening tool for sleep dysfunction in clinical and non-clinical samples: a systematic review and meta-analysis. *Sleep Med. Rev.* 25, 52–73. doi: 10.1016/j.smrv.2015.01.009

National Institute on Alcohol Abuse and Alcoholism (2011). *Alcohol Screening and Brief Intervention for Youth: A Practitioner's Guide. NIH Publications 11-7805*. Bethesda, MD: National Institute on Alcohol Abuse and Alcoholism.

Noone, D. M., Willis, T. A., Cox, J., Harkness, F., Ogilvie, J., Forbes, E., et al. (2014). Catastrophizing and poor sleep quality in early adolescent females. *Behav. Sleep Med.* 12, 41–52. doi: 10.1080/15402002.2013.764528

Paus, T (2010). Growth of white matter in the adolescent brain: myelin or axon? *Brain Cogn.* 72, 26–35. doi: 10.1016/j.bandc.2009.06.002

Pfefferbaum, A., Rohlfing, T., Pohl, K. M., Lane, B., Chu, W., Kwon, D., et al. (2016). Adolescent development of cortical and white matter structure in the NCANDA sample: role of sex, ethnicity, puberty, and alcohol drinking. *Cereb. Cortex* 26, 4101–4121. doi: 10.1093/cercor/bhv205

Pohl, K. M., Sullivan, E. V., Rohlfing, T., Chu, W., Kwon, D., Nichols, B. N., et al. (2016). Harmonizing DTI measurements across scanners to examine the development of white matter microstructure in 803 adolescents of the NCANDA study. *Neuroimage* 130, 194–213. doi: 10.1016/j.neuroimage.2016.01.061

Ragland, J. D., Turetsky, B. I., Gur, R. C., Gunning-Dixon, F., Turner, T., Schroeder, L., et al. (2002). Working memory for complex figures: an fMRI comparison of letter and fractal n-back tasks. *Neuropsychology* 16, 370–379. doi: 10.1037/0894-4105.16.3.370

Riggs, N. R., and Greenberg, M. T. (2009). Neurocognition as a moderator and mediator in adolescent substance misuse prevention. *Am. J. Drug Alcohol Abuse* 35, 209–213. doi: 10.1080/00952990903005940

Rohlfing, T., Cummins, K., Henthorn, T., Chu, W., and Nichols, B. N. (2014). N-CANDA data integration: anatomy of an asynchronous infrastructure for multi-site, multi-instrument longitudinal data capture. *J. Am. Med. Inform. Assoc.* 21, 758–762. doi: 10.1136/amiajnl-2013-002367

Roth, R. M., Erdodi, L. A., McColloch, L. J., and Isquith, P. K. (2015). Much ado about norming: the Behavior Rating Inventory of Executive Function. *Child Neuropsychol.* 21, 225–233. doi: 10.1080/09297049.2014.897318

Samuels, W. E., Tournaki, N., Blackman, S., and Zilinski, C (2016). Executive functioning predicts academic achievement in middle school: a four-year longitudinal study. *J. Educ. Res.* 109, 478–490. doi: 10.1080/00220671.2014.979913

Šidák, Z. K. (1967). Rectangular confidence regions for the means of multivariate normal distributions. *J. Am. Stat. Assoc.* 62, 626–633. doi: 10.1080/01621459.1967.10482935

Shimoni, M., Engel-Yeger, B., and Tirosh, E. (2012). Executive dysfunctions among boys with attention deficit hyperactivity disorder (ADHD): performance-based test and parents report. *Res. Dev. Disabil.* 33, 858–865. doi: 10.1016/j.ridd.2011.12.014

Skranes, J., Lohaugen, G. C., Martinussen, M., Indredavik, M. S., Dale, A. M., Haraldseth, O., et al. (2009). White matter abnormalities and executive function in children with very low birth weight. *Neuroreport* 20, 263–266. doi: 10.1097/WNR.0b013e32832027fe

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* 24, 417–463. doi: 10.1016/S0149-7634(00)00014-2

Sullivan, E., Brumback, T., Tapert, S., Fama, R., Prouty, D., Brown, S. A., et al. (2016). Cognitive, emotion control, and motor baseline performance of adolescents in the NCANDA study: contributions from alcohol consumption, age, puberty, sex, ethnicity, and family history of addiction. *Neuropsychol.* 30, 449–473. doi: 10.1037/neu0000259

Sullivan, E., Brumback, T., Tapert, S., Prouty, D., Fama, R., Brown, S., et al. (2017). Effects of prior testing lasting a full year in NCANDA adolescents: contributions from age, sex, socioeconomic status, ethnicity, site, family history of alcohol or drug abuse, and baseline performance. *Develop. Cogn. Neurosci.* 24, 72–83. doi: 10.1016/j.dcn.2017.01.003

Takahashi, T., Murata, T., Omori, M., Kosaka, H., Takahashi, K., Yonekura, Y., et al. (2004). Quantitative evaluation of age-related white matter microstructural changes on MRI by multifractal analysis. *J. Neurol. Sci.* 225, 33–37. doi: 10.1016/j.jns.2004.06.016

Tapert, S. F., and Brown, S. A. (2000). Substance dependence, family history of alcohol dependence and neuropsychological functioning in adolescence. *Addiction* 95, 1043–1053. doi: 10.1046/j.1360-0443.2000.95710436.x

Tarter, R. E., Kirisci, L., Mezzich, A., Cornelius, J., Pajer, K., Vanyukov, M., et al. (2003). Neurobehavioral disinhibition in childhood predicts early age onset substance use disorder.*Am. J. Psychiatry* 160, 1078–1085. doi: 10.1176/appi.ajp.160.6.1078

Toplak, M. E., West, R. F., and Stanovich, K. E. (2013). Practitioner review: do performance-based measures and ratings of executive function assess the same construct? *J. Child Psychol. Psychiatry* 54, 131–143. doi: 10.1111/jcpp.12001

Vanyukov, M., Kirisci, L., Tarter, R. E., Ridenour, T. A., Reynolds, M. D., Maher, B., et al. (2009). Measurement of the risk for substance use disorders: phenotypic and genetic analysis of an index of common liability. *Behav. Genet.* 39, 233–244. doi: 10.1007/s10519-009-9269-9

Wang, Y., Horst, K. K., Kronenberger, W. G., Hummer, T. A., Mosier, K. M., Kalnin, A. J., et al. (2012). White matter abnormalities associated with disruptive behavior disorder in adolescents with and without attention-deficit./hyperactivity disorder. *Psychiatry Res. Neuroimag.* 202, 245–251. doi: 10.1016/j.pscychresns.2012.01.005

Wilkinson, G. S., and Robertson, G. J. (2006). *WRAT 4: Wide Range Achievement Test: Professional Manual*. Lutz, FL: Psychological Assessment Resources, Incorporated.

Willcutt, E. G., Doyle, A. E., Nigg, J. T., Faraone, S. V., and Pennington, B. F. (2005). Validity of the executive function theory of attention-deficit/hyperactivity disorder: a meta-analytic review. *Biol. Psychiatry* 57, 1336–1346. doi: 10.1016/j.biopsych.2005.02.006

Zhai, Z., Pajtek, S., Luna, B., Geier, C., Ridenour, T., and Clark, D. B. (2015). Reward modulated response inhibition, cognitive shifting, and the orbital frontal cortex in early adolescence. *J. Res. Adolesc.* 24, 753–764. doi: 10.1111/jora.12168

Ziegler, G., Dahnke, R., Winkler, A. D., and Gaser, C. (2013). Partial least squares correlation of multivariate cognitive abilities and local brain structure in children and adolescents. *Neuroimage* 82, 284–294. doi: 10.1016/j.neuroimage.2013.05.088

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: 03 November 2017
doi: 10.3389/fnbeh.2017.00205

Neural Correlates of Rewarded Response Inhibition in Youth at Risk for Problematic Alcohol Use

Brenden Tervo-Clemmens[1,2], Alina Quach[1], Beatriz Luna[1,2,3], William Foran[3], Tammy Chung[3], Michael D. De Bellis[4] and Duncan B. Clark[3]*

[1] Department of Psychology, University of Pittsburgh, Pittsburgh, PA, United States, [2] Center for the Neural Basis of Cognition, University of Pittsburgh, Pittsburgh, PA, United States, [3] Department of Psychiatry, University of Pittsburgh, Pittsburgh, PA, United States, [4] Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC, United States

Risk for substance use disorder (SUD) is associated with poor response inhibition and heightened reward sensitivity. During adolescence, incentives improve performance on response inhibition tasks and increase recruitment of cortical control areas (Geier et al., 2010) associated with SUD (Chung et al., 2011). However, it is unknown whether incentives moderate the relationship between response inhibition and trait-level psychopathology and personality features of substance use risk. We examined these associations in the current project using a rewarded antisaccade (AS) task (Geier et al., 2010) in youth at risk for substance use. Participants were 116 adolescents and young adults (ages 12–21) from the University of Pittsburgh site of the National Consortium on Adolescent Neurodevelopment and Alcohol [NCANDA] study, with neuroimaging data collected at baseline and 1 year follow up visits. Building upon previous work using this task in normative developmental samples (Geier et al., 2010) and adolescents with SUD (Chung et al., 2011), we examined both trial-wise BOLD responses and those associated with individual task-epochs (cue presentation, response preparation, and response) and associated them with multiple substance use risk factors (externalizing and internalizing psychopathology, family history of substance use, and trait impulsivity). Results showed that externalizing psychopathology and high levels of trait impulsivity (positive urgency, SUPPS-P) were associated with general decreases in antisaccade performance. Accompanying this main effect of poor performance, positive urgency was associated with reduced recruitment of the frontal eye fields (FEF) and inferior frontal gyrus (IFG) in both a priori regions of interest and at the voxelwise level. Consistent with previous work, monetary incentive improved antisaccade behavioral performance and was associated with increased activation in the striatum and cortical control areas. However, incentives did not moderate the association between response inhibition behavioral performance and any trait-level psychopathology and personality factor of substance use risk. Reward interactions were observed for BOLD responses at the task-epoch level, however, they were inconsistent across substance use risk types. The

OPEN ACCESS

Edited by:
Shannon Leigh Gourley, Emory University School of Medicine, United States

Reviewed by:
Seth Davin Norrholm, Emory University School of Medicine, United States
Manuela Sellitto, Heinrich Heine Universität Düsseldorf, Germany

***Correspondence:**
Brenden Tervo-Clemmens
bct16@pitt.edu

Received: *02 July 2017*
Accepted: *06 October 2017*
Published: *03 November 2017*

Citation:
Tervo-Clemmens B, Quach A, Luna B, Foran W, Chung T, De Bellis MD and Clark DB (2017) Neural Correlates of Rewarded Response Inhibition in Youth at Risk for Problematic Alcohol Use. Front. Behav. Neurosci. 11:205. doi: 10.3389/fnbeh.2017.00205

results from this study may suggest poor response inhibition and heightened reward sensitivity are not overlapping neurocognitive features of substance use risk. Alternatively, more subtle, common longitudinal processes might jointly explain reward sensitivity and response inhibition deficits in substance use risk.

Keywords: adolescence, response inhibition, reward, substance use, risk factors, functional magnetic resonance imaging (fMRI)

INTRODUCTION

Poor response inhibition and heightened reward sensitivity have been suggested as neurobiological risk factors for problematic substance use (Heitzeg et al., 2015). During adolescence, functional brain development supports the integration of these processes, which may influence risk for substance use initiation (Heitzeg et al., 2015; Luna and Wright, 2016). However, it is unclear how brain processes supporting cognitive-reward integration may incur risk for substance use disorders and whether these are associated with trait-level psychopathology and personality features of substance use risk.

Neuroimaging research in adolescents suggests substance use risk is associated with reduced BOLD activation in cortical brain regions supporting cognitive control. During response inhibition tasks, reduced prefrontal BOLD activation has been observed in youth who would later transition to heavy alcohol use (Norman et al., 2011) (Mahmood et al., 2013) and adolescents with a family history of alcoholism (Schweinsburg et al., 2004; see Heitzeg et al., 2015 for review). Response inhibition continues to improve through adolescence across a range of tasks (e.g., antisaccade; see Luna et al., 2010 for review), accompanied by functional changes in cortical control regions (Ordaz et al., 2013). Accordingly, poor response inhibition (c.f., Nigg et al., 2006) and reduced prefrontal BOLD activation (Norman et al., 2011) during adolescence may serve as risk factors for escalation to problematic substance use later in development.

Reduced engagement of cognitive control circuitry in youth at risk for substance use may underlie common behavioral associations between laboratory measures of response inhibition and trait-level psychopathology features of substance use risk (c.f., Young et al., 2009). To this end, neurobehavioral disinhibition (ND), a latent construct designed to asses an individual's general level of inhibition across affective, personality, and cognitive domains (c.f., Tarter et al., 2003), predicts substance use initiation (Tarter et al., 2003) and has been associated with reductions in BOLD activation in frontal cortex (McNamee et al., 2008).

Although poorer response inhibition and reduced prefrontal function have received support as neurocognitive indicators of adolescent substance use risk, previous work has typically investigated these associations in the absence of incentives on performance. However, a number of studies have demonstrated that adolescents' performance on response inhibition tasks improves when working toward an incentive (Jazbec et al., 2006; Hardin et al., 2007; Geier and Luna, 2012). Furthermore, rewarded response inhibition tasks increase BOLD activation in cortical control regions in adolescents with substance use disorder (SUD; Chung et al., 2011). This enhanced BOLD activation in youth with SUD in the context of reward may occur through enhanced reward signals from the ventral striatum, a brain region involved in the salience of reward cues (Berridge, 2007). Previous work suggests compulsive drug use is associated with a sensitization of ventral-striatal reward pathways (Robinson and Berridge, 1993). Alternatively, reward sensitivity may precede substance use initiation, as trait-level psychopathology features of substance use risk (externalizing symptoms: Bjork et al., 2010a; impulsivity: see Plichta and Scheres, 2014 review) are associated with increased BOLD activation in the ventral striatum.

In the current project, we utilized a rewarded antisaccade (AS) task to examine whether incentives enhanced response inhibition in youth at risk for substance use in a large adolescent neuroimaging sample with two time points. Building on previous work using this rewarded AS task in a normative developmental sample (Geier et al., 2010) and adolescents with SUD (Chung et al., 2011, 2015), we hypothesized that incentives would improve performance and reduce the association between substance use risk and poorer response inhibition. We first examined the relative prediction of multiple substance use risk factors on AS performance and BOLD activation. Based on the neurobehavioral disinhibition model, we predicted substance use risk factors representing poor impulse control (e.g., trait impulsivity and externalizing symptoms) would be associated with poorer AS performance and lower BOLD activation in cortical control areas. We further hypothesized these differences would be moderated by incentives, with at-risk youth showing greater reward-related improvement in response inhibition and smaller differences in inhibition-related BOLD activation during reward, relative to neutral conditions.

METHODS

Participants

Participants were recruited at the University of Pittsburgh site of the National Consortium on Adolescent Neurodevelopment and Alcohol (NCANDA) study, using procedures detailed in Brown et al. (2015). In brief, targeted catchment area calling was used to initially identify eligible youth (e.g., ages 12–21). In order to study prospective prediction for substance use risk, NCANDA recruited youth with limited substance use. However, sampling also prioritized adolescents at increased risk for alcohol use disorder (AUD) (e.g., family history of SUD), such that youth with increased risk represented 47% of the total NCANDA sample. Exclusion criteria included MRI contraindications (e.g., claustrophobia, pregnancy, non-removable metal in the body),

medical history that may influence MRI (e.g., head injury with loss of consciousness), current or persistent psychiatric disorder that may influence study completion (e.g., psychosis), and psychiatric medication (see Brown et al., 2015). The University of Pittsburgh's Institutional Review Board approved the study. Adult participants provided informed consent. For minors, parents provided informed consent and youth provided assent. Participants were compensated for completing research assessments.

Based on study-specific exclusion criteria (see below), the current analyses included 116 participants who provided useable data at baseline and/or 1-year follow up. This final analysis sample spanned adolescence into young adulthood (baseline age: 12.27–21.96, mean = 17.10, $SD = 2.60$); 56.90% ($n = 66$) female; and represented 80.17% White ($n = 93$), 18.10% ($n = 21$) Black, and 1.72 % ($n = 2$) multi-racial or other race/ethnicity (see **Table 1** for sample description). A majority (58%) of participants contributed data at both baseline and follow up (total sessions: $N = 183$; total subjects: $N = 116$; subjects at baseline: $n = 101$; subjects at follow up: $n = 82$; subjects with data at both visits: $n = 67$). As detailed below, we utilize methods robust to missing data (linear and generalized mixed effects models) and therefore include all available data (subject at visit).

Measures

Exceeds Threshold Drinking

At baseline, based on self-report from the Customary Drinking and Drug Use Record (CCDR) (Brown et al., 1998), 31 participants in the analysis sample reported alcohol use that exceeded age-adjusted National Institute on Alcohol Abuse and Alcoholism (NIAAA) guidelines for risky drinking (see Brown et al., 2015), and are considered "exceeds threshold drinkers" (ETD).

Risk Factors for Substance Use

As with other sites, the NCANDA Pittsburgh sample was recruited such that approximately half of the participants were at increased risk for problematic alcohol use. The current sample utilizes participants with increased substance use risk based on one or more of the following: early substance use onset (EOS; $n = 9$), family history of substance use disorder (FH; $n = 21$), externalizing symptoms (EXT, $n = 31$), or internalizing symptoms (INT, $n = 26$). In the current analyses, EOS, FH, EXT, and INT were categorical variables (Risk vs. No Risk) and were defined at the baseline visit as in Brown et al. (2015).

EOS was defined as consuming the first full drink of alcohol prior to age 15, based on the child's report on the CDDR (Brown et al., 1998). FH was defined as having at least one biological parent with a history suggesting a substance use disorder (SUD), based on parent report using the Family History Assessment Module (Rice et al., 1995). EXT was defined as having endorsed at least one symptom of conduct disorder or antisocial personality disorder on the Computerized Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA, Bucholz et al., 1994; Hesselbrock et al., 1999) or having an Achenbach System of Empirically Based Assessment (ASEBA; Achenbach, 2009) externalizing age- and gender-adjusted t-score

TABLE 1 | Subject characteristics in risk categories.

	EXT		INT		FH		ETD		Any Risk (Non-ETD)	
	Risk	No risk	Risk	No risk	Risk	No risk	ETD	Non-ETD	Any risk	No risk
Number of Participants	31	85	26	90	21	95	31	85	40	45
Gender n female	19	47	18	48	9	57	19	47	21	26
Age (years)	17.35 (2.55)	16.98 (2.64)	17.72 (2.25)	16.89 (2.69)	17.38 (2.85)	17.01 (2.57)	19.20 (1.47)	16.30 (2.51)	16.30 (2.34)	16.30 (2.67)
Socioeconomic status (standard score)	81.13 (15.97)	92.73 (12.31)	88.31 (15.83)	89.82 (13.95)	84.80 (15.50)	90.50 (13.97)	89.28 (15.75)	89.53 (13.93)	84.88 (14.74)	94.07 (11.55)
Generalized ability (z-score)	−0.389 (0.967)	0.085 (0.796)	0.191 (0.795)	−0.105 (0.880)	0.038 (0.820)	−0.055 (0.880)	0.199 (0.839)	−0.112 (0.865)	−0.205 (0.938)	−0.049 (0.800)
Positive urgency	1.96 (0.612)	1.77 (0.608)	1.85 (0.629)	1.82 (0.611)	1.89 (0.671)	1.81 (0.602)	1.85 (0.756)	1.82 (0.557)	1.96 (0.562)	1.69 (0.526)
Negative urgency	2.10 (0.621)	1.85 (0.641)	2.03 (0.609)	1.88 (0.652)	1.82 (0.520)	1.93 (0.667)	1.98 (0.699)	1.89 (0.628)	2.06 (0.559)	1.74 (0.657)

*All scores from baseline visit. In addition to EXT, INT, and FH, Any Risk (Non–ETD) additionally excludes EOS (n = 9). See **Table 2** for associations between variables.*

above 60. Similarly, INT was defined as having endorsed two or more symptoms or having an ASEBA internalizing t-score above 60. In the current analysis, we did not examine EOS because of the few participants meeting criteria in the Pittsburgh sample ($n = 9$).

Trait Impulsivity

In addition to risk factors described by Brown et al. (2015), we also examined associations between AS performance and BOLD activation and trait impulsivity as measured by the short version of the UPPS-P (SUPPS-P, Cyders et al., 2014). Trait impulsivity is associated with increased vulnerability to SUD (c.f., Verdejo-García et al., 2008) and has been conceptualized as having shared underlying features with antisocial behavior and substance use (Krueger et al., 2007). Although trait impulsivity and response inhibition have both been considered measures of inhibitory control, it has been suggested trait personality measures of impulsivity likely represent more global differences in impulsive choice whereas behavioral measures of response inhibition reflect specific cognitive processes (Reynolds et al., 2006). We focused on the SUPPS-P scales of negative and positive *urgency* as the urgency domain has been associated with substance use (Zapolski et al., 2009) and BOLD activation in a rewarded stop signal task (Wilbertz et al., 2014). Participants completed the SUPPS-P at both baseline and follow up visits.

Generalized Cognitive Ability

As part of the NCANDA test protocol, participants completed the Penn Computerized Neurocognitive Battery (WebCNP: Gur et al., 2010) and traditional "pencil and paper" neuropsychological tests (Sullivan et al., 2016). We utilized a composite score, generalized ability accuracy (GA; see Sullivan et al., 2016), as an outcome variable and covariate when examining substance use risk factors in our analyses. The GA measure was derived from several domains of neuropsychological function, allowing us to examine whether associations among substance use risk factors, AS performance, and BOLD activation were specific to response inhibition or instead, reflected reduced generalized cognitive ability.

Socioeconomic Status

The socioeconomic status (SES) variable was determined by both parental education and income (see Brown et al., 2015). SES was expressed as a standard score (mean = 100, $SD = 15$) and included as a covariate in secondary analyses.

Baseline and Follow up Coding of Primary Variables of Interest

In order to harmonize our analytic approach with previous work with NCDANDA baseline data (c.f., Brown et al., 2015; Müller-Oehring et al., 2017), at both baseline and follow up we utilized scores from study initiation for the primary substance use risk categories (FH, EXT, INT) and exceeds threshold drinking (ETD) as outlined in Brown et al. (2015). This approach allows our results to be interpreted more readily with other projects describing cognitive differences with baseline risk group definitions (c.f., Sullivan et al., 2016). Continuous primary measures (participant age and trait-impulsivity) were treated as time-varying covariates, with the unique scores entered at baseline and follow up.

Missing Data from Primary Variables of Interest

Based on the random effects structure of our modeling framework (see below), we were able to utilize participants that only had data at one visit (baseline or follow up). However, some participants were missing primary variables of interest. In the final behavioral sample (see below), SUPPS-P data was missing for two participants at baseline and eight participants at follow up. In the final neuroimaging sample two participants were missing SUPPS-P at baseline. As SUPPS-P was collected at both baseline and follow up, missing sessions (subject at visit) were excluded from analyses of SUPPS-P variables. One subject included in both behavioral and neuroimaging analysis was missing GA data. Six participants were missing SES data. As GA and SES were defined at baseline these participants were excluded from all analyses using these variables. No participants were missing FH, EXT, INT, or ETD data.

Rewarded Antisaccade Task

Participants completed the same rewarded antisaccade task as used in Geier et al. (2010) and Chung et al. (2011) (See **Figure 1**). The full protocol included 56 full reward trials and 56 full neutral trials, completed across four neuroimaging runs (14 reward and 14 neutral trials per run), which included three epochs (cue, preparation, and response). Full trials began with a cue epoch (1.5 s), where participants viewed a white central fixation cross, surrounded by a circle of green "$" symbols (reward trials) or blue "#" symbols (neutral trials). Next, a red fixation cross appeared, indicating the preparation epoch (1.5 s), where subjects prepared to stop an impending eye movement to an unknown location. Finally, in the response epoch (1.5 s), a peripheral cue (yellow circle) was presented along the horizontal meridian at 1 of 6 eccentricities (±3, 6, and 9 degrees visual angle, relative to fixation) and participants were instructed to perform a saccade away from the circle toward the mirror location. An additional 24 (12 rewarded; 12 neutral) partial trials that presented either

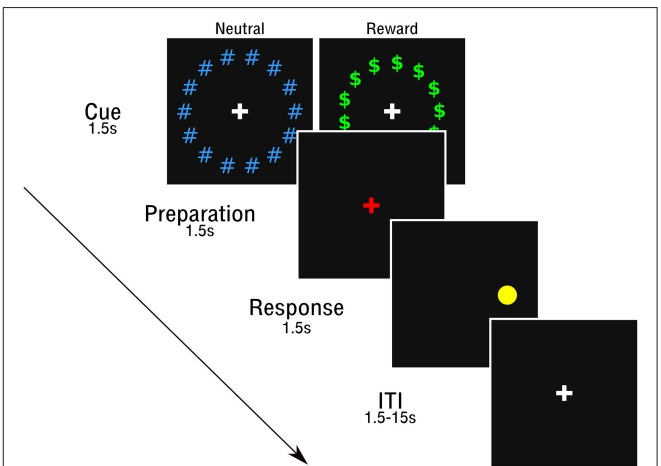


FIGURE 1 | Rewarded antisaccade task.

the cue alone (6 of each reward type) or the cue and prep epoch (6 of each reward type) but not the response epoch were included in order to estimate the hemodynamic response for each trial epoch (c.f., Ollinger et al., 2001).

Prior to the session, participants were informed they could earn a small monetary reward during trials with the "$." As in Chung et al. (2011), participants were told they could win up to $10 for correct antisaccade performance in the session and that there would be no monetary loss for incorrect responses. However, participants were not told the value of a specific response in reward trials and feedback was not provided. This prevented participants from attempting to keep track of an ongoing reward tally. At the end of the session, all participants received the full $10 reward.

Eye Movement Measurement and Scoring

Stimuli from the AS task were presented on a flat screen behind the MRI scanner with E-Prime software (Schneider et al., 2002) and made visible to the subject with a mirror mounted on the head coil. Eye-tracking was performed using a long-range optics eye-tracking system (Applied Science Laboratories, Bedford, MA) and eye-position was measured through corneal reflection. Video monitoring was performed to ensure task compliance. Prior to the test session, a 9-point calibration was performed for each subject.

Eye movements in the response epoch were a scored as a correct AS if the first eye movement during the response epoch had a velocity greater than or equal to 30°/s (Gitelman, 2002) and was made in the mirror location of the peripheral cue and extended at least 2.5° visual angle from central fixation. In contrast, eye movements were marked as an incorrect AS if the first saccade in the response epochs was made toward the peripheral cue and extended at least 2.5° visual angle from central fixation, but then later directed toward the correct location, suggesting task compliance. Trials in which no saccade occurred or if the eye-tracker lost fixation were excluded from all analyses. Scan sessions were excluded from all behavioral and fMRI analysis if the proportion of excluded trials was greater than 33%.

Within the final analysis sample ($N = 116$), the overall proportion of excluded trials was 11.4% at baseline and 12.1% at follow up. Utilizing linear mixed effects models (see below for methods), the proportion of excluded trials was positively associated with the trait impulsivity measure of negative urgency [$t = 2.01$, $\chi^2_{(1)} = 4.02$, $p = 0.045$], suggesting that those with higher levels of negative urgency had more excluded trials. Those with a family history of substance use had a significantly lower proportion of excluded trials [$t = -1.97$, $\chi^2_{(1)} = 3.87$, $p = 0.049$]. Age at visit, EXT, INT, ETD, and positive urgency were not associated with the proportion of excluded eye tracking trials (see Supplemental Table 1).

fMRI Data Acquisition

Imaging data were collected using a 3.0-T Siemens Magnetom TIM Trio at the Magnetic Resonance Research Center at the University of Pittsburgh. Structural images used for functional registration and conversion to a standardized template were collected using a magnetization prepared rapid acquisition gradient-echo (MP-RAGE) pulse sequence with a 160 slices (1.2 × 0.938 × 0.938 mm). Functional data were collected using an echo-planar imaging (EPI) sequence with the following parameters: TR = 1.5 s, TE = 28 ms, Flip Angle = 73°, and 64 × 64 acquisition matrix with a field of view of 200 mm. Twenty-nine slices were collected in the axial plane with 3.125 × 3.125 by 3.200 mm anisotropic voxels.

fMRI Data Preprocessing

Preprocessing of functional data followed standard procedures. This included slice timing correction, despiking (AFNI 3dDespike), motion correction (mcflirt; Jenkinson et al., 2002), brain extraction, non-linear registration of functional data to a standardized anatomical brain (3 mm MNI-152 template: 2009c), spatial smoothing with FWHM of 5 mm (SUSAN; Smith and Brady, 1997), high pass filtering at 80 volumes (0.00625 Hz), and scaling by 10,000 of the global median.

Data Analysis

Associations among Participant Characteristics

Associations among participant characteristics were analyzed for sample description and covariate selection (**Table 2**). In order to provide standardized associations across all

TABLE 2 | Correlations among participant characteristics.

	EXT	INT	FH	ETD	PUG	NUG	Gender	Age	SES	GA
EXT		Polychoric	Polychoric	Polychoric	Polyserial	Polyserial	Polychoric	Polyserial	Polyserial	Polyserial
INT	**0.467****		Polychoric	Polychoric	Polyserial	Polyserial	Polychoric	Polyserial	Polyserial	Polyserial
FH	0.133	0.032		Polychoric	Polyserial	Polyserial	Polychoric	Polyserial	Polyserial	Polyserial
ETD	0.056	−0.085	0.223		Polyserial	Polyserial	Polychoric	Polyserial	Polyserial	Polyserial
PUG	0.176	0.031	0.068	0.032		Pearson	Polyserial	Pearson	Pearson	Pearson
NUG	0.231	0.135	−0.103	0.075	**0.686****		Polyserial	Pearson	Pearson	Pearson
Gender	0.091	0.238	−0.241	0.091	**−0.282***	−0.095		Polyserial	Polyserial	Polyserial
Age	0.086	0.194	0.080	**0.705****	**−0.244***	−0.095	0.136		Pearson	Pearson
SES	**−0.432***	−0.060	−0.210	−0.010	−0.162	−0.109	−0.014	−0.005		Pearson
GA	**−0.306***	0.209	0.062	0.226	**−0.296***	**−0.272***	0.033	**0.433***	**0.411***	

*$p < 0.05$, **$p < 0.01$. Lower triangle displays correlations. Upper triangle displays correlation type. EXT, INT, FH, and ETD were coded with meeting criteria/risk as 1. Gender was coded with women as 1. See (Data Analysis for further discussion). Significant relationships are bolded.

variable types, Pearson (continuous-continuous), polyserial (continuous-categorical), and polychoric (categorical-categorical) correlation values (Fox, 2010) are reported (see **Table 2**). Significance testing utilized Pearson correlation for continuous-continuous associations, Welch's *t*-test for continuous-categorical associations and chi-square testing for categorical-categorical associations.

Antisaccade Behavioral Performance

Behavioral analysis was performed using R 3.1.2 (R Core Team, 2014). Mixed effects models (lme4 package; Bates et al., 2013) were used to examine associations between antisaccade (AS) correct response rates (accuracy) and latency and exceeds threshold drinking (ETD), substance use risk factors (FH, INT, EXT), and trait impulsivity (positive and negative urgency) and whether these associations were moderated by reward.

AS correct response rates were analyzed with generalized mixed-effects models with a logit link function as trial level data was binomially distributed (correct vs. incorrect). AS latencies were analyzed with linear mixed effects models and only included correct trials. Both linear and generalized-linear mixed-effects models used maximum likelihood estimation. Random intercepts were estimated for each subject. Reward condition (reward/neutral), visit (baseline/follow up), age at visit, exceeds threshold drinking (ETD), substance use risk factors (FH, EXT, INT), trait impulsivity measures (positive and negative urgency), generalized ability (GA), and socioeconomic status (SES) were included as fixed effects in three types of models. In the first type (Type A), ETD, FH, EXT, INT, and impulsivity measures were examined as predictors of AS performance in separate models. Second (Type B), ETD, FH, EXT, INT, and impulsivity measures were examined as joint predictors of AS performance in the same model. Third (Type C), GA and SES were included as additional covariates to the joint model. Reward, visit, and age at visit were included in all three model types. Secondary analysis included gender as a covariate in analysis of positive urgency (see below). Significance values for fixed effects were obtained through the car package in R (Fox et al., 2016; Wald chi-square test). Given the correlation between our variables of interest and our goal of defining sensitive and specific associations between substance use risk factors and AS performance, we highlight predictors that are significant across all three model types (A-C).

fMRI

Twelve sessions were excluded from all fMRI analyses due to poor EPI coverage across imaging runs ($n = 4$) or technical errors ($n = 8$). Accordingly, the final neuroimaging sample consisted of 171 scan sessions distributed across 111 participants (participants at baseline, $n = 95$; participants at follow up, $n = 76$; participants with data at both visits, $n = 60$). Subject-level fMRI analyses were performed with Analysis and Visualization of Functional Neuroimages (AFNI, Bethesda, MD) software. In order to estimate the BOLD response at the trial- and individual AS epoch-level (cue, preparation, response), two level 1 GLM analyses were run for each subject at each visit using AFNI's 3dDeconvolve tool.

GLM-1

Reward and neutral trial-level BOLD responses were estimated with a 4,500 ms boxcar convolved with a gamma function (AFNI's block 4) and scaled to have an amplitude of 1. Separate regressors were included for correct, incorrect, and dropped/poor eye-tracking AS trials. Partial trials were modeled in the same manner but with 1,500 ms (cue partial trial) or 3,000 ms (cue and preparation partial trial) boxcars. Six rigid-body head motion parameters and their derivatives and run-wise 0 through 3rd order polynomials were used as nuisance regressors. The current and preceding TR were censored if the Euclidean norm head motion distance surpassed 0.9 mm, based on suggestions for task-based neuroimaging outlined in Siegel et al. (2014). Parameter estimates were examined both as main effects (Task > Fixation) and in comparisons with reward type (Reward > Neutral).

GLM-2

As reward-related changes in BOLD activation have been shown to vary according to demand characteristics (c.f., Geier et al., 2010), we also estimated BOLD activation in individual task-epochs (cue, preparation, and response). Epoch-level BOLD responses were estimated in a second model with individual regressors for cue, preparation, and response epochs. Relevant partial trials were included as examples of the epoch in question to aid in epoch-specific HRF estimation. All three epochs (cue, preparation, and response) were modeled with a 1,500 ms boxcar convolved with a gamma function (AFNI's block 4). The same nuisance regressors and motion censoring were used as in GLM-1.

Regions of Interest

We first examined associations of BOLD activation with ETD and substance use risk (FH, EXT, INT, impulsivity) in a priori regions of interest (ROIs) (**Table 3**). Regions were selected based on their association with the AS task in previous voxelwise analyses (Velanova et al., 2009; Geier et al., 2010), including the frontal and supplementary eye fields (FEF and SEF), pre supplementary motor area (pre-SMA), and executive function more generally (c.f., Wesley and Bickel, 2014), including posterior parietal cortices (PPC), dorsolateral prefrontal cortex (DLPFC), ventrolateral prefrontal cortex (VLPFC), inferior frontal gyrus (IFG) and dorsal anterior cingulate cortex (dACC). Additionally, given the focus on interactions with reward, we also selected regions of interest from the striatum, including the caudate, putamen, and nucleus accumbens (NAcc), which have previously been associated with reward effects and externalizing symptoms (Bjork et al., 2010b) and rewarded response inhibition tasks (Wilbertz et al., 2014).

Cortical ROIs were taken from a previous longitudinal neuroimaging study utilizing an AS task in adolescents (Ordaz et al., 2013), where the full methods are provided. Briefly, central coordinates were identified using topic and term searches in Neurosynth (http://neurosynth.org/), a meta-analysis tool for functional neuroimaging. Minor corrections to coordinates were made to ensure final ROIs overlapped with canonical eye movement regions (c.f., Munoz and Everling, 2004). From

TABLE 3 | Regions of interest and BOLD characteristics.

	MNI X, Y, Z	Radius (mm)	Voxels (*n*)	Sessions excluded (*n*)	Neutral (*t*)	Reward (*t*)	Reward > Neutral (*t*)	Reward by hemisphere (*t*)
SUBCORTICAL								
Caudate		–		0	3.22**	5.56**	3.15**	−0.02
Left	−13.7, 13.5, 9.5		137					
Right	12.0, 13.1, 11.0		154					
Putamen		–		1	18.63**	20.02**	2.01*	<0.01
Left	−25.1, 6.8, 0.5		136					
Right	24.3, 7.0, 0.4	–	150					
NAcc		–		3	−2.28*	1.17	3.86**	0.75
Left	−9.2, 12.4, −6.9		14					
Right	8.5, 13.4, −6.5		14					
CORTICAL								
PPC				0	14.65**	16.23**	2.23*	−0.01
Left	−32, −48, 50	10	85					
Right	32, −54, 48	10	97					
FEF				0	20.18**	21.76**	2.45*	0.08
Left	−25.5, −1.5, 46	10	122					
Right	26.5, −1.5, 58	10	89					
SEF	0.0, −4.6, 62.0	7	46	0	9.37**	10.74**	2.02*	–
pre-SMA	0.0, 5.0, 52.1	7	38	0	10.48**	11.38**	1.24	–
dACC	0.0, 19.5, 40.5	10	156	0	11.43**	13.80**	2.38*	–
DLPFC				0	−0.53	2.04*	2.78**	−0.49
Left	−41.0, 19.0, 41.0	12	103					
Right	42.0, 18.0, 42.0	12	169					
VLPFC				0	6.71**	7.83**	1.36	0.03
Left	−46.5, 10.5, 24.0	10	80					
Right	49.5, 12.0 22.0	10	95					
IFG				1	7.99**	8.11**	0.13	0.17
Left	−40.0, 6.0, 0.0	12	220					
Right	40.0, 10, 2.0	12	230					

*$p < 0.05$, **$p < 0.01$.

each central coordinate, spheres were grown, with the radius determined based on anatomical size and to avoid overlap (see **Table 3**). Striatum ROIs were taken from the Harvard-Oxford Atlas distributed through FSL software (Jenkinson et al., 2012). All ROIs were eroded such that they only included voxels with a 50% or greater probability of being gray matter in the MNI-152 template.

Our final list of ROIs included several pairs of bilateral regions (**Table 3**). In order to reduce the number of comparisons and because our hypotheses were not hemisphere specific, our primary analyses utilized left and right ROI pairs in one model with hemisphere as a within-subject factor. No ROI pair had a significant reward by hemisphere interaction (**Table 3**).

Region of Interest Testing

Nonzero mean BOLD activation (parameter estimates from GLM-1 & 2; see above) from correct trials were extracted from ROIs for each subject at each visit. As in the AS behavioral analysis, linear mixed effects models were utilized to examine the association of BOLD activation with ETD and substance use risk factors (FH, EXT, INT, impulsivity) and whether these associations were moderated by reward. Random intercepts were estimated for each subject and fixed effects were included in three phases (Type A, B, C). Additionally, session-wise motion estimates (proportion of censored volumes due to head motion) were used as a covariate in all models. Secondary analysis of positive urgency included gender as a covariate, as positive urgency was higher in males than females (see below). Scan sessions (subject at a particular visit) were excluded from analysis of an ROI if it did not have at least 90% epi coverage of the ROI or ROI pair. This exclusion never resulted in more than three scan sessions being omitted. In order to maximize the precision of individual subject estimates, we first examined main effects of ETD and risk factors (FH, EXT, INT, impulsivity) and their interactions with reward on trial-wise BOLD responses (GLM-1). Subsequently, we examined whether reward effects varied by AS epoch (reward by epoch interaction) using individual BOLD estimates of cue, preparation, and response from GLM-2. In order to assist in the interpretation of the direction of effects with BOLD activation, we additionally examined the association between BOLD activation and antisaccade correct response rate (accuracy). In all cases, significance values across ROIs were

corrected for multiple comparisons using the false-discovery rate (FDR; $q < 0.05$).

Voxelwise Testing

To ensure the selection of ROIs did not bias our results we also performed voxelwise linear mixed effects analysis (3dLME; Chen et al., 2013) with age at visit and session-wise motion as covariates (model Type A). As in ROI analysis, voxelwise analysis only used data from correct trials. Voxelwise testing was limited to voxels that met each of the following criteria: 50% or greater probability of being gray matter in the MNI-152 template, full EPI coverage in all participants across all runs, and a significant simple effect of the task in reward or neutral trials (task vs. fixation), suggesting activation to the AS task significantly differed (positively or negatively). When examining ETD and risk factors (FH, INT, EXT, impulsivity), multiple comparison correction within this voxelwise space was performed using the intersection of voxelwise FDR correction ($q < 0.05$) and cluster size. AFNI's 3dClustsim program was used to determine cluster size threshold through a Monte Carlo simulation with parameters derived from mean spatial autocorrelation parameters from GLM-1 residuals.

RESULTS

Associations among Participant Characteristics

Based on the significant association of GA with EXT, PUG, and NUG and SES with EXT (**Table 2**), these variables were used as covariates in the step-wise modeling procedure (Type A-C). Additionally given that female participants had lower levels of positive urgency (mean female positive urgency baseline: 1.70; mean male positive urgency baseline: 1.98; polyserial correlation: −0.282, welch's t: −2.49, $p = 0.014$), secondary analysis of positive urgency included gender as a covariate.

Antisaccade Behavioral Performance

Accuracy

Overall, antisaccade (AS) correct response rate (accuracy) (mean = 75.31%, $SD = 16.67\%$) was comparable with previous work (Geier et al., 2010). Consistent with previous work (Geier et al., 2010), participant age was a significant positive predictor of AS accuracy [$z = 5.36$, $\chi^2_{(1)} = 28.70$, $p < 0.001$], with increased performance in older participants. Furthermore, as in Geier et al. (2010), AS accuracy was significantly higher in reward trials compared to neutral [$z = 9.01$, $\chi^2_{(1)} = 81.16$, $p < 0.001$; **Figure 2**].

Substance Use and Risk Factors

Externalizing risk [$z = -2.34$, $\chi^2_{(1)} = 5.50$, $p = 0.019$] and higher levels of the trait impulsivity measure of positive urgency [$z = -4.19$, $\chi^2_{(1)} = 17.53$, $p < 0.001$] were associated with lower AS accuracy while controlling for age and reward condition (model type A; **Figure 2**). Externalizing risk and positive urgency remained significant predictors when ETD and the other substance use risk factors were entered into a multivariate model (model type B; **Table 4**), suggesting that EXT and positive urgency were each independently associated with AS correct response rate controlling for other variables. INT, FH, ETD, and negative urgency were not significantly associated with AS accuracy (see **Table 4**).

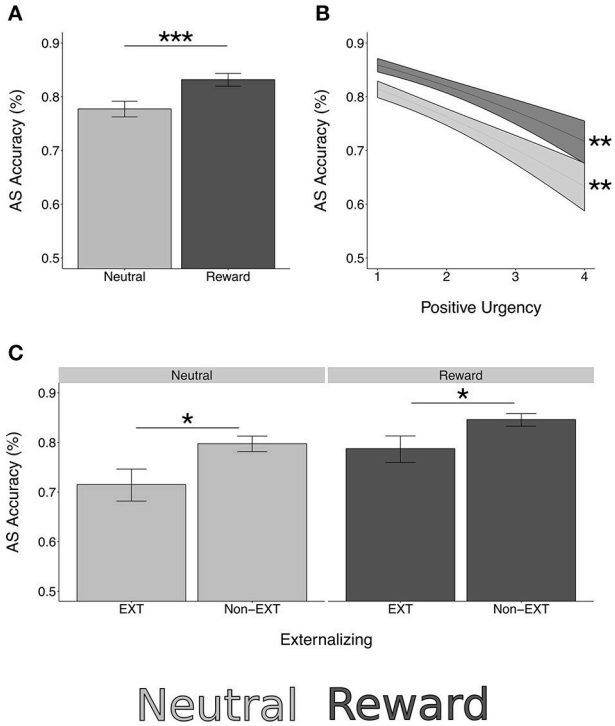


FIGURE 2 | Rewarded antisaccade behavioral performance. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$. **(A)** Antisaccade correct response rate (accuracy) is significantly higher in reward trials compared to neutral [$z = 9.01$, $\chi^2_{(1)} = 81.16$, $p < 0.0001$]. **(B)** Positive urgency is a significant negative predictor of AS accuracy [$z = -4.19$, $\chi^2_{(1)} = 17.53$, $p < 0.001$]. This association is not moderated by reward [$z = 0.32$, $\chi^2_{(1)} = 0.12$, $p = 0.750$]. **(C)** Those with externalizing risk have lower AS accuracy [$z = -2.34$, $\chi^2_{(1)} = 5.50$, $p = 0.019$]. This does not differ by reward type [$z = 0.66$, $\chi^2_{(1)} = 0.43$, $p = 0.510$]. Simple effects testing confirmed significant effects of externalizing risk and positive urgency in both reward and neutral trials (externalizing neutral: $z = -2.43$, $p = 0.015$; externalizing reward: $z = -2.10$, $p = 0.036$; positive urgency neutral: $z = -4.06$, $p = 0.001$; positive urgency reward: $z = -3.73$, $p = 0.002$).

Neither externalizing risk nor positive urgency had significant interaction terms with the AS reward condition [externalizing risk by reward: $z = 0.66$, $\chi^2_{(1)} = 0.43$, $p = 0.510$; positive urgency by reward: $z = 0.32$, $\chi^2_{(1)} = 0.12$, $p = 0.750$], suggesting equivalent performance effects in reward and neutral trials (**Table 4**, **Figure 2**). Supporting this, simple effects testing revealed externalizing risk and positive urgency were significant predictors of AS accuracy in both neutral and reward trials [externalizing neutral: $z = -2.43$, $p = 0.015$; externalizing reward: $z = -2.10$, $p = 0.036$; positive urgency neutral: -4.06, $p < 0.001$; positive urgency reward: $z = -3.73$, $p < 0.001$]. Reward interactions were also not significant for INT, FH, ETD, or negative urgency (**Table 4**).

TABLE 4 | Main effects and reward interactions from models of antisaccade performance.

	EXT	INT	FH	ETD	PUG	NUG	Age	SES	GA
ACCURACY (z)									
Main effect	**−2.34*** [B]	1.12	−0.62	1.33	**−4.19**** [B,C,G]	−1.39	**5.36**** [B,C]	0.82	**2.93****
Reward interaction	0.66	0.58	−0.63	−0.24	0.32	0.66	−1.56	0.42	−0.09
LATENCY (t)									
Main effect	0.04	0.32	−1.14	0.07	**−2.80**** [B,C,G]	−0.26	**−3.32**** [B,C]	0.55	0.25
Reward interaction	0.81	0.56	−1.30	0.45	0.42	0.69	−0.89	1.01	0.11

*$p < 0.05$, **$p < 0.01$. Displayed estimates are test statistics from models with the specific factor, subject age, visit, and reward condition (Type A). [B] $p < 0.05$ (Type B) model with all risk factors, subject age, visit, and reward condition; [C] $p < 0.05$ (Type C) model with all risk factors, subject age, visit, reward condition, and socioeconomic status (SES) and GA (generalized ability). Significant estimates are bolded. [G] Positive Urgency $p < 0.05$ while covarying gender.

Covariate Relationships

SES was not associated with AS accuracy [$z = 0.82$, $\chi^2_{(1)} = 0.68$, $p = 0.410$]. Generalized cognitive ability (GA) was positively associated with AS accuracy [$z = 2.93$, $\chi^2_{(1)} = 8.57$, $p = 0.003$]. When covarying GA (i.e., model type A + GA), the association between externalizing risk and AS accuracy was reduced to a trend [$z = -1.69$, $\chi^2_{(1)} = 2.84$, $p = 0.092$], but positive urgency remained a significant negative predictor of AS accuracy [$z = -3.88$, $\chi^2_{(1)} = 15.09$, $p < 0.001$].These results were unchanged in the full model with all other predictors (model Type C; see **Table 4**). Secondary analysis further showed positive urgency remained a significant negative predictor of AS accuracy while covarying gender [type A + gender: $z = -4.30$, $\chi^2_{(1)} = 18.52$, $p < 0.001$]. Gender was not a significant predictor in this model [$z = 1.17$, $\chi^2_{(1)} = 1.17$, $p = 0.279$].

Latency

AS latency on correct trials (mean = 440.86, *SD* = 59.62) had a negative relationship with participant age [$t = -3.32$, $\chi^2_{(1)} = 11.01$, $p = 0.001$]. AS latency was significantly shorter in rewarded trials compared to neutral trials [$t = -6.99$, $\chi^2_{(1)} = 48.89$, $p < 0.001$].

Substance Use and Risk Factors

Of the substance use and risk measures, only positive urgency was significantly associated with AS latency, where higher positive urgency scores were associated with shorter latencies [$t = -2.80$, $\chi^2_{(1)} = 7.84$, $p = 0.005$] (see **Table 4**). The relationship between positive urgency and AS latency did not vary by reward condition [$t = 0.42$, $\chi^2_{(1)} = 0.18$, $p = 0.676$].

Covariate Relationships

Neither SES [$t = 0.55$, $\chi^2_{(1)} = 0.30$, $p = 0.583$] nor GA [$t = 0.24$, $\chi^2_{(1)} = 0.06$, $p = 0.809$] was associated with AS latency. Gender was associated with AS latency, where males (least-squares mean: 421.74 ms) were faster than females (least-squares mean: 457.55 ms) on correct trials [Type A: $t = -3.55$, $\chi^2_{(1)} = 12.57$, $p = 0.0003$]. When entered as joint predictors (Type A + positive urgency and gender), positive urgency [$t = -2.47$, $\chi^2_{(1)} = 6.13$, $p = 0.013$] and gender [$t = -3.22$, $\chi^2_{(1)} = 10.35$, $p = 0.001$] remained significant predictors of AS latency.

fMRI

Head Motion

The proportion of censored volumes due to head motion was negatively associated with age [$t = -2.07$, $\chi^2_{(1)} = 4.287$, $p = 0.038$] and positively associated with both trait impulsivity measures [positive urgency: $t = 2.86$, $\chi^2_{(1)} = 8.18$, $p = 0.004$; negative urgency: $t = 2.80$ $\chi^2_{(1)} = 7.86$, $p = 0.005$]. There was also trend for those in the externalizing risk group to have more motion [$t = 1.94$ $\chi^2_{(1)} = 3.77$, $p = 0.052$]. In contrast, the proportion of censored volumes due to motion was not associated with exceeding threshold drinking [$t = 0.35$, $\chi^2_{(1)} = 0.12$, $p = 0.728$], internalizing risk [$t = -1.16$, $\chi^2_{(1)} = 1.35$, $p = 0.244$], or family history of SUD [$t = 0.50$, $\chi^2_{(1)} = 0.25$ $p = 0.617$]. As detailed in the method section, the proportion of censored volumes due to head motion was used as a covariate in all fMRI analyses.

Task Effects

Robust activation in response to the task (task > fixation) was observed in canonical AS and cognitive control regions, including bilateral frontal eye fields (FEF), posterior parietal cortices (PPC), as well as in the anterior cingulate cortex (ACC) and striatum (see **Figure 3**). All selected ROIs had significant positive BOLD activation (task > fixation) in either the neutral or reward condition (**Table 3**). Increased activation to reward was observed at the voxelwise level in the striatum, bilateral PPC, and the right middle frontal gyrus/dorsolateral prefrontal cortex (DLPFC; **Figure 3**). The majority of ROIs had greater activation in reward trials, compared to neutral (**Table 3**).

Main Effects of ETD and Substance Use Risk Factors

Within a priori ROIs, positive urgency was associated with reduced BOLD activation in the putamen [$t = -4.68$, $\chi^2_{(1)} = 21.86$, $p < 0.001$, corrected], FEF [$t = -2.64$, $\chi^2_{(1)} = 6.96$, $p = 0.031$, corrected], and inferior frontal gyrus [IFG; $t = -3.00$, $\chi^2_{(1)} = 8.99$, $p = 0.015$, corrected] (**Table 5**). Family history of SUD was associated with reduced activation in the posterior parietal cortex, although this was only a trend after multiple comparison correction [$t = -2.74$, $\chi^2_{(1)} = 7.49$, $p = 0.006$ uncorrected, $p = 0.068$ corrected]. These effects were unchanged with ETD and all substance use risk measures entered in a multivariate model (Type B) or when including SES and GA

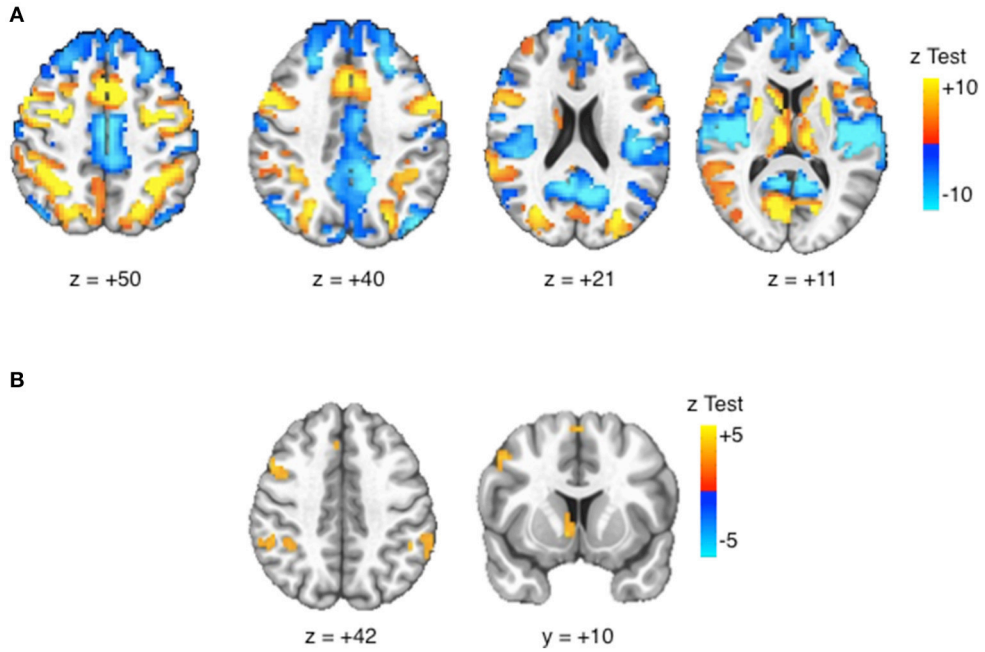


FIGURE 3 | Task BOLD effects. Activation maps displayed at voxelwise threshold $p < 0.005$, number of contiguous voxels (faces touching) > 24, cluster-level alpha <0.05. **(A)** Task > Fixation: Robust BOLD activation is observed in canonical eye movement and executive function areas. **(B)** Reward > Neutral: Increased BOLD activation is observed in striatal reward areas and attentions areas.

TABLE 5 | BOLD main effects in regions of interest (*t*-values): trial-wise (GLM-1).

	EXT	INT	FH	ETD	PUG	NUG	Age	SES	GA	AS Acc
SUBCORTICAL										
Caudate$^{L.R.}$	−1.36	−0.41	−1.03	0.04	−1.00	1.55	−1.65	−0.47	0.41	**2.48***
Putamen$^{L.R.}$	**−2.29**	−1.24	−1.33	−0.19	**−4.68***^{B,C,G}	−1.35	0.87	−0.30	0.44	**3.08***
NAcc$^{L.R.}$	−1.38	−0.21	0.06	0.45	1.39	1.68	−0.86	0.70	0.81	−0.14
CORTICAL										
PPC$^{L.R.}$	−0.91	−0.33	**−2.74+**^{B,C}	**−2.42**	−0.51	−0.20	0.10	−0.58	0.64	1.72
FEF$^{L.R.}$	**−2.05**	−0.79	−0.78	0.20	**−2.64***^{B,C,G}	−0.87	1.03	0.23	**2.09**	**2.34+**
SEF	−1.45	−0.63	−0.23	0.89	−1.06	−0.75	−0.34	−0.77	−0.55	**2.04+**
Pre-SMA	−1.79	0.25	−0.26	−1.22	−1.70	−1.88	0.34	0.06	0.84	**2.14+**
dACC	−1.53	−0.54	−0.88	−0.55	−1.59	−0.93	−0.94	−0.30	1.11	**2.57***
DLPFC$^{L.R.}$	−0.36	−0.10	−1.51	−1.25	−1.65	−0.12	−1.34	−0.62	−1.46	0.49
VLPFC$^{L.R.}$	−0.90	−1.31	−1.04	0.07	−1.34	−0.64	1.35	−1.28	0.47	−1.21
IFG$^{L.R.}$	0.43	−0.48	−1.47	−0.80	**−3.00***^{B,C,G}	−0.26	−0.05	−1.03	−1.31	1.16

$^{+}p < 0.10$ (corrected), $^{*}p < 0.05$ (corrected). Displayed estimates are test statistics from models with the specific factor, subject age, visit, and reward condition (Type A). $^{B}p < 0.05$ (Type B) model with all risk factors, subject age, visit, and reward condition; $^{C}p < 0.05$ (Type C) model with all risk factors, subject age, visit, reward condition, and SES and GA. ^{G}Positive Urgency $p < 0.05$ while covarying gender. $^{L.R.}$, Left/Right ROIs are included within one model. Estimates with uncorrected p's < 0.05 are bolded.

as covariates (Type C). Furthermore, the association between positive urgency and BOLD activation in the putamen, FEF, and IFG, remained significant while covarying gender. No other measures had significant or trending, corrected main effects.

At the voxelwise level, positive urgency had a negative relationship with BOLD activation in the left inferior frontal gyrus (IFG) and right frontal eye field [FEF; voxelwise threshold (p) = 0.0012, $q < 0.05$, number of contiguous voxels (faces touching) > 24] (**Figure 4**), confirming results from a priori ROIs. Negative urgency had two voxels that reached FDR corrected significance ($q < 0.05$). EXT, INT, FH, ETD, and negative urgency did not have significant voxelwise BOLD main effects (minimum q's > 0.072).

Reward Interactions of Substance Use and Risk Factors

Trial-Wise Reward Interactions

With trial-wise BOLD responses (GLM-1), ETD and substance use risk measures did not have a significant, corrected interaction with reward in a priori ROIs (**Table 6**) or at the voxelwise level (minimum q's > 0.876).

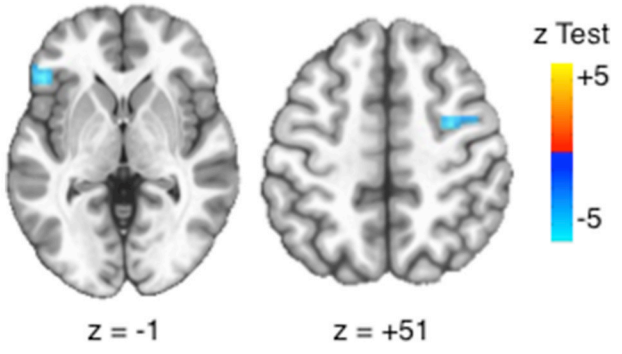


FIGURE 4 | Voxelwise main effects of positive urgency. Positive urgency had a significant, voxelwise main effect with BOLD activation in the left inferior frontal gyrus (IFG: $x = 49.5$, $y = 28.5$, $z = -1.5$; 32 voxels; peak test statistic = −5.93) and right frontal eye field (FEF; $x = -31.5$, $y = 1.5$, $z = 49.5$, 25 voxels, peak test statistic = −5.52). Significance based on intersection of FDR-correction and cluster size [voxelwise threshold (p) = 0.0012, $q < 0.05$, number of contiguous voxels (faces touching) > 24].

Epoch Reward Interactions

Significant BOLD reward interactions for AS task epochs in ROIs are presented in **Table 7**. See Supplemental Tables 2–4 for full list. No reward interactions were observed in the cue epoch. In the preparation epoch, there was a significant interaction between subject age and reward condition in the NAcc ROI [$t = 2.85$, $\chi^2_{(1)} = 8.10$, $p = 0.048$, corrected]. *Post hoc* testing revealed a significant simple effect of age in reward trials [$t_{(204.03)} = 2.38$, $p = 0.018$] but a non-significant simple effect of age in neutral trials [$t_{(204.03)} = -0.45$, $p = 0.651$]. A significant reward interaction was also observed in the preparation epoch for exceeds threshold drinking in the FEF ROI [$t = -2.92$, $\chi^2_{(1)} = 8.54$, $p = 0.038$, corrected] with *post-hoc* testing demonstrating that those who exceeded threshold drinking had higher BOLD activation than those not meeting criteria in the neutral trials [$t_{(150.98)} = 3.47$, $p < 0.001$], but not reward trials [$t_{(150.98)} = 1.41$, $p = 0.158$] (**Figure 5**).

In the response epoch, there was a significant reward interaction for positive urgency in the SEF [$t = 3.06$, $\chi^2_{(1)} = 9.35$, $p = 0.024$, corrected], with *post-hoc* testing demonstrating a significant simple effect of positive urgency in reward trials [$t_{(323.86)} = 2.24$, $p = 0.026$] but a non-significant simple effect in neutral trials [$t_{(323.86)} = -1.10$, $p = 0.274$] (**Figure 5**). A reward interaction was also observed for externalizing risk in the IFG during the response epoch [$t = 2.96$, $\chi^2_{(1)} = 8.75$, $p = 0.034$, corrected], but *post-hoc* testing revealed non-significant simple effects in both reward and neutral trials (p's > 0.0998). However, no reward interactions were significant at the voxelwise level (minimum q's > 0.107).

DISCUSSION

In this study, we examined neural correlates of rewarded response inhibition in youth at risk for problematic substance use. Within a two time point adolescent neuroimaging dataset, we tested whether incentives moderated the association between response inhibition and trait-level psychopathology and personality features of substance use risk. In multivariate models, the trait impulsivity measure of positive urgency and externalizing risk were significant negative predictors of AS correct response rate. Positive urgency was associated with reduced trial-wise BOLD activation in a priori ROIs representing frontal eye fields and inferior frontal gyrus, which were confirmed at the voxelwise level. However, contrary to our hypothesis, limited evidence suggested differential associations between response inhibition and substance use risk factors in the context of reward. Significant interactions between reward and substance use risk factors were not observed with AS behavioral performance or trial-wise BOLD estimates. When examining reward effects in discrete stages of the AS task (e.g., cue, preparation, response), some reward interactions were observed in a priori ROIs, but no reward interaction effects were observed at the voxelwise level.

Substance Use Risk and Brain Systems Supporting Response Inhibition

Consistent with previous work (Young et al., 2009), externalizing risk was associated with poorer response inhibition. However, externalizing risk was also associated with lower general cognitive ability (GA) and when GA was included as a covariate, the association between externalizing risk and AS performance was reduced to a trend. This suggests the association between externalizing risk and AS performance may be driven in part, by aspects of cognition that are not specific to inhibition. In contrast, higher levels of the trait impulsivity measure of positive urgency were associated with poorer AS performance, while accounting for GA. Higher scores of positive urgency also predicted shorter latencies to correct AS responses. This pattern is consistent with the notion of high levels of trait impulsivity predicting a speed-accuracy tradeoff in cognitive tests (Dickman and Meyer, 1988). We further demonstrate positive urgency is associated with reduced BOLD activation in the inferior frontal gyrus (IFG), frontal eye fields (FEF), and putamen. The negative association between urgency and BOLD activation in the IFG is consistent with recent work examining UPPS-P urgency domain score in a rewarded go/no-go task (Wilbertz et al., 2014). We extend this result by demonstrating this association may be specific to positive urgency, rather than negative urgency.

Positive urgency is conceptualized as the tendency to act rashly/impulsively in response to high levels of positive affect (Cyders et al., 2007). Theoretical models implicate the ventromedial prefrontal cortex (VMPFC) in affective instability and impulsivity (see Cyders and Smith, 2008), highlighting evidence from patients with damage to this region (Bechara, 2004). However, in the absence of a significant interaction between positive urgency and reward in AS behavioral performance, our results suggest positive urgency is associated with a general response inhibition deficit. Accordingly, we observed negative associations between positive urgency and BOLD activation in multiple regions associated with response inhibition, including IFG (c.f., Aron et al., 2003) and FEF (c.f.,

TABLE 6 | BOLD reward interactions in regions of interest (*t*-values): trial-wise (GLM-1).

	EXT	INT	FH	ETD	PUG	NUG	Age	SES	GA	AS Acc
SUBCORTICAL										
Caudate$^{L.R.}$	−1.12	1.01	0.85	−0.54	0.27	−1.36	0.31	−1.39	−0.28	−1.11
Putamen$^{L.R.}$	0.47	1.75	−0.05	−0.40	−0.11	−0.88	0.28	−1.77	0.30	−0.44
NAcc$^{L.R.}$	−0.21	0.48	0.91	−0.10	−0.27	−1.42	0.09	−1.39	−0.02	0.58
CORTICAL										
PPC$^{L.R.}$	0.29	1.26	0.56	0.15	0.84	0.12	−0.11	−0.66	−0.53	−0.03
FEF$^{L.R.}$	−0.21	0.79	0.97	−0.43	1.31	0.18	−0.35	−0.76	0.06	−0.28
SEF	−0.55	0.31	1.73	−0.32	**2.11**	0.16	−0.54	−1.02	−0.30	−0.53
Pre-SMA	−0.64	0.71	1.17	−0.62	0.76	−0.35	−0.15	−0.74	−0.21	−0.43
dACC	−0.71	0.84	0.20	−0.05	0.60	−0.77	0.04	−0.82	−0.26	−0.45
DLPFC$^{L.R.}$	−0.68	1.08	0.97	−0.45	0.35	−1.33	−0.22	−0.82	−0.45	−1.37
VLPFC$^{L.R.}$	−0.21	1.14	−0.10	−0.50	−0.74	−1.74	0.07	−0.58	−0.16	−0.70
IFG$^{L.R.}$	−0.35	1.92	−0.56	0.01	−0.24	−1.13	0.30	−1.13	−0.64	−0.64

Displayed estimates are test statistics from models with the specific factor, subject age, visit, and reward condition (Type A). $^{L.R.}$*, Left/Right ROIs are included within one model. Estimates with uncorrected p's < 0.05 are bolded.*

TABLE 7 | BOLD reward interactions of antisaccade epochs in regions of interest (GLM-2).

	Variable	Reward interaction (*t*)	Simple effect: neutral (*t*)	Simple effect: reward (*t*)
PREPARATION				
NAcc$^{L.R.}$	Age	2.85^{*B,C}	−0.45	2.38*
FEF$^{L.R.}$	ETD	−2.92^{*B,C}	3.47**	1.41
RESPONSE				
SEF	PUG	3.06^{*B,C,G}	−1.10	2.24*
IFG$^{L.R.}$	EXT	2.96^{*B,C}	−1.65	0.93

Reward interaction estimates are test statistics from models with the specific variable, subject age, visit, and reward condition (Type A) that were significant after multiple comparison correction. See Supplemental Tables 2–4 for full epoch reward interactions. $^{B}p < 0.05$ *(Type B) model with all risk factors, subject age, visit, and reward condition;* $^{C}p < 0.05$ *(Type C) model with all risk factors, subject age, visit, reward condition, and SES and GA.* ^{G}*Positive Urgency* $p < 0.05$ *while covarying gender.* $^{L.R.}$*, Left/Right ROIs are included within one model. Simple effect neutral and reward refer to test statistics from association between variable and BOLD activation in neutral and reward trials, respectively:* $^{*}p < 0.05$, $^{**}p < 0.01$.

Muggleton et al., 2010) and the putamen ROI (Zandbelt and Vink, 2010).

Both positive urgency and externalizing risk were significant negative predictors of AS correct response rate. This is consistent with an association between disinhibited psychopathology and poorer response inhibition (Young et al., 2009). Moreover, although not significant after correction for multiple comparisons, externalizing risk was associated with lower BOLD activation in a priori FEF and putamen ROIs. To this end, our data provide some support for a notion of common neurocognitive correlates to disinhibited psychopathology and substance use risk. Moreover, associations between positive urgency and externalizing risk and AS response inhibition appear functionally distinct from other forms of psychopathology, as no other risk factors had significant (corrected or uncorrected) associations with AS behavioral performance or BOLD activation in FEF or the putamen.

Reward Interactions and Substance Use Risk

Consistent with previous work utilizing this rewarded AS task (Geier et al., 2010) and other response inhibition tasks (Hardin et al., 2007), the availability of reward improved response inhibition performance (increased correct response rate and reduced latency). However, no substance use risk measures had significant interactions with reward in AS behavioral performance or trial-wise BOLD estimates. This result may suggest that response inhibition deficits and reward sensitivity may be non-overlapping features of substance use risk, which is consistent with factor analytic work suggesting substance use risk is a multidimensional construct (Woicik et al., 2009).

Previous work suggests BOLD activation associated with reward modulation may vary according to the cognitive processes engaged during different epochs of the AS task (c.f., Geier et al., 2010; Chung et al., 2011). To this end, we observed significant BOLD reward modulation when examining specific AS epochs in a priori ROIs, with exceeding threshold drinking associated with increased BOLD activation in FEF during response preparation in neutral trials, but not reward trials. This result is consistent with previous work using this task in adolescents with SUD (Chung et al., 2011) and provides some support for the notion that incentives may normalize response inhibition differences in SUD. Given the pattern of greater differences in neutral trials compared to reward trials was specific to SUD and not substance use risk factors, its possible this effect reflects a sensitization to rewards following substance use initiation. To this end, positive urgency was associated with increased BOLD activation during response preparation in the supplementary eye fields during reward trials, but not neutral trials. However, reward interactions were not observed at the voxelwise level, indicating the need for caution in interpreting possible effects of reward on regional BOLD activation.

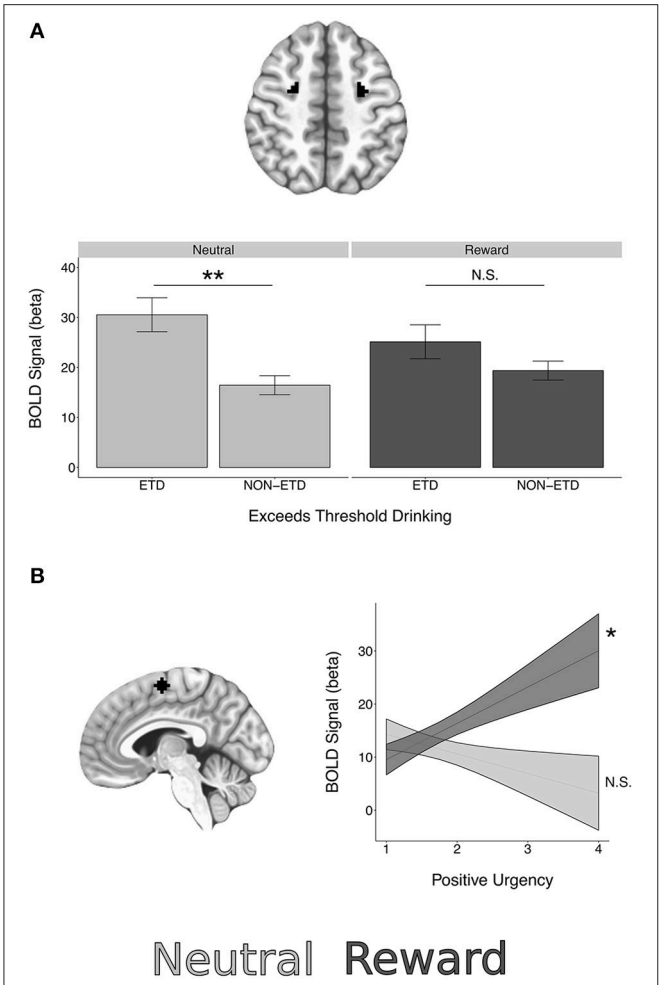


FIGURE 5 | BOLD epoch reward interactions in regions of interest. **(A)** A significant interaction between reward and exceeds threshold drinking was observed in the FEF ROIs during the preparation epoch. **(B)** A significant interaction between reward and positive urgency was observed in the SEF ROI during the response epoch. See **Table 7** for interaction statistics and simple effects testing. $^{*}p < 0.05$, $^{**}p < 0.01$.

A possible explanation for a lack of reward modulation of the association between response inhibition and substance use risk factors in this study may be from our focus on general associations across development. Previous work suggests developmental changes within reward circuitry during adolescence (Larsen and Luna, 2015). Supporting this we found age by reward interactions in the NAcc during the preparation epoch, with greater age-related changes in reward trials compared to neutral. This result is consistent with previous work (Bjork et al., 2010b) suggesting adults have greater NAcc activation during reward anticipation. Accordingly, reward interactions with substance use risk may emerge later in development (Müller et al., 2015). The degree of the moderating effect of reward on the association between substance use risk factors and response inhibition may also depend on the magnitude of the incentive offered. Given the ongoing data-collection within the NCANDA sample, future work may explore within-subject, longitudinal changes during development in relation to reward interactions with substance use risk.

Limitations

This project was characterized by a number of strengths, including a relatively large sample size, multiple time points (baseline and follow up visits), and extensive characterization of psychopathology and personality variables associated with substance use risk. However, it is worth noting a few limitations. First, we coded participants risk scores at both baseline and follow-up visits based on baseline risk factors established by NCANDA. Combined with the use of mixed effects models, this procedure allowed us to increase the precision of between-subject estimates of risk factors groups as defined in previous NCANDA projects (c.f., Brown et al., 2015) and utilize all data from baseline and follow up visits. Nevertheless, subtle variation in meeting criteria for certain risk factors (e.g., externalizing and internalizing risk and exceeding threshold drinking) may have occurred between baseline and follow up visits and we did not examine within-subject change (difference scores between baseline and 1-year follow-up). Accordingly, future work may utilize more complex modeling frameworks with random growth terms to better characterize within-subject changes and joint maturation of substance use risk and brain activation.

Another potential limitation of the analyses is the focus on bivariate relationships between substance use risk factors and AS performance and BOLD activation. Although we examined interactions between substance use risk and reward processes, these were completed iteratively across risk factors. This procedure, combined with multivariate regression, allowed us to examine the specificity of associations between particular substance use risk factors and AS performance and BOLD activation. However, several of our risk factors were significantly correlated with one another. To this end, previous work suggests substance use risk may be characterized by higher-order, latent dimensions that may explain risk factor covariation (c.f., Tarter et al., 2003; Woicik et al., 2009). Future work could utilize latent variable analysis to examine whether higher-order factors of substance use risk display significant interactions with reward on response inhibition tasks.

CONCLUSION

Utilizing a rewarded antisaccade task during fMRI acquisition, the results from this project confirm previous work suggesting substance use risk, and specifically externalizing psychopathology and trait impulsivity, are associated with poorer response inhibition. Furthermore, we found that higher levels of positive urgency are associated with reduced BOLD activation in FEF and IFG. However, we found little evidence that monetary incentive moderated the association between substance use risk factors and AS behavioral performance or BOLD activation. Further work is needed to determine the parameters (e.g., type, magnitude of reward) under which incentives increase response inhibition, examine overlap between substance use

risk factors, and investigate within-subject longitudinal change in the interplay between sensitivity to reward and response inhibition.

AUTHOR CONTRIBUTIONS

BT, BL, and DC designed analysis. BT and AQ completed analysis. BT and WF performed preprocessing and quality assurance on imaging data. BT constructed manuscript with input and feedback from BL, TC, MD, and DC.

FUNDING

National Institute on Alcohol Abuse and Alcoholism (NIAA): U0AA1021690; U01AA021681.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2017.00205/full#supplementary-material

REFERENCES

Achenbach, T. M. (2009). *The Achenbach System of Empirically Based Assessemnt (ASEBA): Development, Findings, Theory, and Applications*. Burlington, VT: University of Vermont Research Center for Children, Youth, and Families.

Aron, A. R., Fletcher, P. C., Bullmore, E. T., Sahakian, B. J., and Robbins, T. W. (2003). Stop-signal inhibition disrupted by damage to right inferior frontal gyrus in humans. *Nat. Neurosci.* 6, 115–116. doi: 10.1038/nn1003

Bates, D., Maechler, M., and Bolker, B. (2013). *lme4: Linear Mixed-Effects Models Using S4 Classes*. Available online at: http://CRAN.R-project.org/package=lme4

Bechara, A. (2004). The role of emotion in decision-making: evidence from neurological patients with orbitofrontal damage. *Brain Cogn.* 55, 30–40. doi: 10.1016/j.bandc.2003.04.001

Berridge, K. C. (2007). The debate over dopamine's role in reward: the case for incentive salience. *Psychopharmacology* 191, 391–431. doi: 10.1007/s00213-006-0578-x

Bjork, J. M., Chen, G., Smith, A. R., and Hommer, D. W. (2010a). Incentive-elicited mesolimbic activation and externalizing symptomatology in adolescents. *J. Child Psychol. Psychiatry* 51, 827–837. doi: 10.1111/j.1469-7610.2009.02201.x

Bjork, J. M., Smith, A. R., Chen, G., and Hommer, D. W. (2010b). Adolescents, adults and rewards: comparing motivational neurocircuitry recruitment using fMRI. *PLoS ONE* 5:e11440. doi: 10.1371/journal.pone.0011440

Brown, S. A., Brumback, T., Tomlinson, K., Cummins, K., Thompson, W. K., and Nagel, B. J., et al. (2015). The National Consortium on Alcohol and NeuroDevelopment in Adolescence (NCANDA): a multisite study of adolescent development and substance use. *J. Stud. Alcohol Drugs* 76, 895–908. doi: 10.15288/jsad.2015.76.895

Brown, S. A., Myers, M. G., Lippke, L., Tapert, S. F., Stewart, D. G., and Vik, P. W. (1998). Psychometric evaluation of the Customary Drinking and Drug Use Record (CDDR): a measure of adolescent alcohol and drug involvement. *J. Stud. Alcohol* 59, 427–438. doi: 10.15288/jsa.1998.59.427

Bucholz, K. K., Cadoret, R., Cloninger, C. R., Dinwiddie, S. H., Hesselbrock, V. M., Nurnberger, J. I., et al. (1994). A new, semi-structured psychiatric interview for use in genetic linkage studies: a report on the reliability of the SSAGA. *J. Stud. Alcohol* 55, 149–158. doi: 10.15288/jsa.1994.55.149

Chen, G., Saad, Z. S., Britton, J. C., Pine, D. S., and Cox, R. W. (2013). Linear mixed-effects modeling approach to FMRI group analysis. *Neuroimage* 73, 176–190. doi: 10.1016/j.neuroimage.2013.01.047

Chung, T., Geier, C., Luna, B., Pajtek, S., Terwilliger, R., Thatcher, D., et al. (2011). Enhancing response inhibition by incentive: comparison of adolescents with and without substance use disorder. *Drug Alcohol Depend.* 115, 43–50. doi: 10.1016/j.drugalcdep.2010.10.017

Chung, T., Paulsen, D. J., Geier, C. F., Luna, B., and Clark, D. B. (2015). Regional brain activation supporting cognitive control in the context of reward is associated with treated adolescents' marijuana problem severity at follow-up: a preliminary study. *Dev. Cogn. Neurosci.* 16, 93–100. doi: 10.1016/j.dcn.2015.05.004

Cyders, M. A., Littlefield, A. K., Coffey, S., and Karyadi, K. A. (2014). Examination of a short English version of the UPPS-P Impulsive Behavior Scale. *Addict. Behav.* 39, 1372–1376. doi: 10.1016/j.addbeh.2014.02.013

Cyders, M. A., and Smith, G. T. (2008). Emotion-based dispositions to rash action: positive and negative urgency. *Psychol. Bull.* 134:807. doi: 10.1037/a0013341

Cyders, M. A., Smith, G. T., Spillane, N. S., Fischer, S., Annus, A. M., and Peterson, C. (2007). Integration of impulsivity and positive mood to predict risky behavior: Development and validation of a measure of positive urgency. *Psychol. Assess.* 19:107. doi: 10.1037/1040-3590.19.1.107

Dickman, S. J., and Meyer, D. E. (1988). Impulsivity and speed-accuracy tradeoffs in information processing. *J. Pers. Soc. Psychol.* 54:274. doi: 10.1037/0022-3514.54.2.274

Fox, J. (2010). *Polycor: Polychoric and Polyserial Correlations. R Package Version 0.7-8*. [*Online*]. Available online at: https://cran.r-project.org/web/packages/polycor/index.html (Accessed August 31, 2010).

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., and Ellison, S., et al. (2016). *Package "car." Companion to Applied Regression*. R Package Version, 2–1.

Geier, C. F., and Luna, B. (2012). Developmental effects of incentives on response inhibition. *Child Dev.* 83, 1262–1274. doi: 10.1111/j.1467-8624.2012.01771.x

Geier, C. F., Terwilliger, R., Teslovich, T., Velanova, K., and Luna, B. (2010). Immaturities in reward processing and its influence on inhibitory control in adolescence. *Cereb. Cortex* 20, 1613–1629. doi: 10.1093/cercor/bhp225

Gitelman, D. R. (2002). ILAB: a program for postexperimental eye movement analysis. *Behav. Res. Methods*, 34, 605–612. doi: 10.3758/BF03195488

Gur, R. C., Richard, J., Hughett, P., Calkins, M. E., Macy, L., Bilker, W. B., et al. (2010). A cognitive neuroscience-based computerized battery for efficient measurement of individual differences: standardization and initial construct validation. *J. Neurosci. Methods* 187, 254–262. doi: 10.1016/j.jneumeth.2009.11.017

Hardin, M. G., Schroth, E., Pine, D. S., and Ernst, M. (2007). Incentive-related modulation of cognitive control in healthy, anxious, and depressed adolescents: development and psychopathology related differences. *J. Child Psychol. Psychiatry Allied Discip.* 48, 446–454. doi: 10.1111/j.1469-7610.2006.01722.x

Heitzeg, M. M., Cope, L. M., Martz, M. E., and Hardee, J. E. (2015). Neuroimaging risk markers for substance abuse: recent findings on inhibitory control and reward system functioning. *Curr. Addict. Rep.* 2, 91–103. doi: 10.1007/s40429-015-0048-9

Hesselbrock, M., Easton, C., Bucholz, K. K., Schuckit, M., and Hesselbrock, V. (1999). A validity study of the SSAGA-a comparison with the SCAN. *Addiction* 94, 1361–1370. doi: 10.1046/j.1360-0443.1999.94913618.x

Jazbec, S., Hardin, M. G., Schroth, E., McClure, E., Pine, D. S., and Ernst, M. (2006). Age-related influence of contingencies on a saccade task. *Exp. Brain Res.* 174, 754–762. doi: 10.1007/s00221-006-0520-9

Jenkinson, M., Bannister, P., Brady, M., and Smith, S. (2002). Improved optimization for the robust and accurate linear registration and motion correction of brain images. *Neuroimage* 17, 825–841. doi: 10.1006/nimg.2002.1132

Jenkinson, M., Beckmann, C. F., Behrens, T. E., Woolrich, M. W., and Smith, S. M. (2012). FSL. *Neuroimage* 62, 782–790. doi: 10.1016/j.neuroimage.2011.09.015

Krueger, R. F., Markon, K. E., Patrick, C. J., Benning, S. D., and Kramer, M. D. (2007). Linking antisocial behavior, substance use, and personality: an integrative quantitative model of the adult externalizing spectrum. *J. Abnorm. Psychol.* 116:645. doi: 10.1037/0021-843X.116.4.645

Larsen, B., and Luna, B. (2015). *In vivo* evidence of neurophysiological maturation of the human adolescent striatum. *Dev. Cogn. Neurosci.* 12, 74–85. doi: 10.1016/j.dcn.2014.12.003

Luna, B., Padmanabhan, A., and O'Hearn, K. (2010). What has fMRI told us about the development of cognitive control through adolescence? *Brain Cogn.* 72, 101–113. doi: 10.1016/j.bandc.2009.08.005

Luna, B., and Wright, C. (2016). "Adolescent brain development: implications for the juvenile criminal justice system," in *APA Handbook of Psychology and Juvenile Justice*, eds K. Heilbrun, D. De-Matteo, and N. Goldstein (Washington, DC: American Psychological Association), 91–116.

Mahmood, O. M., Goldenberg, D., Thayer, R., Migliorini, R., Simmons, A. N., and Tapert, S. F. (2013). Adolescents' fMRI activation to a response inhibition task predicts future substance use. *Addict. Behav.* 38, 1435–1441. doi: 10.1016/j.addbeh.2012.07.012

McNamee, R. L., Dunfee, K. L., Luna, B., Clark, D. B., Eddy, W. F., and Tarter, R. E. (2008). Brain activation, response inhibition, and increased risk for substance use disorder. *Alcohol. Clin. Exp. Res.* 32, 405–413. doi: 10.1111/j.1530-0277.2007.00604.x

Muggleton, N. G., Chen, C. Y., Tzeng, O. J., Hung, D. L., and Juan, C.-H. (2010). Inhibitory control and the frontal eye fields. *J. Cogn. Neurosci.* 22, 2804–2812. doi: 10.1162/jocn.2010.21416

Müller, K. U., Gan, G., Banaschewski, T., Barker, G. J., Bokde, A. L., and Büchel, C., et al. (2015). No differences in ventral striatum responsivity between adolescents with a positive family history of alcoholism and controls. *Addict. Biol.* 20, 534–545. doi: 10.1111/adb.12136

Müller-Oehring, E. M., Kwon, D., Nagel, B. J., Sullivan, E. V., Chu, W., Rohlfing, T., et al. (2017). Influences of age, sex, and moderate alcohol drinking on the intrinsic functional architecture of adolescent brains. *Cereb. Cortex*. doi: 10.1093/cercor/bhx014. [Epub ahead of print].

Munoz, D. P., and Everling, S. (2004). Look away: the anti-saccade task and the voluntary control of eye movement. *Nat. Rev. Neurosci.* 5, 218–228. doi: 10.1038/nrn1345

Nigg, J. T., Wong, M. M., Martel, M. M., Jester, J. M., Puttler, L. I., Glass, J. M., et al. (2006). Poor response inhibition as a predictor of problem drinking and illicit drug use in adolescents at risk for alcoholism and other substance use disorders. *J. Am. Acad. Child Adolesc. Psychiatry* 45, 468–475. doi: 10.1097/01.chi.0000199028.76452.a9

Norman, A. L., Pulido, C., Squeglia, L. M., Spadoni, A. D., Paulus, M. P., and Tapert, S. F. (2011). Neural activation during inhibition predicts initiation of substance use in adolescence. *Drug Alcohol Depend.* 119, 216–223. doi: 10.1016/j.drugalcdep.2011.06.019

Ollinger, J. M., Shulman, G. L., and Corbetta, M. (2001). Separating processes within a trial in event-related functional MRI: I. The method. *Neuroimage* 13, 210–217. doi: 10.1006/nimg.2000.0710

Ordaz, S. J., Foran, W., Velanova, K., and Luna, B. (2013). Longitudinal growth curves of brain function underlying inhibitory control through adolescence. *J. Neurosci.* 33, 18109–18124. doi: 10.1523/JNEUROSCI.1741-13.2013

Plichta, M. M., and Scheres, A. (2014). Ventral–striatal responsiveness during reward anticipation in ADHD and its relation to trait impulsivity in the healthy population: a meta-analytic review of the fMRI literature. *Neurosci. Biobehav. Rev.* 38, 125–134. doi: 10.1016/j.neubiorev.2013.07.012

R Core Team (2014). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Reynolds, B., Ortengren, A., Richards, J. B., and de Wit, H. (2006). Dimensions of impulsive behavior: Personality and behavioral measures. *Pers. Individ. Dif.* 40, 305–315. doi: 10.1016/j.paid.2005.03.024

Rice, J. P., Reich, T., Bucholz, K. K., Neuman, R. J., Fishman, R., Rochberg, N., et al. (1995). Comparison of direct interview and family history diagnoses of alcohol dependence. *Alcoholism* 19, 1018–1023. doi: 10.1111/j.1530-0277.1995.tb00983.x

Robinson, T. E., and Berridge, K. C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res. Rev.* 18, 247–291. doi: 10.1016/0165-0173(93)90013-P

Schneider, W., Eschman, A., and Zuccolotto, A. (2002). *E-Prime: User's Guide*. Pittsburgh, PA: Psychology Software Tools Inc.

Schweinsburg, A. D., Paulus, M. P., Barlett, V. C., Killeen, L. A., Caldwell, L. C., Pulido, C., et al. (2004). An FMRI study of response inhibition in youths with a family history of alcoholism. *Ann. N. Y. Acad. Sci.* 1021, 391–394. doi: 10.1196/annals.1308.050

Siegel, J. S., Power, J. D., Dubis, J. W., Vogel, A. C., Church, J. A., Schlaggar, B. L., et al. (2014). Statistical improvements in functional magnetic resonance imaging analyses produced by censoring high-motion data points. *Hum. Brain Mapp.* 35, 1981–1996. doi: 10.1002/hbm.22307

Smith, S., and Brady, J. (1997). SUSAN - a new approach to low level image processing. *Int. J. Comput. Vis.* 23, 45–78. doi: 10.1023/A:1007963824710

Sullivan, E. V., Brumback, T., Tapert, S. F., Fama, R., Prouty, D., and Brown, S. A., et al. (2016). Cognitive, emotion control, and motor performance of adolescents in the NCANDA study: contributions from alcohol consumption, age, sex, ethnicity, and family history of addiction. *Neuropsychology* 30:449. doi: 10.1037/neu0000259

Tarter, R. E., Kirisci, L., Mezzich, A., Cornelius, J. R., Pajer, K., Vanyukov, M., et al. (2003). Neurobehavioral disinhibition in childhood predicts early age at onset of substance use disorder. *Am. J. Psychiatry* 160, 1078–1085. doi: 10.1176/appi.ajp.160.6.1078

Velanova, K., Wheeler, M. E., and Luna, B. (2009). The maturation of task set-related activation supports late developmental improvements in inhibitory control. *J. Neurosci.* 29, 12558–12567. doi: 10.1523/JNEUROSCI.1579-09.2009

Verdejo-García, A., Lawrence, A. J., and Clark, L. (2008). Impulsivity as a vulnerability marker for substance-use disorders: review of findings from high-risk research, problem gamblers and genetic association studies. *Neurosci. Biobehav. Rev.* 32, 777–810. doi: 10.1016/j.neubiorev.2007.11.003

Wesley, M. J., and Bickel, W. K. (2014). Remember the future II: meta-analyses and functional overlap of working memory and delay discounting. *Biol. Psychiatry* 75, 435–448. doi: 10.1016/j.biopsych.2013.08.008

Wilbertz, T., Deserno, L., Horstmann, A., Neumann, J., Villringer, A., Heinze, H. J., et al. (2014). Response inhibition and its relation to multidimensional impulsivity. *Neuroimage*, 103, 241–248. doi: 10.1016/j.neuroimage.2014.09.021

Woicik, P. A., Stewart, S. H., Pihl, R. O., and Conrod, P. J. (2009). The substance use risk profile scale: a scale measuring traits linked to reinforcement-specific substance use profiles. *Addict. Behav.* 34, 1042–1055. doi: 10.1016/j.addbeh.2009.07.001

Young, S. E., Friedman, N. P., Miyake, A., Willcutt, E. G., Corley, R. P., Haberstick, B. C., et al. (2009). Behavioral disinhibition: liability for externalizing spectrum disorders and its genetic and environmental relation to response inhibition across adolescence. *J. Abnorm. Psychol.* 118, 117–130. doi: 10.1037/a0014657

Zandbelt, B. B., and Vink, M. (2010). On the role of the striatum in response inhibition. *PLoS ONE* 5:e13848. doi: 10.1371/journal.pone.0013848

Zapolski, T. C., Cyders, M. A., and Smith, G. T. (2009). Positive urgency predicts illegal drug use and risky sexual behavior. *Psychol. Addict. Behav.* 23:348. doi: 10.1037/a0014684

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.

The reviewer SN and handling Editor declared their shared affiliation.

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 20 July 2017
doi: 10.3389/fnbeh.2017.00135

Traumatic Brain Injuries during Development: Implications for Alcohol Abuse

Zachary M. Weil and Kate Karelina*

Behavioral Neuroendocrinology Group, Department of Neuroscience, Center for Brain and Spinal Cord Repair, Ohio State University Wexner Medical Center, Columbus, OH, United States

Traumatic brain injuries are strongly related to alcohol intoxication as by some estimates half or more of all brain injuries involve at least one intoxicated individual. Additionally, there is mounting evidence that traumatic brain injuries can themselves serve as independent risk factors for the development of alcohol use disorders, particularly when injury occurs during juvenile or adolescent development. Here, we will review the epidemiological and experimental evidence for this phenomenon and discuss potential psychosocial mediators including attenuation of negative affect and impaired decision making as well as neurochemical mediators including disruption in the glutamatergic, GABAergic, and dopaminergic signaling pathways and increases in inflammation.

Keywords: alcohol, traumatic brain injury, dopamine, inflammation, adolescent

OPEN ACCESS

Edited by:
Jacqueline M. Barker, Medical University of South Carolina, United States

Reviewed by:
Firas H. Kobeissy, University of Florida, United States
Aleksei Yurievich Egorov, Institute of Evolutionary Physiology and Biochemistry (RAS), Russia

****Correspondence:***
Zachary M. Weil
zachary.weil@osumc.edu

Received: *24 April 2017*
Accepted: *07 July 2017*
Published: *20 July 2017*

Citation:
Weil ZM and Karelina K (2017) Traumatic Brain Injuries during Development: Implications for Alcohol Abuse. Front. Behav. Neurosci. 11:135. doi: 10.3389/fnbeh.2017.00135

INTRODUCTION

Traumatic brain injuries (TBI) have received tremendous scientific and public attention in recent years. This attention is commensurate with the enormous societal and economic costs associated with a condition that affects millions of Americans and because of underreporting is likely even larger than current estimates. One important insight that has become clear is that TBIs are more than discrete events but are the start of a lifelong process comprising recovery, adaptation, and vulnerability to a large variety of other disease states (Masel and DeWitt, 2010; Corrigan and Hammond, 2013). In particular, children and adolescents who suffer TBI are less likely to finish school and maintain employment and suffer a greater risk of neurological and psychiatric disorders (among many others; Corrigan et al., 2013).

Alcohol is a prominent component of TBI. A recent assessment of the U.S. national trauma registry revealed alcohol use to be present in as many as 50% of all TBI-related emergency department visits (Chen et al., 2012). Not surprisingly alcohol use in general and binge drinking in particular are powerful risk factors for TBI (Savola et al., 2005; Vaaramo et al., 2014) and contribute substantially to mortality of TBI patients (see reference Opreanu et al., 2010 for a review). Critically, the use of alcohol in patients recovering from TBI is highly deleterious and there is significant evidence that patients that drink after TBI have poorer cognitive, neuropsychiatric and occupational outcomes than those that do not (Corrigan, 1995; Weil et al., 2016a; Unsworth and Mathias, 2017). Drinking after TBI is associated with poor long term outcomes in a variety of domains (Corrigan, 1995). Similar results have been reported in experimental TBI, for example we recently reported that administration of binge-like levels of alcohol in adulthood produces significant functional and neuropathological impairments in mice that had experienced TBI as juveniles (Karelina et al., 2017). Given that both past TBI and drinking are risk factors for future

TBI *and* repeated TBI tend to produce much more severe damage (Guskiewicz et al., 2000; Giza et al., 2013; McCrory et al., 2013) reducing drinking behavior in this patient population will serve to both improve outcomes and reduce the possibility of devastating future injuries.

The strong epidemiological association between brain injuries and pre-injury drinking mean that the TBI population is composed disproportionately of heavy drinkers. However, there is emerging clinical and experimental evidence that TBI may serve as an independent risk factor for the development of alcohol use disorders (AUDs; Weil et al., 2016a; Merkel et al., 2017a). This is particularly apparent among patients that suffer TBIs during childhood or adolescence. The TBI-induced increase in alcohol abuse among patients that suffer injuries during development likely reflects both a greater vulnerability of the developing nervous system to disruption by injury and that children are less likely to already be problem drinkers at the time of their injuries, and thus it is easier to detect independent contributions of TBI to the development of AUDs (Weil et al., 2016a).

DRINKING IN TBI PATIENTS

The relationship between TBI and alcohol abuse is well-known but had been considered to be unidirectional, i.e., drinking was a risk factor for head injuries. The possibility that the opposite was also true, i.e., that brain injuries could under specific conditions increases drinking behavior, was obscured by several factors. First, problem drinking (particularly binge-drinking) is a key predictor and proximate cause of TBI (Corrigan and Mysiw, 2012). Therefore, the TBI population is made up disproportionately of heavy drinkers making it difficult to detect effects of TBI on later alcohol-related outcomes (Corrigan, 1995). Second, patients with the most severe injuries often reduce drinking during the first months after injury. This seems to occur because of a combination of factors but includes lack of access to alcohol because of physical disability and hospitalization (and inpatient rehabilitation; Bombardier et al., 2003). Third, most studies have examined individuals injured as adults but that represents both a relatively small proportion of the total population and includes individuals that have either already begun drinking or are past the age at which new AUD tend to emerge (Grant and Dawson, 1997). Finally, patients that begin (or resume) drinking after injury are more likely to be lost to follow up in longitudinal studies and thus the numbers of these individuals might be underestimated (Corrigan et al., 1997). Indeed, studies of post-TBI drinking in adults have most often reported that there is an initial decrease in alcohol intake followed by some patients gradually returning to problem drinking and others becoming abstainers (Kreutzer et al., 1996; Ponsford et al., 2007). Studies have produced conflicting reports as to whether adult injuries increase the rates of, or vulnerability to, AUDs (Bjork and Grant, 2009).

In contrast, early life injury has been consistently and repeatedly associated with the later development of AUDs (Weil et al., 2016a; Merkel et al., 2017a). For instance, high school students that suffered a TBI were more than twice as likely to meet the diagnostic criteria for AUDs after injury (Ilie et al., 2015). Further the earlier that injuries occur the stronger the association with substance abuse. Children injured before age five were more than 3.6 times as likely to exhibit substance abuse as teenagers than were uninjured children (McKinlay et al., 2014). Patients in an inpatient rehabilitation setting for TBI were more than twice as likely to meet the diagnostic criteria for substance abuse if they had experienced a previous injury before the age of 16 (Corrigan et al., 2013). In addition, among inmates in the South Carolina penitentiary system, age of first brain injury was associated with both severity and earlier age of onset of substance abuse (Fishbein et al., 2016).

A similar age of injury-related discrepancy in drinking after TBI has also been reported in animals. We recently reported that juvenile TBI, but not adult injury, significantly increased alcohol self-administration in a two-bottle choice paradigm in mice (Weil et al., 2016b). Other investigations of drinking behavior in animals injured as adults have produced conflicting results with some studies reporting increases and others reporting decreases (Lowing et al., 2014; Lim et al., 2015; Mayeux et al., 2015). Taken together, it seems that injuries that occur early in life are more likely to produce AUD, however, the specific mechanisms that link TBI to vulnerability to problem drinking remain unspecified.

POTENTIAL MECHANISMS OF INCREASED DRINKING AFTER TBI

In the next section we will discuss two very general classes of potential mediators that underlie the increased drinking behavior observed in pediatric brain injury patients (summarized in **Figure 1**). This is not intended to be an exhaustive discussion but rather to highlight some of the active areas of research.

PSYCHOSOCIAL FACTORS

TBI interacts with or exacerbates many of the traditional risk factors for AUD and may limit some of the moderating factors that serve to reduce AUD risk. Although, the individual contributions of these risk factors are likely relatively minor, when taken together they contribute to the increased likelihood of AUD in these patients. For example, many head injury patients have a family history of AUD, which results in both increased modeling of drinking behavior and genetic risk (Laucht et al., 2007). In addition, head injuries in pediatric populations are strongly, but often indirectly, associated with alcohol intoxication. In children and adolescents, vehicular accidents and physical violence are responsible for a substantial subset of injuries (though the exact percentages shift across age; Keenan and Bratton, 2006; Narang and Clarke, 2014) and extremely large percentages of head injuries related to assaults or incidences of physical abuse are associated with intoxication of either the injured child or more often the assailant, who is often a parent or other close relative (Kraus et al., 1989; Dube et al., 2001). A similar relationship exists for motor vehicle accidents where at least one participant is likely to be intoxicated (Stoduto et al., 1993). Further, male children are more likely to experience

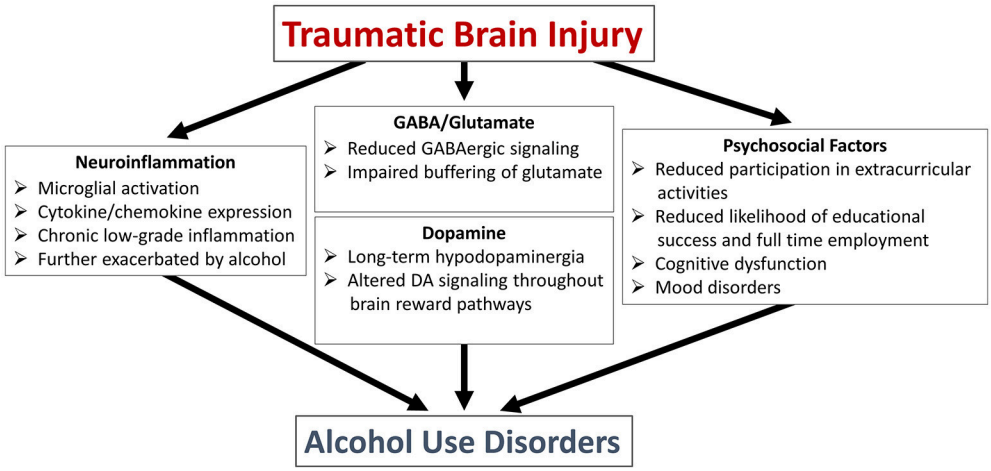


FIGURE 1 | Potential mechanisms linking traumatic brain injury to alcohol use disorders.

abuse-associated head trauma and are also at greater risk of later TBI and adolescent AUD (Costello et al., 1999; Narang and Clarke, 2014). Thus, the pediatric TBI population consists disproportionately of individuals from a family with alcohol use problems and/or a history of abuse, which are both critical risk factors for the development of AUDs (Dube et al., 2001; Barnow et al., 2002).

TBI also reduces many of the negative predictors of substance abuse. For instance, participation in extracurricular activities, forming stable romantic relationships, educational success, and full time employment are associated with reduced risk of alcohol abuse, yet all are less likely in brain injury survivors (Stewart-Scott and Douglas, 1998; Corrigan et al., 2013). Further, more severe TBI is often associated with prolonged absence from school and long-term disability, which often results in alienation from peer groups and increased alcohol use (Glang et al., 1997; Maggs et al., 2008).

The decision to drink has been proposed to reflect a balance between potential negative consequences (hangover, relationship issues, health consequences etc.) and perceived gains (reducing social inhibitions, negative mood states etc.) (Goldman, 1994). TBI can influence both sides of that equation by both reducing the ability to perceive negative consequences and increasing the perception of gains to be derived from drinking. For instance, that framework, termed the incentive motivation theory (Goldman, 1994), would predict that individuals less able to foresee the future consequences of their actions would be more likely to elect to drink. Indeed, TBI survivors show deficits in delay discounting and other cognitive tasks that require evaluation of delayed consequences (Bechara et al., 1994; Kolitz et al., 2003; Graham and Cardon, 2008). Finally, TBI results in more general executive dysfunction that can manifest itself as impulsivity and reduced inhibition, which can serve as predictors of AUD (Laucht et al., 2007; Iacono et al., 2008).

The other component of the incentive motivation theory is that individuals perceive a benefit to drinking. This perceived benefit could be in the form of increasing mood state, or reducing negative emotions or pain, and thus serves as a form of self-medication (King et al., 2004; Bolton et al., 2009). TBI survivors suffer from psychiatric sequelae including anxiety, depression and in a large subset of cases, post-traumatic stress disorder (PTSD; Breslau et al., 1991, 1997; Jorge et al., 2004). TBI often damages corticolimbic structures that regulate mood states, and endocrine and autonomic physiology as well as inducing long-term inflammatory responses, which can all increase the symptoms of affective disorders (Juengst et al., 2015). Even beyond direct damage to the nervous system, patients that acquire long-term cognitive or physical disabilities after brain injury often undergo a significant and prolonged period of adjustment to living with a disability (Smedema and Ebener, 2010). Thus, the very real possibility exists that brain injured patients are drinking to reduce the negative emotional states that are promoted by brain injury (Beresford et al., 2005).

NEUROINFLAMMATORY SIGNALING

The psychosocial and genetic risk factors associated with pediatric TBI also occur in the context of damage to the developing nervous system and likely disruptions of normal brain development. TBI is a complex pathophysiological process that can involve neuronal death, axon disconnection and degeneration, metabolic dysfunction, and aberrant neuroplasticity among other processes depending on the exact type and severity of the injury, comorbidities and age of the patient (Werner and Engelhard, 2007). However, one feature nearly universal to TBI is inflammation (Kelley et al., 2007; Ziebell and Morganti-Kossmann, 2010; Johnson et al., 2013). TBI both directly activates immune cells in the brain and primes cells such that future inflammatory stimuli produce exaggerated inflammatory responses (Fenn et al., 2014) and this is particularly true when inflammatory events occur early in life (Bilbo and Schwarz, 2009). The enhanced basal and stimulus-evoked immune responsiveness of the injured nervous system is important because there is a bidirectional relationship

between neuroinflammation and alcohol intake (Kelley and Dantzer, 2011).

Alcohol produces a central inflammatory response characterized by activation of microglia and induction of inflammatory signaling and cytokines (Valles et al., 2004; Crews et al., 2011). Indeed the brains of long-term alcoholics exhibit evidence of prolonged low-grade inflammatory responses that may contribute to cognitive decline (He and Crews, 2008; Leclercq et al., 2014; Yen et al., 2017). The specific mechanism through which alcohol induces inflammatory responses is not fully understood but likely includes activation of the danger signal detecting molecules toll-like receptors (TLR; Alfonso-Loeches et al., 2010; Pascual et al., 2011). Critically, inflammation and components of inflammatory signaling drive drinking behavior (Crews et al., 2011). Mice treated with the bacterial cell wall component lipopolysaccharide (a molecule that induces a potent inflammatory response) self-administer significantly more alcohol (Blednov et al., 2011). Further, treatment with minocycline, a semisynthetic antibiotic with potent central anti-inflammatory activity produces a prolonged reduction in spontaneous alcohol self-administration (Agrawal et al., 2011). Mice lacking various components of inflammatory signaling cascades also drink less under basal conditions (Blednov et al., 2012).

Thus, TBI produces both acute inflammatory responses *and* primes immune cells like microglia to exhibit exaggerated inflammatory responses to other stimuli later in life. In this manner, TBI can establish a vicious cycle wherein inflammatory responses promote drinking behavior and subsequent drinking further exacerbates inflammatory responses (Mayeux et al., 2015). Critically, the alteration in inflammatory responses from alcohol occur in a brain already impacted by TBI and thus in addition to the deleterious consequences of heavy drinking that occur in otherwise healthy individuals, TBI patients face the potential of enhanced and chronic neuroinflammatory responses.

NEUROCHEMICAL ABNORMALITIES

Dysfunction in neuronal signaling after TBI, during development, can be roughly categorized into several etiologies. First, there is some (although often minimal) frank loss of neurons and associated axonal degeneration that directly disconnects or otherwise impairs neuronal connections. Additionally, as many critical neurodevelopmental events are occurring during these developmental epochs, TBI can result in disruption in the establishment of, or homeostasis in, neurochemical systems.

For instance, the ascending dopaminergic system undergoes significant functional and anatomical plasticity during late childhood and early adolescence (Philpot et al., 2009). This is characterized by changing tonic and stimulus evoked firing, alterations in synthetic machinery, transporter expression, and receptor distribution (Wahlstrom et al., 2010; McCutcheon et al., 2012). Importantly, this period of rapid neurodevelopment coincides temporally with vulnerability to substance abuse (Grant and Dawson, 1998). Early experience with drugs of abuse is a key risk factor for the development of substance abuse disorders and has been shown experimentally to alter the long-term function of the dopamine system (Guerri and Pascual, 2010).

Similarly, dysfunction in dopaminergic signaling is both a common consequence of TBI and a risk factor for the development of AUD (Martinez et al., 2005). There is a large body of experimental animal work indicating that dopamine physiology is significantly altered by TBI, with most studies reporting an acute hyperdopaminergia that resolves into a long-term hypodopaminergic state (Yan et al., 2001, 2002; Wagner et al., 2005a,b, 2009; Hutson et al., 2011).

Although, there is little direct evidence of dopaminergic dysfunction in human TBI patients (Donnemiller et al., 2000), drugs that enhance dopaminergic signaling (either by increasing synaptic dopamine or directly agonizing dopamine receptors) are relatively effective and part of the standard of care for reducing the cognitive deficits experienced by TBI patients (Neurobehavioral Guidelines Working et al., 2006; Bales et al., 2009; Huang et al., 2016). The utility of these drugs does not necessarily indicate that the dopamine system is hypofunctional in human patients (i.e., more dopamine may be helpful to improve cognitive outcomes in patients because of other neurological deficits) but the preponderance of evidence from clinical and experimental sources suggest some level of long term dysfunction in this system (Bales et al., 2009).

Critically, the alterations in dopamine signaling appear to occur beyond what would be expected from frank axonal degeneration or neuronal death (although damage to the ventral tegmental area and striatal targets have been reported following TBI; Dunn-Meynell and Levin, 1997; Ding et al., 2001; Hutson et al., 2011). Rather the alterations in dopamine signaling likely include alterations in network regulation and ongoing inflammation (Merkel et al., 2017a,b). Inflammatory signaling also serves to impair dopaminergic signaling and likely plays a role in the vulnerability to substance abuse in the brain-injured population (Felger and Miller, 2012). For instance, tyrosine hydroxylase production of L-Dopa is the rate-limiting step in dopamine biosynthesis. This enzymatic reaction requires the cofactor, tetrahydrobiopterin (BH4), which is also required for the synthesis of nitric oxide by nitric oxide synthases which are strongly upregulated by inflammatory signals meaning that BH4 can be shunted away from tyrosine hydroxylase when the brain is inflamed (Cunnington and Channon, 2010; Ono et al., 2010; Felger and Miller, 2012).

In contrast, surprisingly little is known about long-term adjustments in the glutamatergic and GABAergic systems after TBI. Acutely, TBI is associated with large, uncontrolled glutamate release that is a key factor in the damage associated with trauma (Katayama et al., 1990; Bullock et al., 1998). Further GABAergic neurons may be disproportionately likely to die and are overall less effective at balancing excitation. Finally, there is often persistent dysfunction in glial cells that typically buffer extracellular glutamate concentrations by expressing transporter proteins. Together, these lead to a net increase in excitatory signaling (Cantu et al., 2015) and the dysregulation in excitatory-inhibitory balance is very often associated with the development of post-traumatic epilepsy, particularly after pediatric injury

(Ates et al., 2006; Pavlov et al., 2011). Over the long term there are compensatory changes that seem to buffer excess excitation but as a consequence of limiting excitatory neurotransmission may limit cognitive recovery (De Beaumont et al., 2012). This is evidenced by the consistent finding that TBI impairs the expression of long-term potentiation (Giza and Prins, 2006; Schwarzbach et al., 2006; Dorsett et al., 2017).

Like TBI, AUD are associated with disruption in the balance in excitatory-inhibitory balance and dysregulation of both glutamatergic and GABAergic signaling (Koob and Volkow, 2016). Alcohol both directly modulates activity of glutamate and GABA receptors, and can induce compensatory adjustments in these systems that perpetuate problem drinking (Chandler, 2003; Fitzgerald et al., 2012). The dynamic role of GABA:glutamate dysregulation and the interaction with normal development of this system in the evolution of drinking after TBI remains unspecified but deserves further attention.

CONCLUDING REMARKS

Alcohol use among adolescents is exceptionally common in western societies. Many individuals can drink heavily during this developmental period without developing AUD. However, patients with a history of TBI are much more likely to develop AUD. This is a major and critical public health problem because drinking after TBI can increase the risk of post-traumatic seizures, impair the efficacy of rehabilitation programs and increase the likelihood of subsequent TBI. The specific mechanisms that link TBI to AUD remain unspecified but it seems highly likely that it involves the coincidence of key psychosocial and neurochemical risk factors with important periods of neurological development. Targeting AUD in this population has the potential to significantly improve long-term outcomes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the Huron Foundation and the Ohio State University Wexner Medical Center Neuroscience Program. Additional support for behavioral experiments was provided by the National Institutes of Health (NINDS NS045758).

REFERENCES

Agrawal, R. G., Hewetson, A., George, C. M., Syapin, P. J., and Bergeson, S. E. (2011). Minocycline reduces ethanol drinking. *Brain Behav. Immun.* 25(Suppl. 1), S165–S169. doi: 10.1016/j.bbi.2011.03.002

Alfonso-Loeches, S., Pascual-Lucas, M., Blanco, A. M., Sanchez-Vera, I., and Guerri, C. (2010). Pivotal role of TLR4 receptors in alcohol-induced neuroinflammation and brain damage. *J. Neurosci.* 30, 8285–8295. doi: 10.1523/JNEUROSCI.0976-10.2010

Ates, O., Ondul, S., Onal, C., Buyukkiraz, M., Somay, H., Cayli, S. R., et al. (2006). Post-traumatic early epilepsy in pediatric age group with emphasis on influential factors. *Child Nerv. Syst.* 22, 279–284. doi: 10.1007/s00381-006-0171-y

Bales, J. W., Wagner, A. K., Kline, A. E., and Dixon, C. E. (2009). Persistent cognitive dysfunction after traumatic brain injury: a dopamine hypothesis. *Neurosci. Biobehav. Rev.* 33, 981–1003. doi: 10.1016/j.neubiorev.2009.03.011

Barnow, S., Schuckit, M. A., Lucht, M., John, U., and Freyberger, H. J. (2002). The importance of a positive family history of alcoholism, parental rejection and emotional warmth, behavioral problems and peer substance use for alcohol problems in teenagers: a path analysis. *J. Stud. Alcohol* 63, 305–315. doi: 10.15288/jsa.2002.63.305

Bechara, A., Damasio, A. R., Damasio, H., and Anderson, S. W. (1994). Insensitivity to future consequences following damage to human prefrontal cortex. *Cognition* 50, 7–15. doi: 10.1016/0010-0277(94)90018-3

Beresford, T. P., Arciniegas, D., Clapp, L., Martin, B., and Alfers, J. (2005). Reduction of affective lability and alcohol use following traumatic brain injury: a clinical pilot study of anti-convulsant medications. *Brain Inj.* 19, 309–313. doi: 10.1080/02699050410001720121

Bilbo, S. D., and Schwarz, J. M. (2009). Early-life programming of later-life brain and behavior: a critical role for the immune system. *Front. Behav. Neurosci.* 3:14. doi: 10.3389/neuro.08.014.2009

Bjork, J. M., and Grant, S. J. (2009). Does traumatic brain injury increase risk for substance abuse? *J. Neurotrauma* 26, 1077–1082. doi: 10.1089/neu.2008.0849

Blednov, Y. A., Benavidez, J. M., Geil, C., Perra, S., Morikawa, H., and Harris, R. A. (2011). Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. *Brain Behav. Immun.* 25(Suppl. 1), S92–S105. doi: 10.1016/j.bbi.2011.01.008

Blednov, Y. A., Ponomarev, I., Geil, C., Bergeson, S., Koob, G. F., and Harris, R. A. (2012). Neuroimmune regulation of alcohol consumption: behavioral validation of genes obtained from genomic studies. *Addict. Biol.* 17, 108–120. doi: 10.1111/j.1369-1600.2010.00284.x

Bolton, J. M., Robinson, J., and Sareen, J. (2009). Self-medication of mood disorders with alcohol and drugs in the National Epidemiologic Survey on Alcohol and Related Conditions. *J. Affect. Disord.* 115, 367–375. doi: 10.1016/j.jad.2008.10.003

Bombardier, C. H., Temkin, N. R., Machamer, J., and Dikmen, S. S. (2003). The natural history of drinking and alcohol-related problems after traumatic brain injury. *Arch. Phys. Med. Rehabil.* 84, 185–191. doi: 10.1053/apmr.2003.50002

Breslau, N., Davis, G. C., Andreski, P., and Peterson, E. (1991). Traumatic events and posttraumatic stress disorder in an urban population of young adults. *Arch. Gen. Psychiatry* 48, 216–222. doi: 10.1001/archpsyc.1991.01810270028003

Breslau, N., Davis, G. C., Peterson, E. L., and Schultz, L. (1997). Psychiatric sequelae of posttraumatic stress disorder in women. *Arch. Gen. Psychiatry* 54, 81–87. doi: 10.1001/archpsyc.1997.01830130087016

Bullock, R., Zauner, A., Woodward, J. J., Myseros, J., Choi, S. C., Ward, J. D., et al. (1998). Factors affecting excitatory amino acid release following severe human head injury. *J. Neurosurg.* 89, 507–518. doi: 10.3171/jns.1998.89.4.0507

Cantu, D., Walker, K., Andresen, L., Taylor-Weiner, A., Hampton, D., Tesco, G., et al. (2015). Traumatic brain injury increases cortical glutamate network activity by compromising GABAergic control. *Cereb. Cortex* 25, 2306–2320. doi: 10.1093/cercor/bhu041

Chandler, L. J. (2003). Ethanol and brain plasticity: receptors and molecular networks of the postsynaptic density as targets of ethanol. *Pharmacol. Ther.* 99, 311–326. doi: 10.1016/S0163-7258(03)00096-2

Chen, C. M., Yi, H.-Y., Yoon, Y.-H., and Dong, C. (2012). Alcohol use at time of injury and survival following traumatic brain injury: results from the National Trauma Data Bank. *J. Stud. Alcohol Drugs* 73, 531–541. doi: 10.15288/jsad.2012.73.531

Corrigan, J. D. (1995). Substance abuse as a mediating factor in outcome from traumatic brain injury. *Arch. Phys. Med. Rehabil.* 76, 302–309. doi: 10.1016/S0003-9993(95)80654-7

Corrigan, J. D., Bogner, J. A., Mysiw, W. J., Clinchot, D., and Fugate, L. (1997). Systematic bias in outcome studies of persons with traumatic brain injury. *Arch. Phys. Med. Rehabil.* 78, 132–137. doi: 10.1016/S0003-9993(97)90253-7

Corrigan, J. D., Bogner, J., Mellick, D., Bushnik, T., Dams-O'Connor, K., Hammond, F. M., et al. (2013). Prior history of traumatic brain injury among persons in the traumatic brain injury model systems national database. *Arch. Phys. Med. Rehabil.* 94, 1940–1950. doi: 10.1016/j.apmr.2013.05.018

Corrigan, J. D., and Hammond, F. M. (2013). Traumatic brain injury as a chronic health condition. *Arch. Phys. Med. Rehabil.* 94, 1199–1201. doi: 10.1016/j.apmr.2013.01.023

Corrigan, J. D., and Mysiw, W. J. (2012). "Substance abuse among persons with TBI," in *Brain Injury Medicine: Principles and Practice, 2nd Edn*, eds N. D. Zasler, D. I. Katz, R. D. Zafonte, D. B. Arciniegas, M. R. Bullock, and J. S. Kreutzer (New York, NY: Demos Medical Publishing), 1315–1328.

Costello, E. J., Erkanli, A., Federman, E., and Angold, A. (1999). Development of psychiatric comorbidity with substance abuse in adolescents: effects of timing and sex. *J. Clin. Child Psychol.* 28, 298–311. doi: 10.1207/S15374424jccp280302

Crews, F. T., Zou, J., and Qin, L. (2011). Induction of innate immune genes in brain create the neurobiology of addiction. *Brain Behav. Immun.* 25, S4–S12. doi: 10.1016/j.bbi.2011.03.003

Cunnington, C., and Channon, K. M. (2010). Tetrahydrobiopterin: pleiotropic roles in cardiovascular pathophysiology. *Heart* 96, 1872–1877. doi: 10.1136/hrt.2009.180430

De Beaumont, L., Tremblay, S., Poirier, J., Lassonde, M., and Theoret, H. (2012). Altered bidirectional plasticity and reduced implicit motor learning in concussed athletes. *Cereb. Cortex* 22, 112–121. doi: 10.1093/cercor/bhr096

Ding, Y., Yao, B., Lai, Q., and McAllister, J. P. (2001). Impaired motor learning and diffuse axonal damage in motor and visual systems of the rat following traumatic brain injury. *Neurol. Res.* 23, 193–202. doi: 10.1179/016164101101198334

Donnemiller, E., Brenneis, C., Wissel, J., Scherfler, C., Poewe, W., Riccabona, G., et al. (2000). Impaired dopaminergic neurotransmission in patients with traumatic brain injury: a SPECT study using 123I-beta-CIT and 123I-IBZM. *Eur. J. Nucl. Med.* 27, 1410–1414. doi: 10.1007/s002590000308

Dorsett, C. R., McGuire, J. L., DePasquale, E. A., Gardner, A. E., Floyd, C. L., and McCullumsmith, R. E. (2017). Glutamate neurotransmission in rodent models of traumatic brain injury. *J. Neurotrauma* 34, 263–272. doi: 10.1089/neu.2015.4373

Dube, S. R., Anda, R. F., Felitti, V. J., Croft, J. B., Edwards, V. J., and Giles, W. H. (2001). Growing up with parental alcohol abuse: exposure to childhood abuse, neglect, and household dysfunction. *Child Abuse Negl.* 25, 1627–1640. doi: 10.1016/S0145-2134(01)00293-9

Dunn-Meynell, A. A., and Levin, B. E. (1997). Histological markers of neuronal, axonal and astrocytic changes after lateral rigid impact traumatic brain injury. *Brain Res.* 761, 25–41. doi: 10.1016/S0006-8993(97)00210-2

Felger, J. C., and Miller, A. H. (2012). Cytokine effects on the basal ganglia and dopamine function: the subcortical source of inflammatory malaise. *Front. Neuroendocrinol.* 33, 315–327. doi: 10.1016/j.yfrne.2012.09.003

Fenn, A. M., Gensel, J. C., Huang, Y., Popovich, P. G., Lifshitz, J., and Godbout, J. P. (2014). Immune activation promotes depression 1 month after diffuse brain injury: a role for primed microglia. *Biol. Psychiatry* 76, 575–584. doi: 10.1016/j.biopsych.2013.10.014

Fishbein, D., Dariotis, J. K., Ferguson, P. L., and Pickelsimer, E. E. (2016). Relationships between traumatic brain injury and illicit drug use and their association with aggression in inmates. *Int. J. Offender Ther. Comp. Criminol.* 60, 575–597. doi: 10.1177/0306624X14554778

Fitzgerald, G. J., Liu, H., and Morzorati, S. L. (2012). Decreased sensitivity of NMDA receptors on dopaminergic neurons from the posterior ventral tegmental area following chronic nondependent alcohol consumption. *Alcohol. Clin. Exp. Res.* 36, 1710–1719. doi: 10.1111/j.1530-0277.2012.01762.x

Giza, C. C., Kutcher, J. S., Ashwal, S., Barth, J., Getchius, T. S., Gioia, G. A., et al. (2013). Summary of evidence-based guideline update: evaluation and management of concussion in sports: report of the Guideline Development Subcommittee of the American Academy of Neurology. *Neurology* 80, 2250–2257. doi: 10.1212/WNL.0b013e31828d57dd

Giza, C. C., and Prins, M. L. (2006). Is being plastic fantastic? Mechanisms of altered plasticity after developmental traumatic brain injury. *Dev Neurosci.* 28, 364–379. doi: 10.1159/000094163

Glang, A., Todis, B., Cooley, E., Wells, J., and Voss, J. (1997). Building social networks for children and adolescents with traumatic brain injury: a school-based intervention. *J. Head Trauma Rehabil.* 12, 32–47. doi: 10.1097/00001199-199704000-00005

Goldman, M. S. (1994). The alcohol expectancy concept: applications to assessment, prevention, and treatment of alcohol abuse. *Appl. Prevent. Psychol.* 3, 131–144. doi: 10.1016/S0962-1849(05)80066-6

Graham, D. P., and Cardon, A. L. (2008). An update on substance use and treatment following traumatic brain injury. *Ann. N.Y. Acad. Sci.* 1141, 148–162. doi: 10.1196/annals.1441.029

Grant, B. F., and Dawson, D. A. (1997). Age at onset of alcohol use and its association with DSM-IV alcohol abuse and dependence: results from the national longitudinal alcohol epidemiologic survey. *J. Subst. Abuse* 9, 103–110. doi: 10.1016/S0899-3289(97)90009-2

Grant, B. F., and Dawson, D. A. (1998). Age of onset of drug use and its association with DSM-IV drug abuse and dependence: results from the National Longitudinal Alcohol Epidemiologic Survey. *J. Subst. Abuse* 10, 163–173. doi: 10.1016/S0899-3289(99)80131-X

Guerri, C., and Pascual, M. (2010). Mechanisms involved in the neurotoxic, cognitive, and neurobehavioral effects of alcohol consumption during adolescence. *Alcohol* 44, 15–26. doi: 10.1016/j.alcohol.2009.10.003

Guskiewicz, K. M., Weaver, N. L., Padua, D. A., and Garrett, W. E. Jr. (2000). Epidemiology of concussion in collegiate and high school football players. *Am. J. Sports Med.* 28, 643–650. doi: 10.1177/03635465000280050401

He, J., and Crews, F. T. (2008). Increased MCP-1 and microglia in various regions of the human alcoholic brain. *Exp. Neurol.* 210, 349–358. doi: 10.1016/j.expneurol.2007.11.017

Huang, C. H., Huang, C. C., Sun, C. K., Lin, G. H., and Hou, W. H. (2016). Methylphenidate on cognitive improvement in patients with traumatic brain injury: a meta-analysis. *Curr. Neuropharmacol.* 14, 272–281. doi: 10.2174/1570159X13666150514233033

Hutson, C. B., Lazo, C. R., Mortazavi, F., Giza, C. C., Hovda, D., and Chesselet, M. F. (2011). Traumatic brain injury in adult rats causes progressive nigrostriatal dopaminergic cell loss and enhanced vulnerability to the pesticide paraquat. *J. Neurotrauma* 28, 1783–1801. doi: 10.1089/neu.2010.1723

Iacono, W. G., Malone, S. M., and McGue, M. (2008). Behavioral disinhibition and the development of early-onset addiction: common and specific influences. *Annu. Rev. Clin. Psychol.* 4, 325–348. doi: 10.1146/annurev.clinpsy.4.022007.141157

Ilie, G., Mann, R. E., Hamilton, H., Adlaf, E. M., Boak, A., Asbridge, M., et al. (2015). Substance use and related harms among adolescents with and without traumatic brain injury. *J. Head Trauma Rehabil.* 30, 293–301. doi: 10.1097/HTR.0000000000000101

Johnson, V. E., Stewart, J. E., Begbie, F. D., Trojanowski, J. Q., Smith, D. H., and Stewart, W. (2013). Inflammation and white matter degeneration persist for years after a single traumatic brain injury. *Brain* 136(Pt 1), 28–42. doi: 10.1093/brain/aws322

Jorge, R. E., Robinson, R. G., Moser, D., Tateno, A., Crespo-Facorro, B., and Arndt, S. (2004). Major depression following traumatic brain injury. *Arch. Gen. Psychiatry* 61, 42–50. doi: 10.1001/archpsyc.61.1.42

Juengst, S. B., Kumar, R. G., Failla, M. D., Goyal, A., and Wagner, A. K. (2015). Acute inflammatory biomarker profiles predict depression risk following moderate to severe traumatic brain injury. *J. Head Trauma Rehabil.* 30, 207–218. doi: 10.1097/HTR.0000000000000031

Karelina, K., Gaier, K. R., Prabhu, M., Wenger, V., Corrigan, T. E., and Weil, Z. M. (2017). Binge ethanol in adulthood exacerbates negative outcomes following juvenile traumatic brain injury. *Brain Behav. Immun.* 60, 304–311. doi: 10.1016/j.bbi.2016.11.009

Katayama, Y., Becker, D. P., Tamura, T., and Hovda, D. A. (1990). Massive increases in extracellular potassium and the indiscriminate release of glutamate following concussive brain injury. *J. Neurosurg.* 73, 889–900. doi: 10.3171/jns.1990.73.6.0889

Keenan, H. T., and Bratton, S. L. (2006). Epidemiology and outcomes of pediatric traumatic brain injury. *Dev. Neurosci.* 28, 256–263. doi: 10.1159/000094152

Kelley, B. J., Lifshitz, J., and Povlishock, J. T. (2007). Neuroinflammatory responses after experimental diffuse traumatic brain injury. *J. Neuropathol. Exp. Neurol.* 66, 989–1001. doi: 10.1097/NEN.0b013e3181588245

Kelley, K. W., and Dantzer, R. (2011). Alcoholism and inflammation: neuroimmunology of behavioral and mood disorders. *Brain Behav. Immun.* 25(Suppl. 1), S13–S20. doi: 10.1016/j.bbi.2010.12.013

King, S. M., Iacono, W. G., and McGue, M. (2004). Childhood externalizing and internalizing psychopathology in the prediction of early substance use. *Addiction* 99, 1548–1559. doi: 10.1111/j.1360-0443.2004.00893.x

Kolitz, B. P., Vanderploeg, R. D., and Curtiss, G. (2003). Development of the Key Behaviors Change Inventory: a traumatic brain injury behavioral outcome assessment instrument. *Arch. Phys. Med. Rehabil.* 84, 277–284. doi: 10.1053/apmr.2003.50100

Koob, G. F., and Volkow, N. D. (2016). Neurobiology of addiction: a neurocircuitry analysis. *Lancet Psychiatry* 3, 760–773. doi: 10.1016/S2215-0366(16)00104-8

Kraus, J. F., Morgenstern, H., Fife, D., Conroy, C., and Nourjah, P. (1989). Blood-alcohol tests, prevalence of involvement, and outcomes following brain injury. *Am. J. Public Health* 79, 294–299. doi: 10.2105/AJPH.79.3.294

Kreutzer, J. S., Witol, A. D., and Marwitz, J. H. (1996). Alcohol and drug use among young persons with traumatic brain injury. *J. Learn. Disabil.* 29, 643–651. doi: 10.1177/002221949602900608

Laucht, M., Becker, K., Blomeyer, D., and Schmidt, M. H. (2007). Novelty seeking involved in mediating the association between the dopamine D4 receptor gene exon III polymorphism and heavy drinking in male adolescents: results from a high-risk community sample. *Biol. Psychiatry* 61, 87–92. doi: 10.1016/j.biopsych.2006.05.025

Leclercq, S., De Saeger, C., Delzenne, N., de Timary, P., and Starkel, P. (2014). Role of inflammatory pathways, blood mononuclear cells, and gut-derived bacterial products in alcohol dependence. *Biol. Psychiatry* 76, 725–733. doi: 10.1016/j.biopsych.2014.02.003

Lim, Y. W., Meyer, N. P., Shah, A. S., Budde, M. D., Stemper, B. D., and Olsen, C. M. (2015). Voluntary alcohol intake following blast exposure in a rat model of mild traumatic brain injury. *PLoS ONE* 10:e0125130. doi: 10.1371/journal.pone.0125130

Lowing, J. L., Susick, L. L., Caruso, J. P., Provenzano, A. M., Raghupathi, R., and Conti, A. C. (2014). Experimental traumatic brain injury alters ethanol consumption and sensitivity. *J. Neurotrauma* 31, 1700–1710. doi: 10.1089/neu.2013.3286

Maggs, J. L., Patrick, M. E., and Feinstein, L. (2008). Childhood and adolescent predictors of alcohol use and problems in adolescence and adulthood in the National Child Development Study. *Addiction* 103, 7–22. doi: 10.1111/j.1360-0443.2008.02173.x

Martinez, D., Gil, R., Slifstein, M., Hwang, D.-R., Huang, Y., Perez, A., et al. (2005). Alcohol dependence is associated with blunted dopamine transmission in the ventral striatum. *Biol. Psychiatry* 58, 779–786. doi: 10.1016/j.biopsych.2005.04.044

Masel, B. E., and DeWitt, D. S. (2010). Traumatic brain injury: a disease process, not an event. *J. Neurotrauma* 27, 1529–1540. doi: 10.1089/neu.2010.1358

Mayeux, J. P., Teng, S. X., Katz, P. S., Gilpin, N. W., and Molina, P. E. (2015). Traumatic brain injury induces neuroinflammation and neuronal degeneration that is associated with escalated alcohol self-administration in rats. *Behav. Brain Res.* 279, 22–30. doi: 10.1016/j.bbr.2014.10.053

McCrory, P., Meeuwisse, W. H., Aubry, M., Cantu, B., Dvorak, J., Echemendia, R. J., et al. (2013). Consensus statement on concussion in sport: the 4th International Conference on Concussion in Sport held in Zurich, November 2012. *Br. J. Sports Med.* 47, 250–258. doi: 10.1136/bjsports-2013-092313

McCutcheon, J. E., Conrad, K. L., Carr, S. B., Ford, K. A., McGehee, D. S., and Marinelli, M. (2012). Dopamine neurons in the ventral tegmental area fire faster in adolescent rats than in adults. *J. Neurophysiol.* 108, 1620–1630. doi: 10.1152/jn.00077.2012

McKinlay, A., Corrigan, J., Horwood, L. J., and Fergusson, D. M. (2014). Substance abuse and criminal activities following traumatic brain injury in childhood, adolescence, and early adulthood. *J. Head Trauma Rehabil.* 29, 498–506. doi: 10.1097/HTR.0000000000000001

Merkel, S. F., Cannella, L. A., Razmpour, R., Lutton, E., Raghupathi, R., Rawls, S. M., et al. (2017a). Factors affecting increased risk for substance use disorders following traumatic brain injury: what we can learn from animal models. *Neurosci. Biobehav. Rev.* 77, 209–218. doi: 10.1016/j.neubiorev.2017.03.015

Merkel, S. F., Razmpour, R., Lutton, E. M., Tallarida, C. S., Heldt, N. A., Cannella, L. A., et al. (2017b). Adolescent traumatic brain injury induces chronic mesolimbic neuroinflammation with concurrent enhancement in the rewarding effects of cocaine in mice during adulthood. *J. Neurotrauma* 34, 165–181. doi: 10.1089/neu.2015.4275

Narang, S., and Clarke, J. (2014). Abusive head trauma: past, present, and future. *J. Child Neurol.* 29, 1747–1756. doi: 10.1177/0883073814549995

Neurobehavioral Guidelines Working, G., Warden, D. L., Gordon, B., McAllister, T. W., Silver, J. M., Barth, J. T., et al. (2006). Guidelines for the pharmacologic treatment of neurobehavioral sequelae of traumatic brain injury. *J. Neurotrauma* 23, 1468–1501. doi: 10.1089/neu.2006.23.1468

Ono, K., Suzuki, H., and Sawada, M. (2010). Delayed neural damage is induced by iNOS-expressing microglia in a brain injury model. *Neurosci. Lett.* 473, 146–150. doi: 10.1016/j.neulet.2010.02.041

Opreanu, R. C., Kuhn, D., and Basson, M. D. (2010). Influence of alcohol on mortality in traumatic brain injury. *J. Am. Coll. Surg.* 210, 997–1007. doi: 10.1016/j.jamcollsurg.2010.01.036

Pascual, M., Fernandez-Lizarbe, S., and Guerri, C. (2011). Role of TLR4 in ethanol effects on innate and adaptive immune responses in peritoneal macrophages. *Immunol. Cell Biol.* 89, 716–727. doi: 10.1038/icb.2010.163

Pavlov, I., Huusko, N., Drexel, M., Kirchmair, E., Sperk, G., Pitkanen, A., et al. (2011). Progressive loss of phasic, but not tonic, gaba(a) receptor-mediated inhibition in dentate granule cells in a model of post-traumatic epilepsy in rats. *Neuroscience* 194, 208–219. doi: 10.1016/j.neuroscience.2011.07.074

Philpot, R. M., Wecker, L., and Kirstein, C. L. (2009). Repeated ethanol exposure during adolescence alters the developmental trajectory of dopaminergic output from the nucleus accumbens septi. *Int. J. Dev. Neurosci.* 27, 805–815. doi: 10.1016/j.ijdevneu.2009.08.009

Ponsford, J., Whelan-Goodinson, R., and Bahar-Fuchs, A. (2007). Alcohol and drug use following traumatic brain injury: a prospective study. *Brain Inj.* 21, 1385–1392. doi: 10.1080/02699050701796960

Savola, O., Niemela, O., and Hillbom, M. (2005). Alcohol intake and the pattern of trauma in young adults and working aged people admitted after trauma. *Alcohol Alcohol.* 40, 269–273. doi: 10.1093/alcalc/agh159

Schwarzbach, E., Bonislawski, D. P., Xiong, G., and Cohen, A. S. (2006). Mechanisms underlying the inability to induce area CA1 LTP in the mouse after traumatic brain injury. *Hippocampus* 16, 541–550. doi: 10.1002/hipo.20183

Smedema, S. M., and Ebener, D. (2010). Substance abuse and psychosocial adaptation to physical disability: analysis of the literature and future directions. *Disabil. Rehabil.* 32, 1311–1319. doi: 10.3109/09638280903514721

Stewart-Scott, A. M., and Douglas, J. M. (1998). Educational outcome for secondary and postsecondary students following traumatic brain injury. *Brain Inj.* 12, 317–331. doi: 10.1080/026990598122629

Stoduto, G., Vingilis, E., Kapur, B. M., Sheu, W. J., Mclellan, B. A., and Liban, C. B. (1993). Alcohol and drug-use among motor-vehicle collision victims admitted to a regional trauma unit - demographic, injury, and crash characteristics. *Accid. Anal. Prev.* 25, 411–420. doi: 10.1016/0001-4575(93)90070-D

Unsworth, D. J., and Mathias, J. L. (2017). Traumatic brain injury and alcohol/substance abuse: a Bayesian meta-analysis comparing the outcomes of people with and without a history of abuse. *J. Clin. Exp. Neuropsychol.* 39, 547–562. doi: 10.1080/13803395.2016.1248812

Vaaramo, K., Puljula, J., Tetri, S., Juvela, S., and Hillbom, M. (2014). Head trauma sustained under the influence of alcohol is a predictor for future traumatic brain injury: a long-term follow-up study. *Eur. J. Neurol.* 21, 293–298. doi: 10.1111/ene.12302

Valles, S. L., Blanco, A. M., Pascual, M., and Guerri, C. (2004). Chronic ethanol treatment enhances inflammatory mediators and cell death in the brain and in astrocytes. *Brain Pathol.* 14, 365–371. doi: 10.1111/j.1750-3639.2004.tb00079.x

Wagner, A. K., Chen, X., Kline, A. E., Li, Y., Zafonte, R. D., and Dixon, C. E. (2005a). Gender and environmental enrichment impact dopamine transporter expression after experimental traumatic brain injury. *Exp. Neurol.* 195, 475–483. doi: 10.1016/j.expneurol.2005.06.009

Wagner, A. K., Drewencki, L. L., Chen, X., Santos, F. R., Khan, A. S., Harun, R., et al. (2009). Chronic methylphenidate treatment enhances striatal dopamine neurotransmission after experimental traumatic brain injury. *J. Neurochem.* 108, 986–997. doi: 10.1111/j.1471-4159.2008.05840.x

Wagner, A. K., Sokoloski, J. E., Ren, D., Chen, X., Khan, A. S., Zafonte, R. D., et al. (2005b). Controlled cortical impact injury affects dopaminergic transmission in the rat striatum. *J. Neurochem.* 95, 457–465. doi: 10.1111/j.1471-4159.2005.03382.x

Wahlstrom, D., Collins, P., White, T., and Luciana, M. (2010). Developmental changes in dopamine neurotransmission in adolescence: behavioral

implications and issues in assessment. *Brain Cogn.* 72, 146–159. doi: 10.1016/j.bandc.2009.10.013

Weil, Z. M., Corrigan, J. D., and Karelina, K. (2016a). Alcohol abuse after traumatic brain injury: experimental and clinical evidence. *Neurosci. Biobehav. Rev.* 62, 89–99. doi: 10.1016/j.neubiorev.2016.01.005

Weil, Z. M., Karelina, K., Gaier, K. R., Corrigan, T. E., and Corrigan, J. D. (2016b). Juvenile traumatic brain injury increases alcohol consumption and reward in female mice. *J. Neurotrauma* 33, 895–903. doi: 10.1089/neu.2015.3953

Werner, C., and Engelhard, K. (2007). Pathophysiology of traumatic brain injury. *Br. J. Anaesth.* 99, 4–9. doi: 10.1093/bja/aem131

Yan, H. Q., Kline, A. E., Ma, X., Hooghe-Peters, E. L., Marion, D. W., and Dixon, C. E. (2001). Tyrosine hydroxylase, but not dopamine beta-hydroxylase, is increased in rat frontal cortex after traumatic brain injury. *Neuroreport* 12, 2323–2327. doi: 10.1097/00001756-200108080-00009

Yan, H. Q., Kline, A. E., Ma, X., Li, Y., and Dixon, C. E. (2002). Traumatic brain injury reduces dopamine transporter protein expression in the rat frontal cortex. *Neuroreport* 13, 1899–1901. doi: 10.1097/00001756-200210280-00013

Yen, C. H., Ho, P. S., Yeh, Y. W., Liang, C. S., Kuo, S. C., Huang, C. C., et al. (2017). Differential cytokine levels between early withdrawal and remission states in patients with alcohol dependence. *Psychoneuroendocrinology* 76, 183–191. doi: 10.1016/j.psyneuen.2016.10.015

Ziebell, J. M., and Morganti-Kossmann, M. C. (2010). Involvement of pro- and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury. *Neurotherapeutics* 7, 22–30. doi: 10.1016/j.nurt.2009.10.016

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: 07 December 2017
doi: 10.3389/fnbeh.2017.00237

Adolescent Corticosterone and TrkB Pharmaco-Manipulations Sex-Dependently Impact Instrumental Reversal Learning Later in Life

Elizabeth T. Barfield[1,2,3,4] *and Shannon L. Gourley*[1,2,3,4]*

[1]Department of Pediatrics, Emory University, Atlanta, GA, United States, [2]Graduate Program in Neuroscience, Emory University, Atlanta, GA, United States, [3]Yerkes National Primate Research Center, Emory University, Atlanta, GA, United States, [4]Department of Psychiatry and Behavioral Sciences, Emory University, Atlanta, GA, United States

Early-life trauma can increase the risk for, and severity of, several psychiatric illnesses. These include drug use disorders, and some correlations appear to be stronger in women. Understanding the long-term consequences of developmental stressor or stress hormone exposure and possible sex differences is critically important. So-called "reversal learning" tasks are commonly used in rodents to model cognitive deficits in stress- and addiction-related illnesses in humans. Here, we exposed mice to the primary stress hormone corticosterone (CORT) during early adolescence (postnatal days 31–42), then tested behavioral flexibility in adulthood using an instrumental reversal learning task. CORT-exposed female, but not male, mice developed perseverative errors. Despite resilience to subchronic CORT exposure, males developed reversal performance impairments following exposure to physical stressors. Administration of a putative tyrosine kinase receptor B (trkB) agonist, 7,8-dihydroxyflavone (7,8-DHF), during adolescence blocked CORT-induced errors in females and improved performance in males. Conversely, blockade of trkB by ANA-12 impaired performance. These data suggest that trkB-based interventions could have certain protective benefits in the context of early-life stressor exposure. We consider the implications of our findings in an extended "Discussion" section.

Keywords: juvenile, impulsivity, BDNF, tropomyosin receptor kinase B, 7,8-dihydroxyflavone

OPEN ACCESS

Edited by:
Nuno Sousa,
Instituto de Pesquisa em Ciências da Vida e da Saúde (ICVS), Portugal

Reviewed by:
Alicia Izquierdo,
University of California, Los Angeles, United States
Nicholas W. Simon,
University of Pittsburgh, United States
Caitlin Anne Orsini,
University of Florida, United States

***Correspondence:**
Shannon L. Gourley
shannon.l.gourley@emory.edu

Received: *28 July 2017*
Accepted: *14 November 2017*
Published: *07 December 2017*

Citation:
Barfield ET and Gourley SL (2017) Adolescent Corticosterone and TrkB Pharmaco-Manipulations Sex-Dependently Impact Instrumental Reversal Learning Later in Life. Front. Behav. Neurosci. 11:237. doi: 10.3389/fnbeh.2017.00237

INTRODUCTION

Reversal tasks assess the ability of mice or rats to flexibly modify behaviors when reinforcement contingencies change. These tasks are commonly used to model behavioral inflexibility associated with addiction and other disorders in humans (for recent review Izquierdo et al., 2017). Typically, animals are trained to associate specific actions or stimuli with reward (e.g., food), then, the association is modified such that a previously non-predictive contingency could be used to obtain reinforcement, while the original contingency is no longer predictive. Thus, these tasks require animals to inhibit a familiar response strategy and deploy a new strategy to obtain reinforcement.

Behavioral inflexibility in these tasks may result from (Ersche et al., 2008; Jentsch et al., 2002; Schoenbaum et al., 2004) and also *predispose* organisms to (Dalley et al., 2007; Belin et al., 2008; Groman et al., 2009) drug-seeking behaviors. For example, in humans, deficits in reversal

learning correlate with the severity of cocaine use (Moreno-López et al., 2015), and a "behavioral disinhibition" trait is associated with later substance use disorders (Tarter et al., 2004; Nigg et al., 2006). Further, rats or mice exhibiting low inhibitory control over impulsive responding more rapidly escalate rates of cocaine self-administration and are more prone to developing drug taking characterized as compulsive (Dalley et al., 2007; Belin et al., 2008; Cervantes et al., 2013). Thus, individual differences in behavioral flexibility and inhibitory control may influence the progression from initial drug use to habitual or compulsive drug-seeking (Cervantes et al., 2013).

Adverse experiences early in life are linked with negative psychiatric outcomes in adulthood, including increased risk for drug use disorders (Fergusson et al., 1996; Kessler et al., 1997; Dube et al., 2003; Green et al., 2010; Afifi et al., 2012) and greater lifetime severity of substance abuse (Hyman et al., 2006; Sacks et al., 2008; Enoch, 2011). Furthermore, several studies report that this association is stronger in women (Widom and White, 1997; MacMillan et al., 2001; Simpson and Miller, 2002; Hyman et al., 2008). The neurobehavioral processes that translate developmental stressor exposure into psychiatric vulnerabilities later in life are incompletely understood, but may involve disruption of prefrontal cortex (PFC)-dependent executive functions, such as behavioral flexibility and inhibitory control (Elton et al., 2014). Indeed, early-life trauma is associated with deficits in inhibitory control (Lewis et al., 2007; Mueller et al., 2010; Skowron et al., 2014; Marshall et al., 2016), and in at least one study, trait impulsivity mediated the relationship between childhood trauma and substance dependence severity in adulthood (Schwandt et al., 2013). Comparing susceptibility to early-life stress-induced inhibitory control deficits between males and females may provide insight into mechanisms of sex differences in risk for stress-related psychopathology.

In the present study, we manipulated levels of the primary glucocorticoid, corticosterone (CORT), and determined long-term consequences in an instrumental reversal task. We exposed mice to exogenous CORT from postnatal day (P) 31–42, corresponding to early adolescence in rodents (Spear, 2000), and a period of considerable structural maturation in the PFC (Shapiro et al., 2017b). Mice were then trained as adults to perform food-reinforced nose poke responses. We found that subchronic CORT exposure induced habit-biased behavior, despite the cessation of CORT exposure, and these findings are reported in Barfield et al. (in press). We then tested mice in an instrumental reversal task, and the results of those tests are reported here.

In some groups, we stimulated tyrosine kinase receptor B (trkB), the high-affinity receptor for brain-derived neurotrophic factor (BDNF), which regulates dendritic spine structure and function (Yoshii and Constantine-Paton, 2010). BDNF-trkB systems are impacted by stress (Gray et al., 2013; Numakawa et al., 2013), implicated in addiction-related behaviors (Li and Wolf, 2015), and significantly modify reward-related decision making (Pitts et al., 2016). Loss of neurotrophic support following prolonged exposure to elevated glucocorticoids is thought to contribute to structural and functional alterations in the PFC that are associated with stress-related psychopathology (Duman et al., 1997). Moreover, recent findings implicate down-regulation of BDNF-trkB signaling in the long-term behavioral consequences of adolescent stress exposure (Xu et al., 2016; Zhang et al., 2017; Barfield et al., in press). Thus, we hypothesized that stimulating trkB may have protective benefits in animals exposed to CORT during adolescence, blocking enduring behavioral deficits.

We report sex-dependent long-term consequences of both CORT and trkB manipulations in an instrumental reversal task. Namely, a history of early-adolescent CORT exposure in females, but not males, induced perseverative responding. Despite apparent resilience to early-adolescent CORT, males developed behavioral inflexibilities following repeated stressor exposure or trkB blockade during early adolescence. Further, a putative trkB agonist improved performance in both sexes. We consider the implications of our findings in an extended "Discussion" section.

MATERIALS AND METHODS

Subjects

Subjects were male and female wild-type C57BL/6 mice (Jackson Labs, Bar Harbor, ME, USA) or mice expressing *thy1*-derived yellow fluorescent protein (YFP; Feng et al., 2000) that were fully back-crossed onto a C57BL/6 background. Mice were not handled, other than for routine veterinary care, until P31. Mice were group-housed, maintained on a 12-h light cycle (0700 on) and provided food and water *ad libitum* except during instrumental conditioning when body weights were maintained at 90%–93% of baseline to motivate responding. Animal numbers for each experiment are indicated in the respective figure captions. This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Emory University IACUC.

CORT Exposure

CORT hemisuccinate (4-pregnen-11β 21-DIOL-3 20-DIONE 21-hemisuccinate; Steraloids) was dissolved in tap water (25 μg/mL free base; Gourley et al., 2008a,b, 2012b). CORT-exposed mice were given CORT in place of normal drinking water, while control mice consumed tap water. CORT solutions were changed every 3 days. Water bottles were weighed daily, and mice weighed every other day to calculate average doses (∼5–9 mg/kg/day) of CORT. Mice were exposed to CORT or water from P31 to 42, corresponding to early adolescence in rodents (Spear, 2000). After a 2-week washout period, when mice reached young adulthood (P56), instrumental conditioning began. Timelines are in the figures.

Forced Swim Stress

Mice were exposed to forced swim stress daily from P31 to 42. Mice were placed in a glass cylinder (24 cm × 15.5 cm

diameter) filled with 25°C water in a dimly lit room. After 6 min, mice were dried in a warm cage lined with paper towels, then returned to the home cage. Water was changed between mice. Control mice were handled but not exposed to swim stress.

Instrumental Conditioning

Mice were trained to nose poke for food reinforcement (20 mg grain-based pellets; Bio-Serv, Flemington, NJ, USA) using Med Associates conditioning chambers equipped with two nose-poke recesses, a retractable lever, and a food magazine. Responding on each of two nose-poke recesses was reinforced using a fixed ratio 1 (FR1) schedule of reinforcement; 30 reinforcers were available for responding on each aperture, resulting in 60 reinforcers/session. Sessions ended when mice acquired all 60 reinforcers or at 70 min. Five to seven daily training sessions were conducted. Response acquisition curves represent both nose poke responses/min. Mice were then tested in an instrumental contingency degradation task, followed by a reversal task (see timeline in **Figure 1A**). Given our focus on reversal conditioning here, only reversal performance is shown.

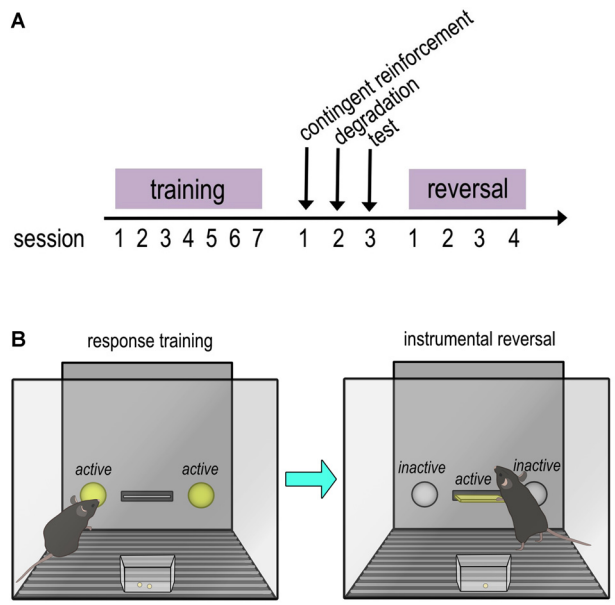


FIGURE 1 | Timeline and schematic of instrumental training and reversal task. **(A)** Timeline of training and tasks. Mice were trained to nose poke in operant conditioning chambers for food reinforcers. Next, an action-outcome contingency degradation task was performed, in which the contingency between an action and outcome are "degraded" by providing food pellets independently of the mouse's actions. The data from these tests are reported in Barfield et al. (in press). An instrumental reversal task followed, and the data from these tests are reported in the present manuscript. **(B)** Schematic of response training and instrumental reversal. In the instrumental reversal task, the reinforced response was "reversed" from a nose poke on the sides of the chamber to a lever press located in the center of the chamber that had not previously been available. Acquisition of the newly reinforced response (lever press) and inhibition of the previously reinforced response (nose poke) were measured over multiple sessions.

Instrumental Reversal Test

In this task, the reinforced response was "reversed" to a lever press; there were no consequences for generating the previously reinforced nose poke response (**Figure 1B**). Lever pressing was reinforced using an FR1 schedule, with one 25-min session/day for four consecutive days. Lever press rates reflect acquisition of a new response, while nose poke rates reflect inhibition of the previously reinforced response. The percent of correct responses made during session 1 was calculated by dividing total lever presses by total responses (lever presses + nose pokes).

Drugs (Dosing and Timing)

Male and female mice were administered (*i.p.*) the putative trkB agonist, 7,8-dihydroxyflavone (7,8-DHF; Sigma; 3 mg/kg; dissolved in 17% DMSO and saline; Zhang et al., 2014), or vehicle daily from P39 to 47, overlapping with the end of the adolescent CORT exposure period. This period is marked by significant pruning of dendritic spines on excitatory pyramidal neurons in the mouse orbital PFC (oPFC; Shapiro et al., 2017b). Additionally, expression levels of trkB in the oPFC increase during this time (Shapiro et al., 2017b), potentially facilitating activity-dependent refinement of synaptic connections and stabilizing synapses that are not pruned. This period was also determined based on prior work (Barfield et al., in press).

The trkB antagonist, ANA-12 (Sigma; 0.5 mg/kg, 1% DMSO), or vehicle was administered (*i.p.*) daily from P31 to 42, to match the period of adolescent CORT exposure.

Statistical Analyses

Two-tailed statistical analyses with $\alpha \leq 0.05$ were performed using SPSS. Response rates were compared by two-factor (group × session) or three-factor (CORT × 7,8-DHF × session) mixed analysis of variance (ANOVA) with session as a within-subjects (repeated measure) factor. Tukey's *post hoc* tests were used following interactions or main effects between greater than two groups, and results are indicated graphically. The percent of responses that were reinforced ("correct") were compared by one-factor or two-factor (CORT × 7,8-DHF) ANOVA or Student's *t*-tests. In additional comparisons, CORT-exposed mice were split into "vulnerable" and "non-vulnerable" groups based on a median split of percent correct values from session 1 of the instrumental reversal task. Throughout, values >2 SDs from the mean were excluded.

RESULTS

Early-Adolescent Corticosteroid Exposure in Females Impairs Response Inhibition in Adulthood

We exposed female and male mice to CORT in the drinking water from P31 to 42, equivalent to early adolescence in humans (Spear, 2000). Some mice also received daily *i.p.* injections of vehicle (17% DMSO and saline) or 7,8-DHF (3 mg/kg) from P39 to P47. In the interest of clarity, only

vehicle-treated and non-injected mice are shown in **Figure 2**, and then all injected groups are represented in **Figures 3**, **4**, respectively.

As adults, female mice acquired the nose poke responses without group differences (main effect and interaction $Fs < 1$; **Figure 2A**). When the response requirement was "reversed" to a lever press, females with a history of adolescent CORT exposure made more perseverative errors than control mice—responding on the previously reinforced nose poke apertures despite non-reinforcement—during session 1 (interaction $F_{(3,69)} = 4.0$, $p = 0.01$; **Figure 2B**). Meanwhile, acquisition of the newly reinforced lever response was unaffected (main effect and interaction $Fs < 1$; **Figure 2C**).

We also calculated the percentage of total responses that were reinforced during session 1. While this measure did not significantly differ between groups ($t_{(23)} = 1.3$, $p = 0.2$; **Figure 2D**), mice with a history of CORT exposure qualitatively appeared to segregate into two groups. As an exploratory analysis, we applied a median split to CORT mice. CORT-exposed "vulnerable" mice made significantly fewer correct responses relative to both control and CORT-exposed "non-vulnerable" mice (main effect group $F_{(2,22)} = 11.1$, $p < 0.001$; **Figure 2E**). Thus, in females, a history of CORT exposure during early adolescence increases perseverative errors, and considerable individual differences are noted.

We repeated this experiment in male mice. Mice acquired the food-reinforced nose poke responses in adulthood, without group differences (main effect and interaction $Fs < 1$; **Figure 2F**). In contrast to females, early-adolescent CORT exposure in males did not impact response reversal (errors, main effect and interaction $Fs < 1$; **Figure 2G**; lever response acquisition, main effect and interaction $Fs < 1$; **Figure 2H**). There were no differences in the percentage of responses that were reinforced ($t_{(12)} = 0.8$, $p = 0.5$; **Figure 2I**), and a median split of values revealed no differences between control mice and either of the two CORT-exposed subgroups (main effect group $F_{(2,11)} = 2.0$, $p = 0.2$; **Figure 2J**). Thus, females may be especially vulnerable to the long-term effects of CORT exposure during early adolescence on response inhibition.

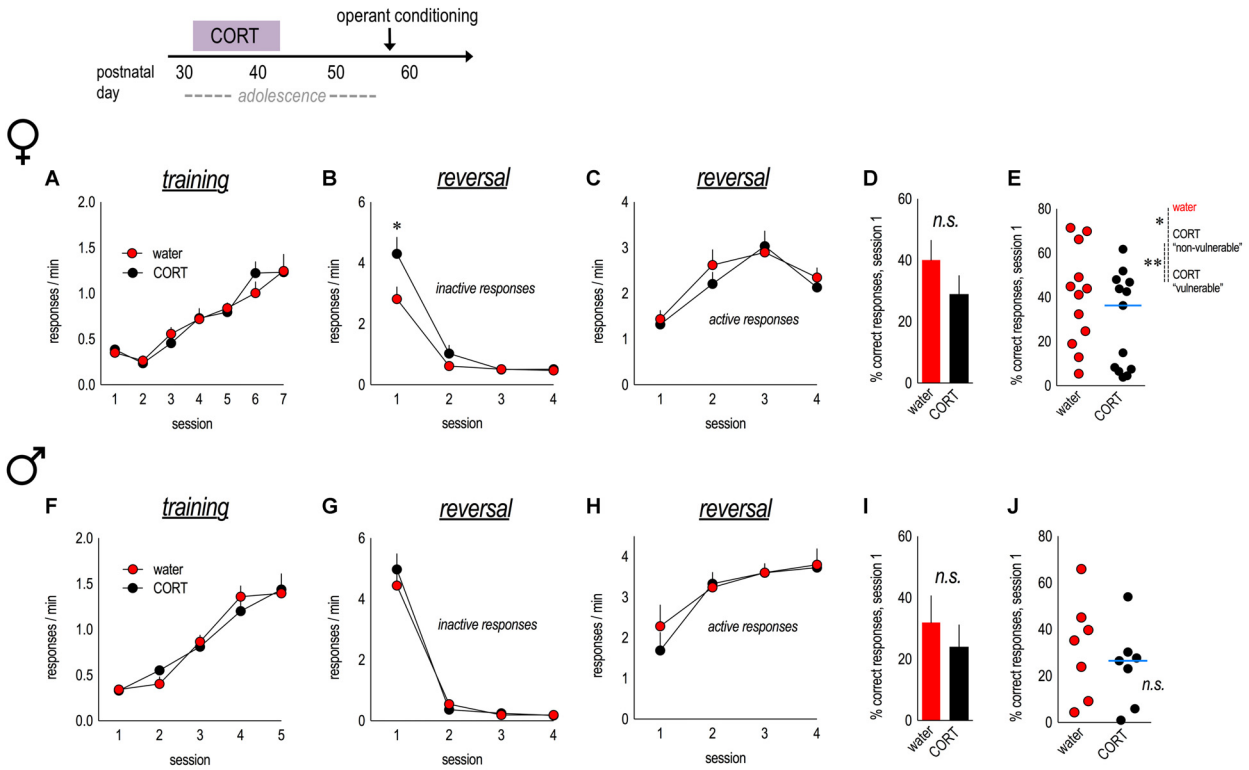


FIGURE 2 | Corticosterone (CORT) exposure in adolescent females induces reversal errors in adulthood. An experimental timeline is at top. **(A)** Female mice with a history of CORT exposure were trained to nose poke for food reinforcers, with no group differences. **(B)** In an instrumental reversal task, CORT-exposed mice made more perseverative errors, generating the previously-reinforced response. **(C)** CORT did not impact the acquisition of the new lever press response. **(D)** The percent of correct responses made during session 1 did not differ between groups. **(E)** However, a median split revealed that CORT-exposed females separated into two distinct subgroups; the "vulnerable" group made significantly fewer correct responses. $n = 12$–13/group. **(F)** Male mice exposed to CORT during adolescence acquired the nose poke responses without group differences. **(G)** A history of early-adolescent CORT exposure did not impact inhibition of the previously reinforced, now-inactive, response. **(H)** Acquisition of the newly reinforced response was also not impacted. **(I,J)** The percent of correct responses made during session 1 also did not differ between groups. $n = 7$/group. Symbols and bars represent means + SEMs, except for scatter plots, where symbols represent individual mice, and blue lines indicate the median. $^{*}p \leq 0.05$. $^{**}p \leq 0.001$.

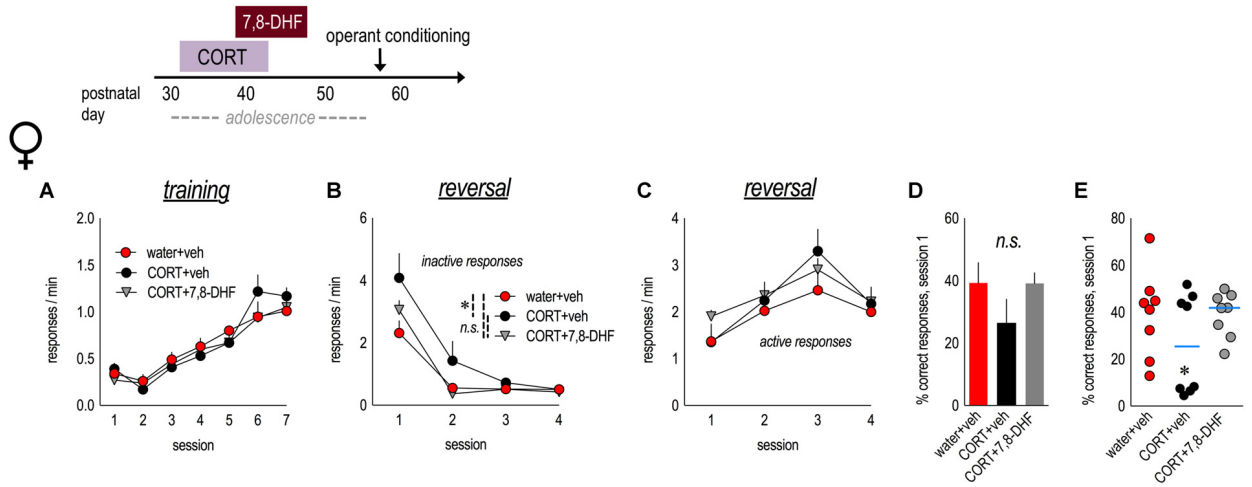


FIGURE 3 | Tyrosine kinase receptor B (TrkB) stimulation blocks perseverative errors in CORT-exposed female mice. Experimental timeline is at top. Female mice were exposed to CORT from P31 to 42 and administered the trkB agonist, 7,8-dihydroxyflavone (7,8-DHF), from P39 to 47. **(A)** As adults, mice were trained to nose poke for food reinforcers, with no differences between groups. **(B)** CORT-exposed mice made more perseverative errors, and 7,8-DHF mitigated this effect. **(C)** Groups generated the newly reinforced response at similar rates. **(D)** There were no gross effects on the percent of correct responses made during session 1. **(E)** CORT-exposed mice were divided into two subgroups on the basis of a median split of percent values, revealing two populations, including a subgroup that made significantly fewer correct responses. 7,8-DHF eliminated these differences. n = 8/group. Symbols and bars represent means + SEMs. $^*p \leq 0.05$.

TrkB Stimulation Blocks CORT-Induced Errors

We attempted to block the long-term behavioral consequences of adolescent CORT exposure in females by administering 7,8-DHF (3 mg/kg) during mid-adolescence (P39–47). A 7,8-DHF-only group was omitted to conserve animal usage. We found no group differences in response acquisition (main effect and interaction Fs < 1; **Figure 3A**). As before, mice with a history of adolescent CORT exposure generated more non-reinforced responses in reversal (main effect group $F_{(2,21)} = 3.6$, $p = 0.046$; session × group interaction $F_{(6,63)} = 2.1$, $p = 0.07$), and treatment with 7,8-DHF mitigated this impairment in response inhibition (**Figure 3B**). Acquisition of the newly reinforced lever press did not differ (main effect and interaction Fs ≤ 1; **Figure 3C**). Groups did not differ in the percentage of responses that were reinforced during session 1 (main effect group $F_{(2,21)} = 1.5$, $p = 0.3$; **Figure 3D**). Further, treatment with 7,8-DHF eliminated the existence of "vulnerable" and "non-vulnerable" populations within the CORT-exposed group (ANOVA with CORT+vehicle and CORT+7,8-DHF mice separated into two subgroups based on a median split (five groups total): main effect group $F_{(4,19)} = 7.9$, $p = 0.001$; **Figure 3E**). Thus, a trkB agonist occludes long-term deficits in response inhibition following early-life CORT exposure.

TrkB Stimulation in Adolescence Improves Behavioral Flexibility in Adulthood

Male mice exposed to water or CORT during early adolescence were also administered either vehicle or 7,8-DHF. As adults, all mice acquired the nose poke responses for food reinforcement, without group differences (main effect and interaction Fs ≤ 1.2; **Figure 4A**). In instrumental reversal, a history of CORT exposure did not impact response inhibition (Fs < 1) or acquisition of the newly reinforced response (Fs < 1), as described above. Nevertheless, 7,8-DHF reduced perseverative responding during session 1 in both control and CORT-exposed animals (session × 7,8-DHF interaction $F_{(3,72)} = 7.1$, $p < 0.001$; **Figure 4B**) and increased responding on the newly reinforced lever in session 1 (session × 7,8-DHF interaction $F_{(3,72)} = 6.6$, $p = 0.001$; **Figure 4C**). 7,8-DHF also increased the percentage of responses that were correct during session 1 (main effect $F_{(1,24)} = 7.7$, $p = 0.01$; CORT × 7,8-DHF interaction $F_{(1,24)} = 1.8$, $p = 0.2$; **Figure 4D**), further indicating that stimulation of trkB during adolescence enhances behavioral flexibility in adulthood, well beyond the period of drug treatment.

Adolescent Stressor Exposure and TrkB Inhibition Impair Reversal Performance

The apparent resilience of male mice to early-adolescent CORT exposure was somewhat surprising, so we exposed another group of males to swim stress during early adolescence (daily from P31 to 42). In adulthood, we detected no group differences in the acquisition of the nose poke responses (main effect $F < 1$; interaction $F_{(4,68)} = 1.8$, $p = 0.1$; **Figure 5A**). When mice were required to shift responding to a previously non-reinforced lever, control and stressor-exposed mice did not differ in responding on the previously reinforced nose poke apertures (main effect $F_{(1,16)} = 1.3$, $p = 0.3$; interaction $F_{(3,48)} = 1.3$, $p = 0.3$; **Figure 5B**). However, mice with a history of adolescent stressor exposure responded less on the newly reinforced lever (main effect $F_{(1,16)} = 8.1$, $p = 0.01$; session × group interaction $F_{(3,48)} = 1.2$, $p = 0.3$; **Figure 5C**), making fewer reinforced responses during

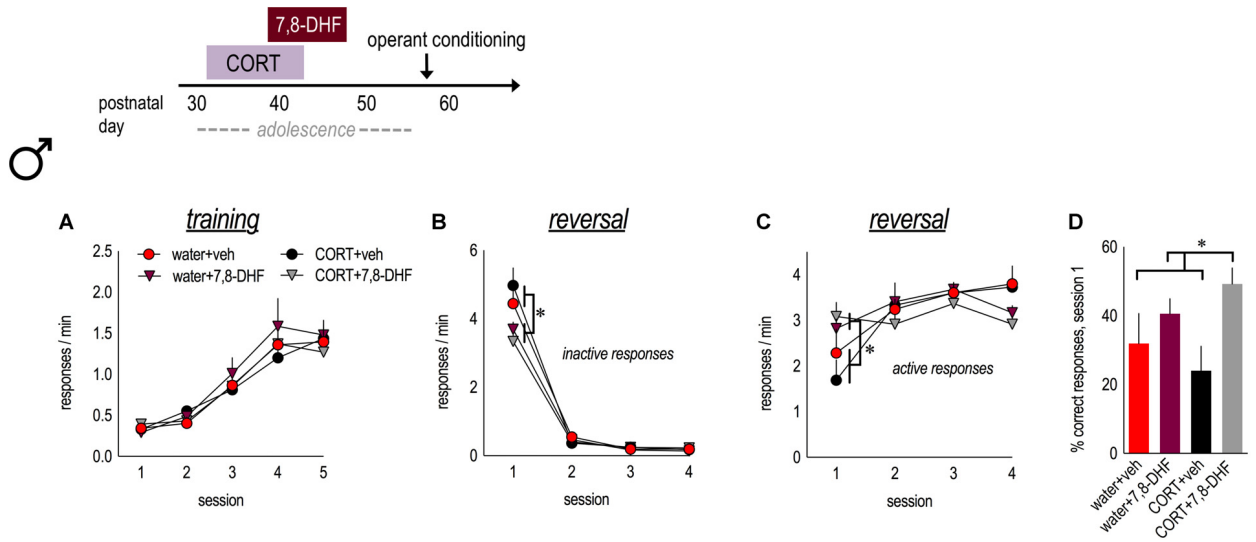


FIGURE 4 | TrkB stimulation during adolescence enhances behavioral flexibility in adulthood. Experimental timeline is at top. Male mice were exposed to CORT from P31 to 42 and administered the trkB agonist, 7,8-DHF, from P39 to 47. **(A)** Mice acquired the nose poke responses in adulthood, with no group differences. **(B)** In both water and CORT-exposed mice, 7,8-DHF reduced perseverative errors in the first reversal session. **(C)** Further, 7,8-DHF increased active response rates on the first day of reversal. **(D)** 7,8-DHF also increased the percent of correct responses made during session 1 (asterisk signifies a main effect of 7,8-DHF). n = 7/group. Symbols and bars represent means + SEMs. $^*p \leq 0.05$.

session 1 ($t_{(17)}$ = 2.3, p = 0.04; **Figure 5D**). Thus, stressor exposure in early adolescence impairs the ability of male mice to develop novel response strategies in adulthood. This deficiency is similar to those following lateral oPFC (loPFC) ablation (Gourley et al., 2010; summarized in **Table 1**).

Prolonged stressor or glucocorticoid exposure can down-regulate *Bdnf* or BDNF-trkB signaling in the PFC, and these alterations are associated with impairments in PFC-dependent behavioral tasks (Gourley et al., 2009a, 2012b; Xu et al., 2016; Zhang et al., 2017). Thus, we also tested whether inhibiting trkB during early adolescence would induce reversal deficits in male mice.

Male mice received the trkB antagonist, ANA-12 (0.5 mg/kg), daily from P31 to 42, matching the period of early-adolescent CORT exposure. As adults, mice acquired the nose poke responses for food reinforcement, without group differences (main effect $F_{(1,12)}$ = 3.6, p = 0.08; interaction $F_{(4,48)}$ = 2.2, p = 0.09; **Figure 5E**). ANA-12 impaired response inhibition (interaction $F_{(3,33)}$ = 10.4, $p < 0.001$; **Figure 5F**) and also acquisition (interaction $F_{(3,30)}$ = 4.1, p = 0.01; **Figure 5G**) during session 1. ANA-12-treated mice made fewer correct responses overall during session 1 ($t_{(12)}$ = 3.1, p = 0.01; **Figure 5H**). Thus, trkB inhibition during adolescence recapitulated the effects of CORT exposure in females (perseverative responding) and also stressor exposure in males (response acquisition impairments). Our findings are summarized in **Table 1**.

DISCUSSION

Adolescents who experience chronic stress have higher incidences of stress-related psychiatric illnesses and behaviors associated with addiction as adults. These behaviors may result in part from disruption of the PFC-dependent ability to flexibly modify behavior when environmental contingencies change (Elton et al., 2014; Zhang et al., 2016; see for review Watt et al., 2017). BDNF signaling through trkB regulates cellular maturational processes occurring in the PFC during adolescence (Xu et al., 2000; Shapiro et al., 2017b), and is disrupted by exposure to stress or elevated glucocorticoids (Bath et al., 2013; Numakawa et al., 2013; Suri and Vaidya, 2013). Using an oPFC-dependent instrumental reversal task in mice (see Gourley et al., 2010), we evaluated the long-term consequences of adolescent exposure to CORT, physical stress, trkB stimulation, or trkB inhibition, on behavioral flexibility. In females, adolescent CORT exposure increased perseverative errors in adulthood, and this was blocked by a trkB agonist. By contrast, behavioral flexibility was not impacted by CORT exposure in males, but was enhanced by trkB stimulation, as in females. Males did, however, develop reversal deficits in adulthood following repeated stressor exposure or trkB antagonism during adolescence. These findings add to evidence that adverse events or elevated glucocorticoid levels during adolescence can impair behavioral flexibility later in life. Moreover, our data suggest that trkB-based interventions may prevent adolescent stress-related behavioral impairments later in life.

Long-Term, Sex-Dependent Behavioral Consequences of Adolescent CORT Exposure

The majority of rodent studies examining the long-term neurobehavioral effects of early-life adversity have utilized male subjects, despite evidence for sex differences in the

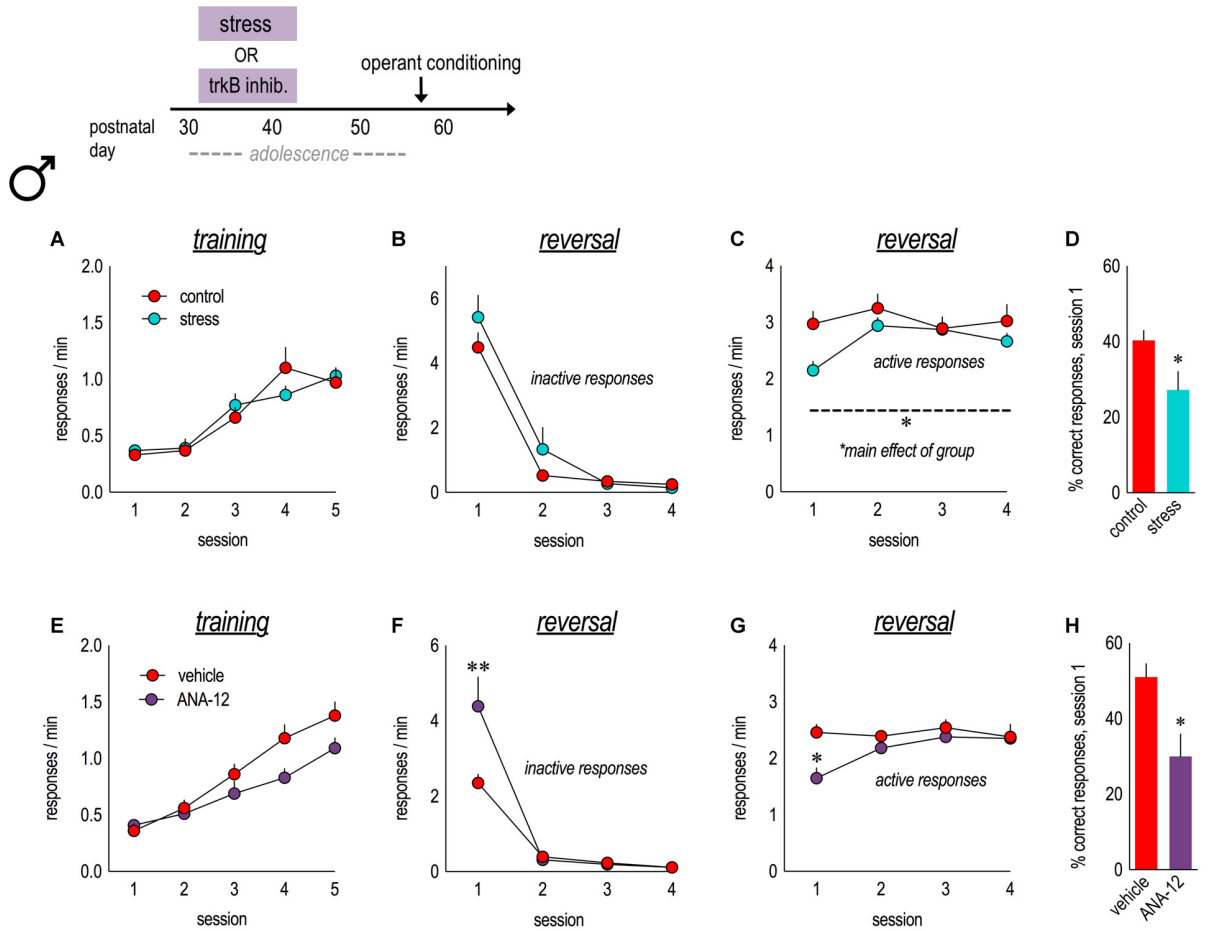


FIGURE 5 | Physical stressors and pharmacological inhibition of trkB in adolescence impair instrumental reversal in adulthood. Experimental timeline is at top. **(A)** A history of stressor exposure did not impact responding for food reinforcement during training in adulthood. **(B)** In an instrumental reversal task, both groups performed the now-inactive nose poke response at similar rates. **(C)** By contrast, stressor-exposed mice performed the now-active lever press response at lower rates. **(D)** Adolescent stressor exposure also reduced the percent of correct responses made during session 1. n = 9–10/group. **(E)** In parallel experiments, mice received daily injections of the trkB antagonist, ANA-12, from P31 to 42. Mice learned to nose poke for food reinforcement in adulthood, with no group differences. **(F)** TrkB blockade during adolescence increased perseverative errors during the first reversal session. **(G)** ANA-12 also reduced responding on the now-active lever during session 1. **(H)** ANA-12-treated mice made fewer correct responses during session 1. n = 7/group. Symbols and bars represent means + SEMs. $^{*}p \leq 0.05$. $^{**}p \leq 0.001$.

prevalence and severity of stress-related psychiatric illnesses (Kessler et al., 1993; MacMillan et al., 2001; Holbrook et al., 2002; Kuehner, 2003; Becker et al., 2012; Fattore et al., 2014). We report that CORT dissolved in the drinking water of mice during a period equivalent to early adolescence in humans (P31–42; Spear, 2000) impaired performance in an instrumental reversal task in adulthood in female mice, but not males. Specifically, adolescent CORT impaired the inhibition of a previously reinforced response, increasing perseverative errors. Similarly, exposure to stress-level cortisol in young-adult or older-adult female squirrel monkeys increases response inhibition errors in a detour-reaching task that assesses the ability to inhibit prepotent responses when reinforcement contingencies change (Lyons et al., 2000).

We also calculated the percent of responses that were reinforced ("correct") during the first reversal session, revealing a "CORT vulnerable" subgroup (approximately half of the group) with significantly impaired behavioral flexibility relative to a CORT-exposed "non-vulnerable" group and control mice. What mediates this vulnerability is unclear, but one factor may be dopamine D2-family receptors (D2, D3, D4). D2/3 antagonists disrupt reversal performance in monkeys and rodents (Ridley et al., 1981; Kruzich and Grandy, 2004; Floresco et al., 2006; Lee et al., 2007; De Steno and Schmauss, 2009), and variation in D2 receptor density in the ventral PFC (including oPFC; Gourley et al., 2009b) and midbrain (Laughlin et al., 2011) correlates with behavioral flexibility. Furthermore, adolescent stress exposure decreases PFC D2 expression in adulthood (Wright et al., 2008). Whether vulnerabilities to

TABLE 1 | Failures in response acquisition and errors of perseveration in an instrumental reversal task can be dissociated.

Manipulation	Region	Sex	Reference
Impairments of response acquisition			
Early-adolescent forced swim stress	n/a	M	**Figure 5C**
Early-adolescent trkB antagonism	n/a	M	**Figure 5G**
Lesion	loPFC	M	Gourley et al. (2010)
Inhibition of Abl-family kinases	loPFC	M	Gourley et al. (2012a)
Bdnf knockdown	loPFC	M	Gourley et al. (2013a)
Disconnection of the loPFC and striatum	loPFC	M	Gourley et al. (2013a)
Impairments of response inhibition			
Early-adolescent CORT exposure	n/a	F	**Figure 2B**
Early-adolescent trkB antagonism	n/a	M	**Figure 5F**
Lesion	moPFC+loPFC	M	Boulougouris et al. (2007)
Lesion	moPFC	M	Gourley et al. (2010)

Errors of perseveration (repeatedly performing an "old" response, despite reversal) or response acquisition (failing to develop a new strategy) can be dissociated in reversal tasks that rely on spatial or instrumental learning and memory. Here we detected failures in response acquisition and errors of perseveration (highlighted in blue). These deficits are associated with manipulations of the lateral oPFC (loPFC) and medial oPFC (moPFC), respectively (highlighted in gray).

corticosteroid-related modifications in dopamine D2 levels influence the behavioral patterns reported here could be tested in future experiments.

In contrast to females, males exposed to early-adolescent CORT failed to develop instrumental reversal impairments in adulthood. However, some studies report long-term deficits in reversal learning and extradimensional set-shifting (EDS; a medial PFC (mPFC)-dependent function) in male rodents following social stress during early adolescence (Snyder et al., 2015; Zhang et al., 2016). Accordingly, daily forced swim stress during early adolescence (P31–42) impaired the acquisition of the "reversed" response contingency here, resembling the effects of loPFC lesions (Gourley et al., 2010). These findings suggest that in males exposed to repeated stress during adolescence, components of the stress response besides elevations in CORT (e.g., increased noradrenergic tone) may be responsible for response acquisition deficiencies. Indeed, under conditions of chronic stress in adult rats, stress-induced release of norepinephrine in the mPFC impairs certain forms of behavioral flexibility, while noradrenergic receptor blockade in the mPFC prevents stress-induced deficits (Jett and Morilak, 2013). On the other hand, 3 weeks of CORT exposure in adolescent male mice causes errors in a similar instrumental reversal task (Shapiro et al., 2017a), indicating that males are not entirely resilient to CORT.

One possibility is that higher concentrations of CORT would have triggered reversal deficiencies in males. Females have higher basal CORT levels than males, an effect that develops during puberty (Netherton et al., 2004; McCormick and Mathews, 2007; Stroud et al., 2011). Because puberty in rodents typically occurs around P35 in females and P40 in males (Korenbrot et al., 1977; Evans, 1986), administering exogenous CORT during a time when endogenous levels in females are increasing may result in higher total CORT levels in females. While we did not measure blood serum CORT in these experiments, it is possible that recapitulating "female-like" CORT levels in males may have induced comparable behavioral inflexibility.

Because the PFC undergoes significant structural remodeling during adolescence, and PFC-dependent cognitive functions continue to develop throughout this period, prolonged stressor or CORT exposure during adolescence may be more impactful than exposure in adulthood. Consistent with this possibility, male rats exposed to 5 days of social defeat stress during early adolescence (P28–32), then housed in isolation, exhibit deficits in EDS and reversal learning in an attentional set-shifting task 6 weeks later. However, rats exposed to the same stress in mid-adolescence (P38–47) or adulthood (P70–79) do not show these cognitive impairments (Zhang et al., 2016). Furthermore, adolescent stress- or CORT-induced alterations in PFC neuronal morphology, like behavioral deficits, can persist into adulthood. For example, social isolation stress during early adolescence (P30–35) in male rats reduces synaptic density in the mPFC in adulthood (Leussis et al., 2008), suggestive of a long-term loss of dendritic spines, which house the majority of excitatory synapses in the brain. Gourley et al. (2013b) also reported dendritic spine loss on pyramidal neurons in the oPFC immediately following CORT exposure during adolescence (P35–56) that is still evident 1 week after the cessation of CORT. Meanwhile, CORT-induced spine changes in the infralimbic mPFC, hippocampus, and amygdala recover. Consistent with these findings in rodents, early-life adversity in humans is associated with reduced oPFC volume and morphological alterations in adulthood (Holz et al., 2015; Teicher and Samson, 2016). Our findings add to these and other studies reporting long-term consequences of stressor or CORT exposure during early adolescence.

TrkB Manipulations in Adolescence Influence Behavioral Flexibility in Adulthood

One mechanism by which CORT or stressor exposure during adolescence may impact PFC-dependent behaviors later in life is by reducing BDNF-dependent stimulation of trkB (Duman et al., 1997; Blugeot et al., 2011). Consistent with this possibility, the trkB agonist 7,8-DHF blocked perseverative responding

in females with a history of adolescent CORT exposure. Additionally, while a subgroup of vehicle-treated CORT-exposed females was especially vulnerable to impairments in behavioral flexibility, 7,8-DHF eliminated these individual differences, and in males, 7,8-DHF improved performance above control levels.

How might 7,8-DHF have long-term behavioral consequences? One possibility is that it prevents CORT-induced disruptions in PFC synaptic maturation by augmenting or restoring trkB activity. BDNF and trkB increase in adolescence/young adulthood (Webster et al., 2002; Shapiro et al., 2017b), and BDNF-trkB signaling is critical for the maintenance of stabile dendritic spines in the postnatal brain (Vigers et al., 2012). Chronic stress or CORT exposure can reduce PFC BDNF and trkB (e.g., see Gourley et al., 2009a, 2012b), potentially altering the trajectory of dendritic spine maturation (Leussis and Andersen, 2008; Eiland et al., 2012) and behavioral outcomes. Consistent with this possibility, we found that administration of the trkB antagonist, ANA-12, during early adolescence impaired behavioral flexibility in adulthood.

Our trkB antagonism experiments were performed in males, but it is worth noting that prior reports indicate that knockdown of *Bdnf* beginning in the preadolescent period or in adulthood in female mice produces behavioral consequences that are comparable to, or more severe than, those in male mice. For example, Vigers et al. (2012) report that forebrain-specific knockdown of *Bdnf* (progressive knockdown begins at ∼P21) induces depressive-like immobility in the forced swim test and contextual fear generalization in both male and female adult mice. However, Monteggia et al. (2007) report that this knockdown strategy results in depression-like behavior in female, but not male, mice. Similarly, inducible knockdown of *Bdnf* in adulthood increases susceptibility to stress-induced depression-like behavior in female mice only (Autry et al., 2009).

Recent findings in addition to ours interestingly implicate BDNF-trkB in the long-term behavioral consequences of early-adolescent stress. Male rats exposed to chronic mild stress (CMS) during early adolescence (P28–37) exhibit impairments in EDS and decreased levels of BDNF protein, the ratio of p-ERK42/44/ERK42/44, and p-CREB in the mPFC 6 weeks after stressor exposure (Zhang et al., 2017). Additionally, EDS is correlated with p-ERK/ERK ratios in mPFC, and the antidepressant duloxetine ameliorates stress-induced alterations in EDS and mPFC BDNF levels in tandem. *Impairing* BDNF-trkB signaling can also alter PFC-dependent functions and drug-taking behaviors. For example, mutant mice with disruption of promoter IV-driven (activity-dependent) BDNF expression are impaired in a spatial reversal task (Sakata et al., 2013). Knock-in mice expressing a homolog of the human *BDNF* gene polymorphism *Val66Met*, which decreases the activity-dependent release of BDNF (Chen et al., 2004), develop excessive and compulsive alcohol drinking despite adverse consequences, and this phenotype is reversible by a trkB agonist (Warnault et al., 2016). Furthermore, early-life stress decreases *Bdnf* exon IV mRNA in the PFC and increases cocaine-induced conditioned place preference (CPP) in periadolescent (P45) mice (Viola et al., 2016). Interestingly, higher CPP scores correlate with *Bdnf* expression, with higher place preference associated with lower *Bdnf*. Thus, by disrupting trkB signaling, early-life stress may increase vulnerability to compulsive drug taking.

Broader Considerations: Sex Differences in Reversal Performance

Here, we found that adolescent CORT-exposed females and adolescent stress-exposed males exhibited impairments in reversal performance in adulthood; however, the specific behavioral impairments were sexually dimorphic—females failed to inhibit the previously reinforced response, while males were unable to acquire a newly reinforced response (summarized in **Table 1**). In the context of addiction, poor control over perseverative behavior could modulate the progression from recreational drug use to abuse and dependence, increase relapse rates, and impair treatment response (Carroll et al., 2011). Meanwhile, deficits in the acquisition of a "reversed" response contingency could be comparable to impairments in the ability to adjust behavior according to positive feedback and potentially, to substitute new healthy behaviors for maladaptive ones.

The association between early-life trauma and the risk for, and severity of, drug dependence is stronger in women (Widom and White, 1997; MacMillan et al., 2001; Simpson and Miller, 2002; Hyman et al., 2008), and at least one study reports that a greater proportion of stimulant-dependent women, compared to men, have clinically significant "disinhibition" scores prior to stimulant abuse (Winhusen and Lewis, 2013). In that same study, physical abuse was associated with greater disinhibition in women, but not men. Thus, females may be more vulnerable to chronic stress-induced inhibitory control deficits, in line with our findings. Further, Elton et al. (2014) report that childhood maltreatment results in enduring, sex-dependent changes in the functional connectivity of a network mediating inhibitory behavioral control, also in line with sex-dependent effects of adolescent CORT exposure on perseverative responding here. Future work should examine whether adolescent CORT-induced deficits in response inhibition increase vulnerability to compulsive drug taking later in life. CORT exposure during adolescence increases cue-induced reinstatement of ethanol seeking in adult female, but not male, rats (Bertholomey et al., 2016), suggesting that females are especially vulnerable to adolescent CORT-induced neurobehavioral changes that increase susceptibility to drug relapse later in life.

Our findings may also point to sexually dimorphic stress-related vulnerabilities in specific brain regions, given that lesions of the medial oPFC (moPFC) impair response inhibition in this task (like CORT-exposed females), while loPFC lesions impair response acquisition (like stressor-exposed males; Gourley et al., 2010). These patterns are interesting when considered alongside studies reporting structural changes in the oPFC of substance-dependent males and females. For example, cocaine-dependent females and males exhibit decreased cerebral blood flow in the moPFC and loPFC, respectively (Adinoff et al., 2006). Adolescent females with substance dependence exhibit decreased cortical thickness and gray matter volume in regions involved

in inhibitory control, decision-making, reward, and risk-taking, including the moPFC and dorsolateral PFC (DLPFC; Dalwani et al., 2015; Boulos et al., 2016), while substance-dependent adolescent males show decreased gray matter volume in DLPFC (Dalwani et al., 2011). Regner et al. (2015) report decreased oPFC gray matter volume in abstinent stimulant-dependent women, but not men. However, in a population of predominantly male treatment-seeking individuals with alcohol dependence, loPFC surface area and volume were smaller in individuals who subsequently relapsed in a 1-year period compared to individuals who remained abstinent (Cardenas et al., 2011; Durazzo et al., 2011). Together, these findings suggest that PFC subregions disrupted by both adolescent stress or glucocorticoid exposure and drugs of abuse may be sex-dependent (potentially: loPFC in males and both moPFC and loPFC in females; see **Table 1**).

CONCLUSION

PFC dysfunction and associated deficits in inhibitory control and behavioral flexibility are hallmarks of stress-related illnesses, including addiction. Substance-dependent individuals report loss of control over drug use and have difficulty modifying behaviors when reward contingencies change, potentially contributing to persistent drug seeking despite adverse consequences (Garavan and Hester, 2007; Everitt and Robbins, 2016). Being able to implement new strategies to promote behavioral change and control impulsive responses may be critical to treatment response—among cocaine-dependent individuals undergoing cognitive-behavioral therapy, higher impulsivity/risk-taking before treatment is associated with poorer treatment retention and higher relapse rates (Carroll et al., 2011).

Substance-dependent individuals exhibit structural and functional alterations in the oPFC, including blood flow abnormalities (London et al., 2000; Adinoff et al., 2006), decreased gray matter volume (Matochik et al., 2003; Tanabe et al., 2009; Dalwani et al., 2015), and decreased cortical thickness (Boulos et al., 2016). In animal models, adolescent CORT and cocaine exposure result in long-term changes in oPFC neuron structure (Gourley et al., 2012a, 2013b; DePoy et al., 2014, 2016, 2017) that are associated with maladaptive behaviors symptomatic of addiction (Lucantonio et al., 2012). Further, poor inhibitory control may be a *predisposing factor* for addiction (Jentsch and Taylor, 1999; Tarter et al., 2004; Groman et al., 2009; Moffitt et al., 2011), as is a history of early-life adversity (Fergusson et al., 1996; Kessler et al., 1997; Dube et al., 2003; Green et al., 2010; Afifi et al., 2012). These and other findings have led to the hypothesis that early-life adversity may affect addiction liability by impairing oPFC-mediated inhibitory control. That is, in individuals with a history of stressor exposure during sensitive developmental periods like adolescence, repeated drug use may further impair inhibitory control, exacerbating the development, persistence, and severity of addiction. Indeed, social stress during adolescence in rats causes binge-like cocaine self-administration in adulthood (Burke and Miczek, 2015).

Using an oPFC-dependent instrumental reversal task, we find that exposure to CORT during early adolescence induces perseverative errors in female, but not male, mice in adulthood. Further, intervention with a trkB agonist in mid-adolescence, when dendritic spines in the PFC undergo activity-dependent pruning or stabilization (Petanjek et al., 2011; Gourley et al., 2012a; Selemon, 2013; Chung et al., 2017) corrected decision-making strategies in CORT-exposed mice. With the caveat that the sexes were tested in independent cohorts here—precluding direct comparisons between them—we nevertheless argue that further related research may advance our understanding of how sex differences in the neurobehavioral response to adolescent adversities contribute to sex differences in vulnerability to, and clinical course of, stress-related illnesses. Our findings additionally suggest that pharmacotherapies augmenting trkB may ameliorate the enduring consequences of stressful life events occurring in adolescence.

AUTHOR CONTRIBUTIONS

ETB performed the experiments, analyzed the data and wrote the manuscript. ETB and SLG designed the experiments, interpreted the data and edited the manuscript.

FUNDING

This work was supported by an National Institutes of Health (NIH) BRAINS award (MH101477) and the National Science Foundation Graduate Research Fellowship Program under grant number DGE-1444932. The Yerkes National Primate Research Center is supported by the Office of Research Infrastructure Programs/OD P51OD011132.

ACKNOWLEDGMENTS

We thank A. Allen for assisting with injections.

REFERENCES

Adinoff, B., Williams, M. J., Best, S. E., Harris, T. S., Chandler, P., and Devous, M. D. (2006). Sex differences in medial and lateral orbitofrontal cortex hypoperfusion in cocaine-dependent men and women. *Gend. Med.* 3, 206–222. doi: 10.1016/s1550-8579(06)80209-3

Afifi, T. O., Henriksen, C. A., Asmundson, G. J., and Sareen, J. (2012). Childhood maltreatment and substance use disorders among men and women in a nationally representative sample. *Can. J. Psychiatry* 57, 677–686. doi: 10.1177/070674371205701105

Autry, A. E., Adachi, M., Cheng, P., and Monteggia, L. M. (2009). Gender specific impact of BDNF signaling on stress-induced depression-like behavior. *Biol. Psychiatry* 66, 84–90. doi: 10.1016/j.biopsych.2009.02.007

Barfield, E. T., Gerber, K. J., Zimmermann, K. S., Ressler, K. J., Parsons, R. G., and Gourley, S. L. (in press). Bidirectional regulation of actions and habits by ventral hippocampal trkB and adolescent corticosteroid exposure. *PLoS Biol.*

Bath, K. G., Schilit, A., and Lee, F. S. (2013). Stress effects on BDNF expression: effects of age, sex, and form of stress. *Neuroscience* 239, 149–156. doi: 10.1016/j.neuroscience.2013.01.074

Becker, J. B., Perry, A. N., and Westenbroek, C. (2012). Sex differences in the neural mechanisms mediating addiction: a new synthesis and hypothesis. *Biol. Sex Differ.* 3:14. doi: 10.1186/2042-6410-3-14

Belin, D., Mar, A. C., Dalley, J. W., Robbins, T. W., and Everitt, B. J. (2008). High impulsivity predicts the switch to compulsive cocaine-taking. *Science* 320, 1352–1355. doi: 10.1126/science.1158136

Bertholomey, M. L., Nagarajan, V., and Torregrossa, M. M. (2016). Sex differences in reinstatement of alcohol seeking in response to cues and yohimbine in rats with and without a history of adolescent corticosterone exposure. *Psychopharmacology* 233, 2277–2287. doi: 10.1007/s00213-016-4278-x

Blugeot, A., Rivat, C., Bouvier, E., Molet, J., Mouchard, A., Zeau, B., et al. (2011). Vulnerability to depression: from brain neuroplasticity to identification of biomarkers. *J. Neurosci.* 31, 12889–12899. doi: 10.1523/JNEUROSCI.1309-11.2011

Boulos, P. K., Dalwani, M. S., Tanabe, J., Mikulich-Gilbertson, S. K., Banich, M. T., Crowley, T. J., et al. (2016). Brain cortical thickness differences in adolescent females with substance use disorders. *PLoS One* 11:e0152983. doi: 10.1371/journal.pone.0152983

Boulougouris, V., Dalley, J. W., and Robbins, T. W. (2007). Effects of orbitofrontal, infralimbic and prelimbic cortical lesions on serial spatial reversal learning in the rat. *Behav. Brain Res.* 179, 219–228. doi: 10.1016/j.bbr.2007.02.005

Burke, A. R., and Miczek, K. A. (2015). Escalation of cocaine self-administration in adulthood after social defeat of adolescent rats: role of social experience and adaptive coping behavior. *Psychopharmacology* 232, 3067–3079. doi: 10.1007/s00213-015-3947-5

Cardenas, V. A., Durazzo, T. C., Gazdzinski, S., Mon, A., Studholme, C., and Meyerhoff, D. J. (2011). Brain morphology at entry into treatment for alcohol dependence is related to relapse propensity. *Biol. Psychiatry* 70, 561–567. doi: 10.1016/j.biopsych.2011.04.003

Carroll, K. M., Kiluk, B. D., Nich, C., Babuscio, T. A., Brewer, J. A., Potenza, M. N., et al. (2011). Cognitive function and treatment response in a randomized clinical trial of computer-based training in cognitive-behavioral therapy. *Subst. Use Misuse* 46, 23–34. doi: 10.3109/10826084.2011.521069

Cervantes, M. C., Laughlin, R. E., and Jentsch, J. D. (2013). Cocaine self-administration behavior in inbred mouse lines segregating different capacities for inhibitory control. *Psychopharmacology* 229, 515–525. doi: 10.1007/s00213-013-3135-4

Chen, Z. Y., Patel, P. D., Sant, G., Meng, C. X., Teng, K. K., Hempstead, B. L., et al. (2004). Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. *J. Neurosci.* 24, 4401–4411. doi: 10.1523/JNEUROSCI.0348-04.2004

Chung, D. W., Wills, Z. P., Fish, K. N., and Lewis, D. A. (2017). Developmental pruning of excitatory synaptic inputs to parvalbumin interneurons in monkey prefrontal cortex. *Proc. Natl. Acad. Sci. U S A* 114, E629–E637. doi: 10.1073/pnas.1610077114

Dalley, J. W., Fryer, T. D., Brichard, L., Robinson, E. S., Theobald, D. E., Laane, K., et al. (2007). Nucleus accumbens D2/3 receptors predict trait impulsivity and cocaine reinforcement. *Science* 315, 1267–1270. doi: 10.1126/science.1137073

Dalwani, M., Sakai, J. T., Mikulich-Gilbertson, S. K., Tanabe, J., Raymond, K., McWilliams, S. K., et al. (2011). Reduced cortical gray matter volume in male adolescents with substance and conduct problems. *Drug Alcohol Depend.* 118, 295–305. doi: 10.1016/j.drugalcdep.2011.04.006

Dalwani, M. S., McMahon, M. A., Mikulich-Gilbertson, S. K., Young, S. E., Regner, M. F., Raymond, K. M., et al. (2015). Female adolescents with severe substance and conduct problems have substantially less brain gray matter volume. *PLoS One* 10:e0126368. doi: 10.1371/journal.pone.0126368

DePoy, L. M., Allen, A. G., and Gourley, S. L. (2016). Adolescent cocaine self-administration induces habit behavior in adulthood: sex differences and structural consequences. *Transl. Psychiatry* 6:e875. doi: 10.1038/tp.2016.150

DePoy, L. M., Perszyk, R. E., Zimmermann, K. S., Koleske, A. J., and Gourley, S. L. (2014). Adolescent cocaine exposure simplifies orbitofrontal cortical dendritic arbors. *Front. Pharmacol.* 5:228. doi: 10.3389/fphar.2014.00228

DePoy, L. M., Zimmermann, K. S., Marvar, P. J., and Gourley, S. L. (2017). Induction and blockade of adolescent cocaine-induced habits. *Biol. Psychiatry* 81, 595–605. doi: 10.1016/j.biopsych.2016.09.023

De Steno, D. A., and Schmauss, C. (2009). A role for dopamine D2 receptors in reversal learning. *Neuroscience* 162, 118–127. doi: 10.1016/j.neuroscience.2009.04.052

Dube, S. R., Felitti, V. J., Dong, M., Chapman, D. P., Giles, W. H., and Anda, R. F. (2003). Childhood abuse, neglect, and household dysfunction and the risk of illicit drug use: the adverse childhood experiences study. *Pediatrics* 111, 564–572. doi: 10.1542/peds.111.3.564

Duman, R. S., Heninger, G. R., and Nestler, E. J. (1997). A molecular and cellular theory of depression. *Arch. Gen. Psychiatry* 54, 597–606. doi: 10.1001/archpsyc.1997.01830190015002

Durazzo, T. C., Tosun, D., Buckley, S., Gazdzinski, S., Mon, A., Fryer, S. L., et al. (2011). Cortical thickness, surface area and volume of the brain reward system in alcohol dependence: relationships to relapse and extended abstinence. *Alcohol. Clin. Exp. Res.* 35, 1187–1200. doi: 10.1111/j.1530-0277.2011.01452.x

Eiland, L., Ramroop, J., Hill, M. N., Manley, J., and McEwen, B. S. (2012). Chronic juvenile stress produces corticolimbic dendritic architectural remodeling and modulates emotional behavior in male and female rats. *Psychoneuroendocrinology* 37, 39–47. doi: 10.1016/j.psyneuen.2011.04.015

Elton, A., Tripathi, S. P., Mletzko, T., Young, J., Cisler, J. M., James, A. G., et al. (2014). Childhood maltreatment is associated with a sex-dependent functional reorganization of a brain inhibitory control network. *Hum. Brain Mapp.* 35, 1654–1667. doi: 10.1002/hbm.22280

Enoch, M. A. (2011). The role of early life stress as a predictor for alcohol and drug dependence. *Psychopharmacology* 214, 17–31. doi: 10.1007/s00213-010-1916-6

Ersche, K. D., Roiser, J. P., Robbins, T. W., and Sahakian, B. J. (2008). Chronic cocaine but not chronic amphetamine use is associated with perseverative responding in humans. *Psychopharmacology* 197, 421–431. doi: 10.1007/s00213-007-1051-1

Evans, A. M. (1986). Age at puberty and first litter size in early and late paired rats. *Biol. Reprod.* 34, 322–326. doi: 10.1095/biolreprod34.2.322

Everitt, B. J., and Robbins, T. W. (2016). Drug addiction: updating actions to habits to compulsions ten years on. *Annu. Rev. Psychol.* 67, 23–50. doi: 10.1146/annurev-psych-122414-033457

Fattore, L., Melis, M., Fadda, P., and Fratta, W. (2014). Sex differences in addictive disorders. *Front. Neuroendocrinol.* 35, 272–284. doi: 10.1016/j.yfrne.2014.04.003

Feng, G., Mellor, R. H., Bernstein, M., Keller-Peck, C., Nguyen, Q. T., Wallace, M., et al. (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 28, 41–51. doi: 10.1016/s0896-6273(00)00084-2

Fergusson, D. M., Horwood, L. J., and Lynskey, M. T. (1996). Childhood sexual abuse and psychiatric disorder in young adulthood: II. Psychiatric outcomes of childhood sexual abuse. *J. Am. Acad. Child Adolesc. Psychiatry* 35, 1365–1374. doi: 10.1097/00004583-199610000-00024

Floresco, S. B., Magyar, O., Ghods-Sharifi, S., Vexelman, C., and Tse, M. T. (2006). Multiple dopamine receptor subtypes in the medial prefrontal cortex of the rat regulate set-shifting. *Neuropsychopharmacology* 31, 297–309. doi: 10.1038/sj.npp.1300825

Garavan, H., and Hester, R. (2007). The role of cognitive control in cocaine dependence. *Neuropsychol. Rev.* 17, 337–345. doi: 10.1007/s11065-007-9034-x

Gourley, S. L., Kedves, A. T., Olausson, P., and Taylor, J. R. (2009a). A history of corticosterone exposure regulates fear extinction and cortical NR2B, GluR2/3, and BDNF. *Neurpsychopharmacology* 34, 707–716. doi: 10.1038/npp.2008.123

Gourley, S. L., Koleske, A. J., and Taylor, J. R. (2009b). Loss of dendrite stabilization by the Abl-related gene (Arg) kinase regulates behavioral flexibility and sensitivity to cocaine. *Proc. Natl. Acad. Sci. U S A* 106, 16859–16864. doi: 10.1073/pnas.0902286106

Gourley, S. L., Kiraly, D. D., Howell, J. L., Olausson, P., and Taylor, J. R. (2008a). Acute hippocampal BDNF restores motivational and forced swim performance after corticosterone. *Biol. Psychiatry* 64, 884–890. doi: 10.1016/j.biopsych.2008.06.016

Gourley, S. L., Wu, F. J., Kiraly, D. D., Ploski, J. E., Kedves, A. T., Duman, R. S., et al. (2008b). Regionally specific regulation of ERK MAP kinase in a model of antidepressant-sensitive chronic depression. *Biol. Psychiatry* 63, 353–359. doi: 10.1016/j.biopsych.2007.07.016

Gourley, S. L., Lee, A. S., Howell, J. L., Pittenger, C., and Taylor, J. R. (2010). Dissociable regulation of instrumental action within mouse prefrontal cortex. *Eur. J. Neurosci.* 32, 1726–1734. doi: 10.1111/j.1460-9568.2010.07438.x

Gourley, S. L., Olevska, A., Warren, M. S., Taylor, J. R., and Koleske, A. J. (2012a). Arg kinase regulates prefrontal dendritic spine refinement and cocaine-induced plasticity. *J. Neurosci.* 32, 2314–2323. doi: 10.1523/JNEUROSCI.2730-11.2012

Gourley, S. L., Swanson, A. M., Jacobs, A. M., Howell, J. L., Mo, M., DiLeone, R. J., et al. (2012b). Action control is mediated by prefrontal BDNF and glucocorticoid receptor binding. *Proc. Natl. Acad. Sci. U S A* 109, 20714–20719. doi: 10.1073/pnas.1208342109

Gourley, S. L., Olevska, A., Zimmermann, K. S., Ressler, K. J., Dileone, R. J., and Taylor, J. R. (2013a). The orbitofrontal cortex regulates outcome-based decision-making via the lateral striatum. *Eur. J. Neurosci.* 38, 2382–2388. doi: 10.1111/ejn.12239

Gourley, S. L., Swanson, A. M., and Koleske, A. J. (2013b). Corticosteroid-induced neural remodeling predicts behavioral vulnerability and resilience. *J. Neurosci.* 33, 3107–3112. doi: 10.1523/JNEUROSCI.2138-12.2013

Gray, J. D., Milner, T. A., and McEwen, B. S. (2013). Dynamic plasticity: the role of glucocorticoids, brain-derived neurotrophic factor and other trophic factors. *Neuroscience* 239, 214–227. doi: 10.1016/j.neuroscience.2012.08.034

Green, J. G., McLaughlin, K. A., Berglund, P. A., Gruber, M. J., Sampson, N. A., Zaslavsky, A. M., et al. (2010). Childhood adversities and adult psychiatric disorders in the national comorbidity survey replication I: associations with first onset of DSM-IV disorders. *Arch. Gen. Psychiatry* 67, 113–123. doi: 10.1001/archgenpsychiatry.2009.186

Groman, S. M., James, A. S., and Jentsch, J. D. (2009). Poor response inhibition: at the nexus between substance abuse and attention deficit/hyperactivity disorder. *Neurosci. Biobehav. Rev.* 33, 690–698. doi: 10.1016/j.neubiorev.2008.08.008

Holbrook, T. L., Hoyt, D. B., Stein, M. B., and Sieber, W. J. (2002). Gender differences in long-term posttraumatic stress disorder outcomes after major trauma: women are at higher risk of adverse outcomes than men. *J. Trauma* 53, 882–888. doi: 10.1097/00005373-200211000-00012

Holz, N. E., Boecker, R., Hohm, E., Zohsel, K., Buchmann, A. F., Blomeyer, D., et al. (2015). The long-term impact of early life poverty on orbitofrontal cortex volume in adulthood: results from a prospective study over 25 years. *Neuropsychopharmacology* 40, 996–1004. doi: 10.1038/npp.2014.277

Hyman, S. M., Garcia, M., and Sinha, R. (2006). Gender specific associations between types of childhood maltreatment and the onset, escalation and severity of substance use in cocaine dependent adults. *Am. J. Drug Alcohol Abuse* 32, 655–664. doi: 10.1080/10623320600919193

Hyman, S. M., Paliwal, P., Chaplin, T. M., Mazure, C. M., Rounsaville, B. J., and Sinha, R. (2008). Severity of childhood trauma is predictive of cocaine relapse outcomes in women but not men. *Drug Alcohol Depend.* 92, 208–216. doi: 10.1016/j.drugalcdep.2007.08.006

Izquierdo, A., Brigman, J. L., Radke, A. K., Rudebeck, P. H., and Holmes, A. (2017). The neural basis of reversal learning: an updated perspective. *Neuroscience* 345, 12–26. doi: 10.1016/j.neuroscience.2016.03.021

Jentsch, J. D., Olausson, P., De La Garza, R. II., and Taylor, J. R. (2002). Impairments of reversal learning and response perseveration after repeated, intermittent cocaine administrations to monkeys. *Neuropsychopharmacology* 26, 183–190. doi: 10.1016/s0893-133x(01)00355-4

Jentsch, J. D., and Taylor, J. R. (1999). Impulsivity resulting from frontostriatal dysfunction in drug abuse: implications for the control of behavior by reward-related stimuli. *Psychopharmacology* 146, 373–390. doi: 10.1007/pl00005483

Jett, J. D., and Morilak, D. A. (2013). Too much of a good thing: blocking noradrenergic facilitation in medial prefrontal cortex prevents the detrimental effects of chronic stress on cognition. *Neuropsychopharmacology* 38, 585–595. doi: 10.1038/npp.2012.216

Kessler, R. C., Davis, C. G., and Kendler, K. S. (1997). Childhood adversity and adult psychiatric disorder in the US National Comorbidity Survey. *Psychol. Med.* 27, 1101–1119. doi: 10.1017/s0033291797005588

Kessler, R. C., McGonagle, K. A., Swartz, M., Blazer, D. G., and Nelson, C. B. (1993). Sex and depression in the National Comorbidity Survey. I: lifetime prevalence, chronicity and recurrence. *J. Affect. Disord.* 29, 85–96. doi: 10.1016/0165-0327(93)90026-g

Korenbrot, C. C., Huhtaniemi, I. T., and Weiner, R. I. (1977). Preputial separation as an external sign of pubertal development in the male rat. *Biol. Reprod.* 17, 298–303. doi: 10.1095/biolreprod17.2.298

Kruzich, P. J., and Grandy, D. K. (2004). Dopamine D_2 receptors mediate two-odor discrimination and reversal learning in C57BL/6 mice. *BMC Neurosci.* 5:12. doi: 10.1186/1471-2202-5-12

Kuehner, C. (2003). Gender differences in unipolar depression: an update of epidemiological findings and possible explanations. *Acta Psychiatr. Scand.* 108, 163–174. doi: 10.1034/j.1600-0447.2003.00204.x

Laughlin, R. E., Grant, T. L., Williams, R. W., and Jentsch, J. D. (2011). Genetic dissection of behavioral flexibility: reversal learning in mice. *Biol. Psychiatry* 69, 1109–1116. doi: 10.1016/j.biopsych.2011.01.014

Lee, B., Groman, S., London, E. D., and Jentsch, J. D. (2007). Dopamine D2/D3 receptors play a specific role in the reversal of a learned visual discrimination in monkeys. *Neuropsychopharmacology* 32, 2125–2134. doi: 10.1038/sj.npp.1301337

Leussis, M. P., and Andersen, S. L. (2008). Is adolescence a sensitive period for depression? Behavioral and neuroanatomical findings from a social stress model. *Synapse* 62, 22–30. doi: 10.1002/syn.20462

Leussis, M. P., Lawson, K., Stone, K., and Andersen, S. L. (2008). The enduring effects of an adolescent social stressor on synaptic density, part II: poststress reversal of synaptic loss in the cortex by adinazolam and MK-801. *Synapse* 62, 185–192. doi: 10.1002/syn.20483

Lewis, E. E., Dozier, M., Ackerman, J., and Sepulveda-Kozakowski, S. (2007). The effect of placement instability on adopted children's inhibitory control abilities and oppositional behavior. *Dev. Psychol.* 43, 1415–1427. doi: 10.1037/0012-1649.43.6.1415

Li, X., and Wolf, M. E. (2015). Multiple faces of BDNF in cocaine addiction. *Behav. Brain Res.* 0, 240–254. doi: 10.1016/j.bbr.2014.11.018

London, E. D., Ernst, M., Grant, S., Bonson, K., and Weinstein, A. (2000). Orbitofrontal cortex and human drug abuse: functional imaging. *Cereb. Cortex* 10, 334–342. doi: 10.1093/cercor/10.3.334

Lucantonio, F., Stalnaker, T. A., Shaham, Y., Niv, Y., and Schoenbaum, G. (2012). The impact of orbitofrontal dysfunction on cocaine addiction. *Nat. Neurosci.* 15, 358–366. doi: 10.1038/nn.3014

Lyons, D. M., Lopez, J. M., Yang, C., and Schatzberg, A. F. (2000). Stress-level cortisol treatment impairs inhibitory control of behavior in monkeys. *J. Neurosci.* 20, 7816–7821.

MacMillan, H. L., Fleming, J. E., Streiner, D. L., Lin, E., Boyle, M. H., Jamieson, E., et al. (2001). Childhood abuse and lifetime psychopathology in a community sample. *Am. J. Psychiatry* 158, 1878–1883. doi: 10.1176/appi.ajp.158.11.1878

Marshall, D. F., Passarotti, A. M., Ryan, K. A., Kamali, M., Saunders, E. F., Pester, B., et al. (2016). Deficient inhibitory control as an outcome of childhood trauma. *Psychiatry Res.* 235, 7–12. doi: 10.1016/j.psychres.2015.12.013

Matochik, J. A., London, E. D., Eldreth, D. A., Cadet, J. L., and Bolla, K. I. (2003). Frontal cortical tissue composition in abstinent cocaine abusers: a magnetic resonance imaging study. *Neuroimage* 19, 1095–1102. doi: 10.1016/s1053-8119(03)00244-1

McCormick, C. M., and Mathews, I. Z. (2007). HPA function in adolescence: role of sex hormones in its regulation and the enduring consequences of exposure to stressors. *Pharmacol. Biochem. Behav.* 86, 220–233. doi: 10.1016/j.pbb.2006.07.012

Moffitt, T. E., Arseneault, L., Belsky, D., Dickson, N., Hancox, R. J., Harrington, H., et al. (2011). A gradient of childhood self-control predicts health, wealth, and public safety. *Proc. Natl. Acad. Sci. U S A* 108, 2693–2698. doi: 10.1073/pnas.1010076108

Monteggia, L. M., Luikart, B., Barrot, M., Theobold, D., Malkovska, I., Nef, S., et al. (2007). Brain-derived neurotrophic factor conditional knockouts show gender differences in depression-related behaviors. *Biol. Psychiatry* 61, 187–197. doi: 10.1016/j.biopsych.2006.03.021

Moreno-López, L., Perales, J. C., van Son, D., Albein-Urios, N., Soriano-Mas, C., Martinez-Gonzalez, J. M., et al. (2015). Cocaine use severity and cerebellar

gray matter are associated with reversal learning deficits in cocaine-dependent individuals. *Addict. Biol.* 20, 546–566. doi: 10.1111/adb.12143

Mueller, S. C., Maheu, F. S., Dozier, M., Peloso, E., Mandell, D., Leibenluft, E., et al. (2010). Early-life stress is associated with impairment in cognitive control in adolescence: an fMRI study. *Neuropsychologia* 48, 3037–3044. doi: 10.1016/j.neuropsychologia.2010.06.013

Netherton, C., Goodyear, I., Tamplin, A., and Herbert, J. (2004). Salivary cortisol and dehydroepiandrosterone in relation to puberty and gender. *Psychoneuroendocrinology* 29, 125–140. doi: 10.1016/S0306-4530(02)00150-6

Nigg, J. T., Wong, M. M., Martel, M. M., Jester, J. M., Puttler, L. I., Glass, J. M., et al. (2006). Poor response inhibition as a predictor of problem drinking and illicit drug use in adolescents at risk for alcoholism and other substance use disorders. *J. Am. Acad. Child Adolesc. Psychiatry* 45, 468–475. doi: 10.1097/01.chi.0000199028.76452.a9

Numakawa, T., Adachi, N., Richards, M., Chiba, S., and Kunugi, H. (2013). Brain-derived neurotrophic factor and glucocorticoids: reciprocal influence on the central nervous system. *Neuroscience* 239, 157–172. doi: 10.1016/j.neuroscience.2012.09.073

Petanjek, Z., Judaš, M., Šimic, G., Rasin, M. R., Uylings, H. B., Rakic, P., et al. (2011). Extraordinary neoteny of synaptic spines in the human prefrontal cortex. *Proc. Natl. Acad. Sci. U S A* 108, 13281–13286. doi: 10.1073/pnas.1105108108

Pitts, E. G., Taylor, J. R., and Gourley, S. L. (2016). Prefrontal cortical BDNF: a regulatory key in cocaine- and food-reinforced behaviors. *Neurobiol. Dis.* 91, 326–335. doi: 10.1016/j.nbd.2016.02.021

Regner, M. F., Dalwani, M., Yamamoto, D., Perry, R. I., Sakai, J. T., Honce, J. M., et al. (2015). Sex differences in gray matter changes and brain-behavior relationships in patients with stimulant dependence. *Radiology* 277, 801–812. doi: 10.1148/radiol.2015142541

Ridley, R. M., Haystead, T. A., and Baker, H. F. (1981). An analysis of visual object reversal learning in the marmoset after amphetamine and haloperidol. *Pharmacol. Biochem. Behav.* 14, 345–351. doi: 10.1016/0091-3057(81)90401-9

Sacks, J. Y., McKendrick, K., and Banks, S. (2008). The impact of early trauma and abuse on residential substance abuse treatment outcomes for women. *J. Subst. Abuse Treat.* 34, 90–100. doi: 10.1016/j.jsat.2007.01.010

Sakata, K., Martinowich, K., Woo, N. H., Schloesser, R. J., Jimenez, D. V., Ji, Y., et al. (2013). Role of activity-dependent BDNF expression in hippocampal-prefrontal cortical regulation of behavioral perseverance. *Proc. Natl. Acad. Sci. U S A* 110, 15103–15108. doi: 10.1073/pnas.1222872110

Schoenbaum, G., Saddoris, M. P., Ramus, S. J., Shaham, Y., and Setlow, B. (2004). Cocaine-experienced rats exhibit learning deficits in a task sensitive to orbitofrontal cortex lesions. *Eur. J. Neurosci.* 19, 1997–2002. doi: 10.1111/j.1460-9568.2004.03274.x

Schwandt, M. L., Heilig, M., Hommer, D. W., George, D. T., and Ramchandani, V. A. (2013). Childhood trauma exposure and alcohol dependence severity in adulthood: mediation by emotional abuse severity and neuroticism. *Alcohol. Clin. Exp. Res.* 37, 984–992. doi: 10.1111/acer.12053

Selemon, L. D. (2013). A role for synaptic plasticity in the adolescent development of executive function. *Transl. Psychiatry* 3:e238. doi: 10.1038/tp.2013.7

Shapiro, L. P., Omar, M. H., Koleske, A. J., and Gourley, S. L. (2017a). Corticosteroid-induced dendrite loss and behavioral deficiencies can be blocked by activation of Abl2/Arg kinase. *Mol. Cell. Neurosci.* 85, 226–234. doi: 10.1016/j.mcn.2017.10.007

Shapiro, L. P., Parsons, R. G., Koleske, A. J., and Gourley, S. L. (2017b). Differential expression of cytoskeletal regulatory factors in the adolescent prefrontal cortex: implications for cortical development. *J. Neurosci. Res.* 95, 1123–1143. doi: 10.1002/jnr.23960

Simpson, T., and Miller, W. (2002). Concomitance between childhood sexual and physical abuse and substance use problems: a review. *Clin. Psychol. Rev.* 22, 27–77. doi: 10.1016/s0272-7358(00)00088-x

Skowron, E. A., Cipriano-Essel, E., Gatzke-Kopp, L. M., Teti, D. M., and Ammerman, R. T. (2014). Early adversity, RSA, and inhibitory control: evidence of children's neurobiological sensitivity to social context. *Dev. Psychobiol.* 56, 964–978. doi: 10.1002/dev.21175

Snyder, K. P., Barry, M., and Valentino, R. J. (2015). Cognitive impact of social stress and coping strategy throughout development. *Psychopharmacology* 232, 185–195. doi: 10.1007/s00213-014-3654-7

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* 24, 417–463. doi: 10.1016/s0149-7634(00)00014-2

Stroud, L. R., Papandonatos, G. D., Williamson, D. E., and Dahl, R. E. (2011). Sex differences in cortisol response to corticotropin releasing hormone challenge over puberty: pittsburgh Pediatric Neurobehavioral Studies. *Psychoneuroendocrinology* 36, 1226–1238. doi: 10.1016/j.psyneuen.2011.02.017

Suri, D., and Vaidya, V. A. (2013). Glucocorticoid regulation of brain-derived neurotrophic factor: relevance to hippocampal structure and functional plasticity. *Neuroscience* 239, 196–213. doi: 10.1016/j.neuroscience.2012.08.065

Tanabe, J., Tregellas, J. R., Dalwani, M., Thompson, L., Owens, E., Crowley, T., et al. (2009). Medial orbitofrontal cortex gray matter is reduced in abstinent substance-dependent individuals. *Biol. Psychiatry* 65, 160–164. doi: 10.1016/j.biopsych.2008.07.030

Tarter, R. E., Kirisci, L., Habeych, M., Reynolds, M., and Vanyukov, M. (2004). Neurobehavior disinhibition in childhood predisposes boys to substance use disorder by young adulthood: direct and mediated etiologic pathways. *Drug Alcohol Depend.* 73, 121–132. doi: 10.1016/j.drugalcdep.2003.07.004

Teicher, M. H., and Samson, J. A. (2016). Annual research review: enduring neurobiological effects of childhood abuse and neglect. *J. Child Psychol. Psychiatry* 57, 241–266. doi: 10.1111/jcpp.12507

Vigers, A. J., Amin, D. S., Talley-Farnham, T., Gorski, J. A., Xu, B., and Jones, K. R. (2012). Sustained expression of brain-derived neurotrophic factor is required for the maintenance of dendritic spines and normal behavior. *Neuroscience* 212, 1–18. doi: 10.1016/j.neuroscience.2012.03.031

Viola, T. W., Wearick-Silva, L. E., De Azeredo, L. A., Centeno-Silva, A., Murphy, C., Mashall, P., et al. (2016). Increased cocaine-induced conditioned place preference during periadolescence in maternally separated male BALB/c mice: the role of cortical BDNF, microRNA-212, and MeCP2. *Psychopharmacology* 233, 3279–3288. doi: 10.1007/s00213-016-4373-z

Warnault, V., Darcq, E., Morisot, N., Phamluong, K., Wilbrecht, L., Massa, S. M., et al. (2016). The BDNF valine 68 to methionine polymorphism increases compulsive alcohol drinking in mice that is reversed by tropomyosin receptor kinase B activation. *Biol. Psychiatry* 79, 463–473. doi: 10.1016/j.biopsych.2015.06.007

Watt, M. J., Weber, M. A., Davies, S. R., and Forster, G. L. (2017). Impact of juvenile chronic stress on adult cortico-accumbal function: implications for cognition and addiction. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 79, 136–154. doi: 10.1016/j.pnpbp.2017.06.015

Webster, M. J., Weickert, C. S., Herman, M. M., and Kleinman, J. E. (2002). BDNF mRNA expression during postnatal development, maturation and aging of the human prefrontal cortex. *Dev. Brain Res.* 139, 139–150. doi: 10.1016/s0165-3806(02)00540-0

Widom, C. S., and White, H. R. (1997). Problem behaviors in abused and neglected children grown-up: prevalence and co-occurrence of substance abuse, crime and violence. *Crim. Behav. Ment. Health* 7, 287–310. doi: 10.1002/cbm.191

Winhusen, T., and Lewis, D. (2013). Sex differences in disinhibition and its relationship to physical abuse in a sample of stimulant-dependent patients. *Drug Alcohol Depend.* 129, 158–162. doi: 10.1016/j.drugalcdep.2012.09.014

Wright, L. D., Hebert, K. E., and Perrot-Sinal, T. S. (2008). Periadolescent stress exposure exerts long-term effects on adult stress responding and expression of prefrontal dopamine receptors in male and female rats. *Psychoneuroendocrinology* 33, 130–142. doi: 10.1016/j.psyneuen.2007.10.009

Xu, B., Zang, K., Ruff, N. L., Zhang, Y. A., McConnell, S. K., Stryker, M. P., et al. (2000). Cortical degeneration in the absence of neurotrophin signaling: dendritic retraction and neuronal loss after removal of the receptor TrkB. *Neuron* 26, 233–245. doi: 10.1016/S0896-6273(00)81153-8

Xu, H., Zhang, Y., Zhang, F., Yuan, S. N., Shao, F., and Wang, W. (2016). Effects of duloxetine treatment on cognitive flexibility and BDNF expression in the mPFC of adult male mice exposed to social stress during adolescence. *Front. Mol. Neurosci.* 9:95. doi: 10.3389/fnmol.2016.00095

Yoshii, A., and Constantine-Paton, M. (2010). Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. *Dev. Neurobiol.* 70, 304–322. doi: 10.1002/dneu.20765

Zhang, Y., Shao, F., Wang, Q., Xie, X., and Wang, W. (2017). Neuroplastic correlates in the mPFC underlying the impairment of stress-coping ability and cognitive flexibility in adult rats exposed to chronic mild stress during adolescence. *Neural Plast.* 2017:9382797. doi: 10.1155/2017/9382797

Zhang, J. C., Wu, J., Fujita, Y., Yao, W., Ren, Q., Yang, C., et al. (2014). Antidepressant effects of trkB ligands on depression-like behavior and dendritic changes in mice after inflammation. *Int. J. Neuropsychopharmacol.* 18:pyu077. doi: 10.1093/ijnp/pyu077

Zhang, F., Yuan, S., Shao, F., and Wang, W. (2016). Adolescent social defeat induced alterations in social behavior and cognitive flexibility in adult mice: effects of developmental stage and social condition. *Front. Behav. Neurosci.* 10:149. doi: 10.3389/fnbeh.2016.00149

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 Pharmacology

ORIGINAL RESEARCH
published: 07 July 2017
doi: 10.3389/fphar.2017.00450

Age and Sex Interact to Mediate the Effects of Intermittent, High-Dose Ethanol Exposure on Behavioral Flexibility

Jacqueline M. Barker, Kathleen G. Bryant, Jennifer I. Osborne and L. J. Chandler**

Department of Neuroscience, Medical University of South Carolina, Charleston, SC, United States

Human alcoholics have been shown to have impaired cognitive control over actions and increased reliance on habitual response strategies. While it is unclear in humans whether these differences predate ethanol exposure or result from chronic drinking, data from animal studies suggest that ethanol acts to promote the development of inflexible behaviors. Here, we investigated how intermittent exposure to high doses of ethanol impacts the ability to flexibly regulate behavior in a habit model. As adolescence, may represent a period of increased drug taking and developmental vulnerability that may impact adult behavior, we compared the effects of high-dose ethanol exposure during adolescence to exposure during adulthood in male and female rats. Our findings indicated that the effects of intermittent, high-dose ethanol exposure on habitual behavior is mediated by age and sex such that ethanol exposure during adolescence promoted the use of habitual response strategies in adult females, but not males, and that the opposite pattern emerged following intermittent, high-dose ethanol exposure in adult rats.

Keywords: adolescence, habit, behavioral flexibility, ethanol exposure, rat models, sex differences

OPEN ACCESS

Edited by:
Tod Edward Kippin, University of California, Santa Barbara, United States

Reviewed by:
Karen K. Szumlinski, University of California, Santa Barbara, United States
Scott Edwards, LSU Health Sciences Center New Orleans, United States

****Correspondence:***
Jacqueline M. Barker
barker.jacqui@gmail.com
L. J. Chandler
chandj@musc.edu

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *26 May 2017*
Accepted: *22 June 2017*
Published: *07 July 2017*

Citation:
Barker JM, Bryant KG, Osborne JI and Chandler LJ (2017) Age and Sex Interact to Mediate the Effects of Intermittent, High-Dose Ethanol Exposure on Behavioral Flexibility. Front. Pharmacol. 8:450. doi: 10.3389/fphar.2017.00450

INTRODUCTION

The development of alcohol use disorders is associated with a reduction in the ability to flexibly regulate behaviors. The ability to balance efficiency and flexibility in order to alter and adapt behavior is a critical function in changing environments. Shifting between cognitive regulation of behavior and inflexible stimulus-driven behavior can be modeled by the transition between goal-directed and habitual response strategies. When a new behavior is learned, it is typically performed in a goal-directed fashion. That is, behavior is performed in relationship to its outcome. After time and repeated performance, a transition to habitual response strategies may occur in which behavior is no longer mediated by its outcome.

Inflexible behavior is implicated in a number of neuropsychiatric illnesses characterized by deficits in cognitive control, such as addiction and obsessive compulsive disorder. Indeed, data from human neuroimaging studies revealed impairments in behavioral flexibility and

an overreliance on habitual response strategies in abstinent alcoholics (Sjoerds et al., 2013). Though deficits in cognitive control are a hallmark of addictive disorders, it is unclear whether this phenotype is the result of chronic ethanol exposure in humans, or rather is a risk factor predating the development of alcohol use disorders. Evidence from animal models has suggested that innate individual differences in cue reactivity and incentive motivation can predict subsequent differences in drug self-administration (Flagel et al., 2009; Saunders and Robinson, 2013) as well as the development of inflexible behaviors (Barker et al., 2012, 2014). In addition, sex differences in the propensity to develop habits have been reported such that while female rodents develop sucrose-seeking habits more quickly than males (Quinn et al., 2007), male rodents show more rapid development of habitual ethanol seeking (Barker et al., 2010). It is possible that sex differences in habit formation relate to differences in motivation to consume ethanol or differences in ethanol reinforcement. While sex differences in ethanol consumption have been observed, females generally self-administer ethanol at higher rates than males (e.g., Barker et al., 2010). It is alternatively possible that sub-chronic ethanol exposure differentially impacts loss of cognitive control over actions in males versus females. Indeed, while a growing body of literature indicates that ethanol exposure itself may act to promote the development of inflexible behaviors, including habits (Corbit et al., 2012), the bulk of this research has been performed in males.

In humans, the onset of drinking behavior during adolescence is highly predictive of subsequent development of alcohol use disorders, and as such, adolescence may mark a period of unique vulnerability to the effects of alcohol on cognitive control. Adolescence is characterized by increased risk-taking (Laviola et al., 2003) and goal-seeking behavior (Doremus-Fitzwater and Spear, 2016), and adolescent males are resistant to the development of habitual ethanol seeking (Serlin and Torregrossa, 2014). Binge-like ethanol exposure during adolescence has been shown to have long-lasting effects on behavior, including impairments in fear learning and extinction (Broadwater and Spear, 2013) and in set-shifting (Gass et al., 2014). These findings suggest that the impact of ethanol exposure on the development of habitual reward seeking may not only be sex-specific, but may also depend upon the developmental age when exposure occurs. Here, we investigated how intermittent, high-dose ethanol exposure during either adolescence or adulthood impacted the development of habitual reward seeking in male and female rats.

MATERIALS AND METHODS

Animals

Male and female Long Evans rats (Harlan Labs) were delivered to the Medical University of South Carolina animal facility at either postnatal day (PND) 22 (adolescent ethanol exposure) or 60 (adult ethanol exposure). Rats were housed on a reverse light cycle with 12 h light:12 h dark. All behavioral procedures occurred during the dark cycle. Adolescent rats were housed with litter-mates of the same sex, and adult rats were pair housed unless separation was required because of injury or fighting. All procedures were conducted with approval of the Medical University of South Carolina Institutional Animal Care and Use Committee.

Intermittent Ethanol Exposure Procedure

Adolescent and adult rats assigned to undergo ethanol exposure were subjected to five cycles of ethanol exposure via vapor inhalation (**Figure 1A**). Control rats were moved to the chamber exposure room each day but did not undergo vapor inhalation of ethanol. Each cycle of ethanol exposure consisted of two consecutive episodes of exposure, with each exposure lasting 14 h in the vapor chambers (**Figure 1B**). Vapor chamber exposure began 3 h prior to the onset of the light cycle, and rats are removed from vapor chambers 1 h prior to the onset of the dark cycle at which time intoxication ratings and tail-vein blood samples are obtained. Adolescent ethanol exposure spanned the period from early to mid- adolescence (PND 28–44) and involved exposure to ethanol vapor on PND 28 and 29 (Cycle I), 31 and 32 (Cycle II), 35 and 36 (Cycle III), 38 and 39 (Cycle IV), and 42 and 43 (Cycle V). Following ethanol exposure, rats were returned to the colony where they were maintained on *ad libitum* food and water until at least PND 80. To investigate the effects of intermittent, high-dose ethanol exposure during adulthood, exposure began at PND 70 and rats were exposed to ethanol vapor on PND 70 and 71 (Cycle I), 73 and 74 (Cycle II), 77 and 78 (Cycle III), 80 and 81 (Cycle IV), and 84 and 85 (Cycle V). Rats that underwent intermittent, high-dose ethanol exposure in adulthood were maintained on *ad libitum* food and water for 36 days prior to any behavioral testing in order to match the time between adolescent ethanol exposure and assessment of the development of habitual reward seeking in adulthood. Tail vein blood was collected on a subset of ethanol vapor exposure sessions for both adolescent and adult-exposed animals in order to measure blood ethanol concentration (BEC), and the level of intoxication was determined at the end of each vapor chamber exposure using a previously described 5-point behavioral intoxication rating scale (Nixon and Crews, 2002; Gass et al., 2014) as follows: 1 = not intoxicated (no apparent signs of intoxication); 2 = low intoxication (slight motor impairment); 3 = moderate intoxication (obvious motor impairment, but able to walk); 4 = high intoxication (loss of righting reflex for >30 s, dragging of abdomen when walking, severe motor impairment, maintains eye blink reflex); 5 = extreme intoxication (loss of righting reflex, loss of eye blink reflex, may be unconscious, or difficult to arouse). We targeted intoxication ratings between 2 and 3 (low to moderate intoxication levels; **Figures 2C,D**). Targeted BECs between 250 and 350 mg/dL and low to moderate intoxication ratings were chosen to model ethanol consumption patterns in young adults drinkers. Recent reports suggest that in natural environments (i.e., at a bar) adolescent and young adults achieve estimated blood alcohol concentrations within this range (Treloar et al., 2017). Intoxication ratings have been shown previously to correlate with BEC, and indeed our findings revealed that intoxication ratings in both adolescent and adult ethanol exposed animals significantly correlated with BECs (**Figures 2E,F**). Since obtaining tail vein blood from

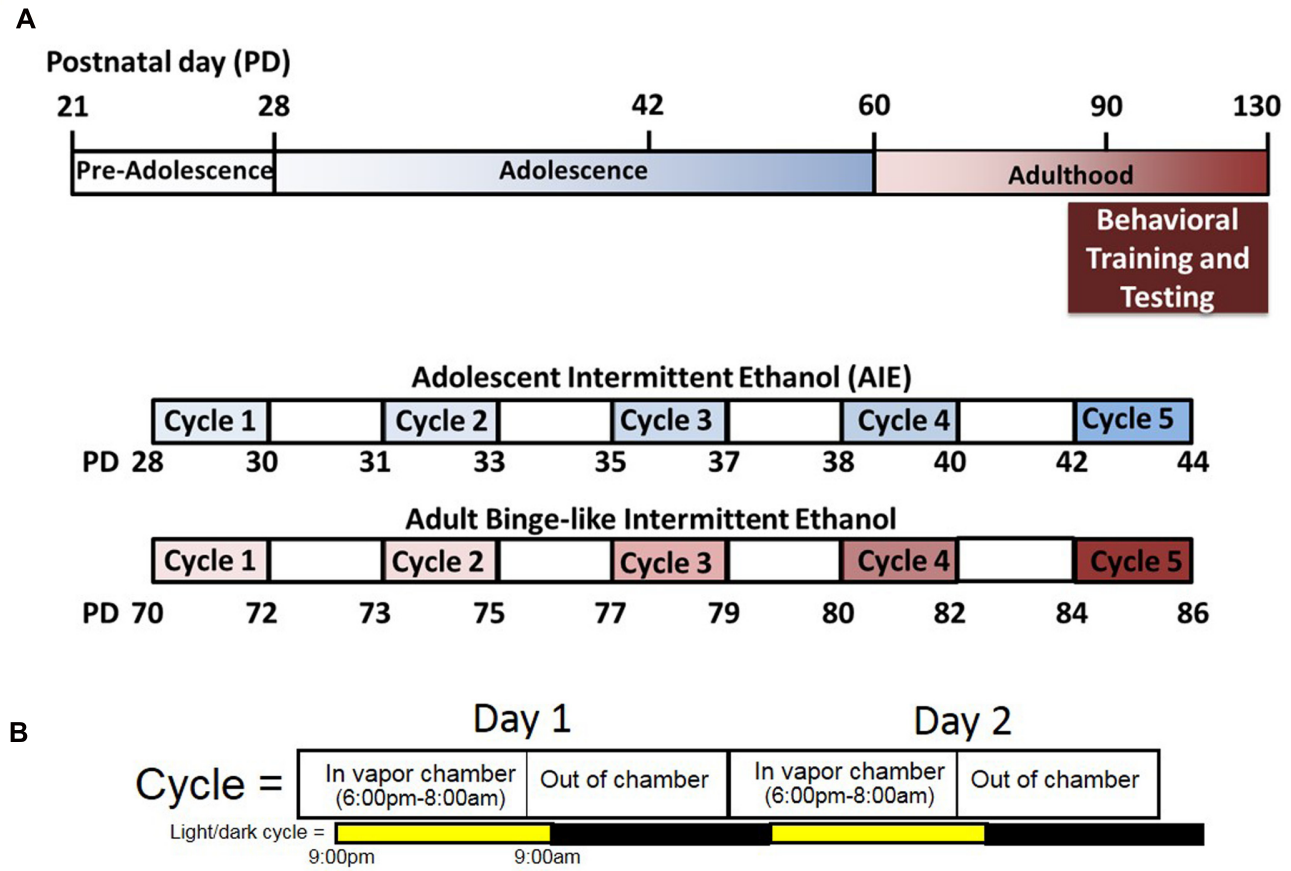


FIGURE 1 | Experimental design and time-line. **(A)** Male and female rats were exposed to intermittent ethanol exposure during either adolescence (blue) or adulthood (red). All rats underwent five cycles of exposure to ethanol. For adolescent ethanol exposed rats, exposure occurred between PND 28–44 and testing occurred after PND 80. For adult exposed rats, exposure occurred between PND 70–86 and testing occurred after PND 120. **(B)** Each cycle of ethanol exposure consisted of two 14-h exposures that began 3 h prior to the start of the light cycle and ended 1 h prior to the start of the dark cycle.

unintoxicated rats is a much more stressful procedure than it is with intoxicated rats, blood was not obtained from control rats so as not to introduce this stress as an experimental confound.

Behavioral Training and Testing

After at least 36 days from the last cycle of ethanol exposure, rats were food restricted to approximately 90% of their free feeding weights and then trained to self-administer 10% sucrose via two levers in a standard rat operant chamber from Med-Associates (St. Albans, VT, United States). Operant chambers were housed within a sound-attenuating box. Each chamber was equipped with a 28 V house light and had two retractable levers on the left and right panels of the right wall. A magazine was located at the bottom of the middle panel on the right side of the wall. Sucrose was delivered to a well in the magazine via a syringe pump housed within the sound attenuating chamber but outside of the operant box. A fan provided background noise and ventilation. No additional tones or cues were presented.

Each day, rats had a 30-min training session during which each lever was presented separately. Each lever was only available for 15 min and the order of lever availability was alternated daily. A press on either lever was initially reinforced on a fixed ratio 1 (FR1) schedule in which each lever press resulted in a single delivery of sucrose. After stable responding ($\geq$ to 3 consecutive days) of at least 30 presses per lever, reinforcement schedules were transitioned to either an action-promoting random ratio (RR) 5 or a habit-promoting random interval (RI) 30 schedule (Adams and Dickinson, 1981; Gremel and Costa, 2013). The use of these schedules enabled within-subjects assessment of response strategy under conditions where either goal-directed actions (RR schedule) or habitual response strategies were expected (RI schedule). On the RR5 schedule, the number of lever presses controlled reinforcer delivery. On average, the fifth response was reinforced, but the program software randomly generated the actual response requirement. On the RI30 schedule, the first lever pressed after a randomly determined interval (averaging 30 s) had elapsed was reinforced. Unlike the RR schedule, reinforcer delivery on the RI schedule was not related to the number of lever presses. The response schedule assigned to each lever was consistent across training for each animal (i.e., left lever was always the

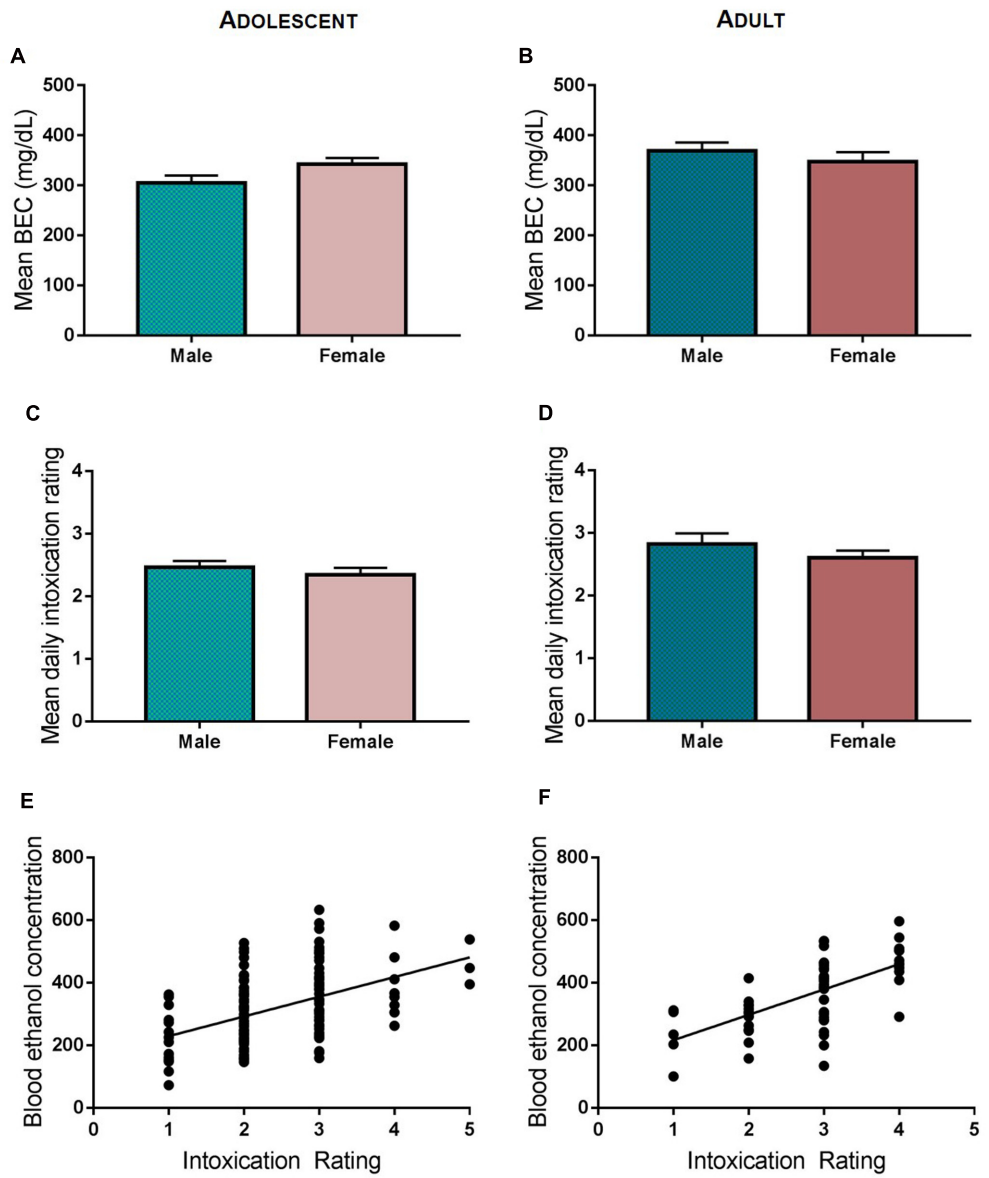


FIGURE 2 | Ethanol vapor exposure. Mean intoxication ratings were comparable for adolescent **(A)** and adult **(B)** ethanol exposed rats and did not differ between male and female rats. Male and female rats had similar blood ethanol levels during adolescent **(C)** or adult **(D)** ethanol vapor exposure. BECs and intoxication ratings were significantly correlated in both adolescent exposed **(E)** and adult exposed rats **(F)**.

'interval' lever and right lever was always the 'ratio' lever). After two sessions on the RR5/RI30 schedules, these were transitioned to RR10 and RI60 schedules for an additional eight sessions.

Following eight sessions on the RR10/RI60 schedule, response strategy was assessed using a specific satiety outcome devaluation procedure. Because habits are by definition insensitive to changes in outcome value, devaluation procedures enable the determination of whether rats are relying on habitual response strategies in which responding is unaffected by devaluation, or are using goal-directed strategies in which reward seeking behavior is reduced following devaluation. Rats underwent both a 'non-devalued' and 'devalued' testing procedure for the RR and RI levers. During the 'devalued' condition, rats received access to a 10% sucrose bottle for 1 h prior to a 10 min test session in a novel cage. During these test sessions, conditions were identical to training except that they were performed in extinction in which lever presses did not result in reinforcer delivery. Following this test session, rats were again provided 1-h access to a 10% sucrose bottle to confirm devaluation. In the 'non-devalued' condition, rats received 1-h access to home cage chow prior to testing. Between all devaluation tests, rats had 1 additional training session on the RR10/RI60 schedule and the order of testing was counterbalanced.

Statistical Analyses

Analysis was performed in SPSS using repeated measures ANOVA and *t*-tests as appropriate. *Post hoc t*-test comparisons were Bonferroni corrected.

RESULTS

Intoxication Ratings

Comparison of daily intoxication ratings with BEC values across groups revealed no difference between male and females, or between adult and adolescent rats. Data were collapsed across exposure cycles for analysis. Neither mean BECs nor intoxication ratings significantly differed between males and females in adolescent rats ($t = 0.1828$, $p > 0.5$; $t = 0.755$, $p > 0.4$; **Figures 2A,C**) or in adult rats ($t = 0.876$, $p > 0.3$; $t = 1.084$, $p > 0.2$, respectively; **Figures 2B,D**). Intoxication ratings and BECs were positively correlated on days that BEC's were taken, indicating that intoxication ratings reliably reflect BEC (adolescent ethanol exposure, Pearson's correlation, $r^2 = 0.208$, $p < 0.001$; adult ethanol exposure, Pearson's correlation, $r^2 = 0.407$; $p < 0.001$; **Figures 2E,F**).

Acquisition of Sucrose Seeking

Adolescent Ethanol Exposure

A rmANOVA on total lever presses across acquisition indicated main effects of reinforcement schedule [$F(1,37) = 26.233$, $p < 0.001$] and day of training [$F(12,444) = 50.527$, $p < 0.001$]. In addition, significant interactions between schedule, day of training, and sex were observed [$F(12, 444) = 2.427$, $p < 0.01$], as well as schedule x day x ethanol exposure [$F(12,444) = 3.104$, $p < 0.001$]. Follow-up rmANOVA indicated that in male rats, reinforcement schedule, ethanol exposure, and day of training interact to moderate responding [$F(12,228) = 2.656$, $p < 0.01$]. *Post hoc* comparisons further indicated that in air control males, responding on the RR vs RI levers was significantly different on days 3, 10, 11, 12, and 13 of training (p-values < 0.05; **Figure 3A**). In contrast, in ethanol exposed rats, responding differed on RR and RI levers only on day 2 and 12 of training (p-values < 0.05). In females, ethanol exposure did not impact responding during acquisition [main effect $p > 0.2$, no significant interactions], but a schedule by day interaction was present [$F(12, 216) = 5.070$, $p < 0.001$]. Follow-up comparisons indicated differences in responses on the RI vs RR lever on days 11, 12, and 13 for female rats ($p < 0.05$; **Figure 3B**).

Adult Ethanol Exposure

A rmANOVA on total lever presses across acquisition indicated a main effect of schedule on responding [$F(1,26) = 4.723$, $p < 0.05$] and a main effect of day of training [$F(12,312) = 17.437$, $p < 0.001$], consistent with an increase in responding across training. In addition, a significant day x sex interaction was observed [$F(12, 312) = 2.843$, $p < 0.01$] as well as a reinforcement schedule x day of training interaction [$F(12,312) = 3.102$, $p < 0.001$]. No significant main effect of ethanol exposure was observed [$F(1,26) = 0.243$, $p > 0.6$], and no significant ethanol exposure interactions were observed with any other measures (p-values > 0.4). In male rats, no main effect of schedule [$F(1,12) = 0.505$, $p > 0.4$] or schedule × day interaction were observed [$F(12,180) = 0.605$, $p > 0.8$] indicating comparable levels of responding on the RR and RI schedules. However, a main effect of day was observed [$F(12,180) = 5.529$, $p < 0.001$] consistent with increased responding across training (**Figure 3C**). In female rats, in addition to a significant effect of day [$F(12,156) = 12.724$, $p < 0.001$], a significant schedule × day interaction was observed [$F(12,156) = 3.4$, $p < 0.001$], indicating that response rates were distinct on RI and RR schedules. While a main effect of schedule [$F(1,156) = 4.591$, $p = 0.05$] is consistent with a higher response rate on the RR schedule than the RI schedule, post-hoc comparisons did not indicate significant differences in responding on the RR versus RI schedule on individual days of training (**Figure 3D**).

Adolescent Ethanol Exposure Promoted Used of Habitual Response Strategies in Females

For adult rats exposed to alcohol during adolescence, a rmANOVA of response rates during the outcome devaluation test indicated a reinforcement schedule × devaluation × ethanol exposure interaction [$F(1,41) = 5.621$, $p < 0.05$] as well as a significant schedule × devaluation interaction [$F(1,41) = 9.791$, $p < 0.01$]. Main effects of reinforcement schedule [$F(1,41) = 11.947$, $p < 0.01$] and devaluation [$F(1,41) = 27.303$, $p < 0.001$] were also observed. Follow-up comparisons indicated that in males, no main effect of ethanol exposure [$F(1,21) = 0.035$, $p > 0.8$] or interactions with ethanol exposure were observed (p-values > 0.2). A significant schedule × devaluation was observed [$F(1,21) = 7.928$, $p < 0.01$], as well as main effects of schedule [$F(1,21) = 8.407$, $p < 0.01$] and devaluation [$F(1,21) = 15.417$, $p < 0.001$]. *Post hoc* analyses indicated no difference between response rates on the RI lever during valued versus devalued conditions ($p > 0.3$), consistent with habitual behavior. In contrast, response rates on the RR lever were lower during the devalued test than the valued test ($p < 0.01$; **Figure 4A**), consistent with goal-directed behavior. In female rats, a schedule × devaluation × ethanol exposure interaction was indicated by rmANOVA [$F(1,20) = 4.709$, $p < 0.05$], as well as a devaluation × ethanol exposure interaction [$F(1,20) = 2.37$, $p < 0.05$). In addition, a significant main effect of devaluation was observed [$F(1,20) = 12.013$, $p < 0.01$]. Subsequent comparisons indicated that adolescent air exposed female rats exhibited habitual responding on the RI lever and did not change response rates when the outcome was devalued ($p > 0.4$). In contrast, air exposed females exhibited goal-directed reward seeking on the RR lever and lower response rates on the RR lever ($p < 0.05$). Female rats that had been subjected to intermittent, high-dose ethanol exposure during adolescence, however, showed habitual behavior on both the RR and RI levers ($p > 0.8$ and $p > 0.1$, respectively), consistent with increased reliance on habitual

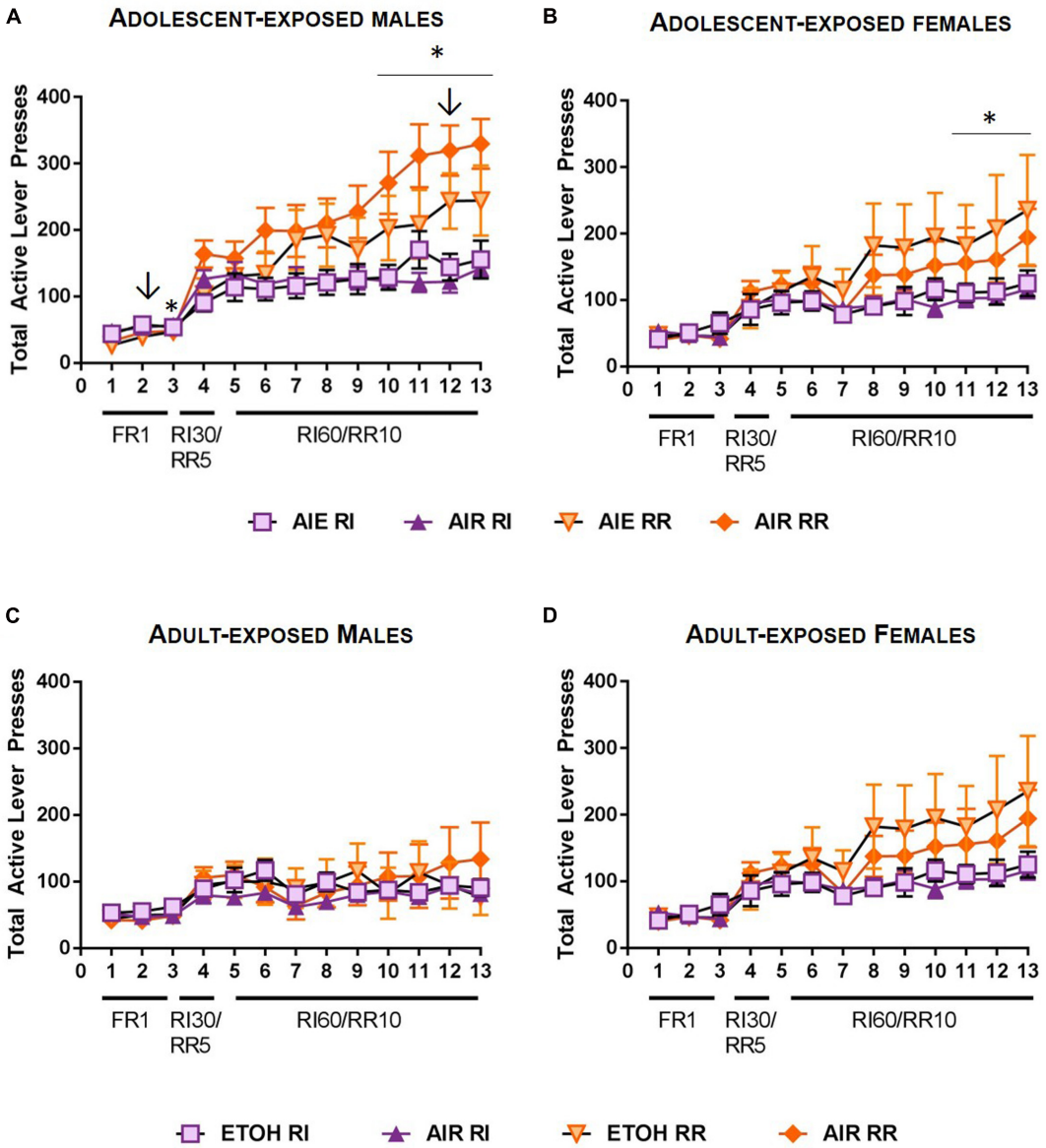


FIGURE 3 | Acquisition of sucrose seeking in rats exposed to ethanol. **(A)** In adult male rats that had been subject to adolescent intermittent ethanol (AIE), responding on the RR lever was significantly higher than responding on the RI lever on days 2 and 12 (arrows). In control (AIR) male rats, responding was significantly higher on the RR lever on days 3, and 10–13. **(B)** In adult female rats that had been subject to AIE, no differences in response rates were observed between AIE and AIR exposed rats. Responding was significantly higher on the RR lever than the RI lever on the final 3 days or RI60/RR10 responding for both groups. **(C)** In male rats subjected to intermittent ethanol (ETOH) exposure in adulthood, no differences in responding between ethanol-exposed and control rats were observed, nor did adult ethanol exposure alter response rates on the RR and RI levers. **(D)** In female rats exposed to ethanol in adulthood, no significant differences in responding on RR versus RI levers were observed. $\downarrow p < 0.05$; $^{*}p < 0.05$.

response strategies in female rats following adolescent ethanol exposure (**Figure 4B**).

Adult Ethanol Exposure Promoted the Use of Habitual Response Strategies in Males

For rats exposed to ethanol during adulthood, a rmANOVA of response rates during the outcome devaluation tests indicated a four-way interaction between schedule × sex × ethanol exposure × devaluation [$F(1,26) = 4.276$, $p < 0.05$], as well as a main effect of schedule [$F(1,26) = 13.378$, $p < 0.01$], a schedule × sex interaction [$F(1,26) = 9.216$, $p < 0.01$] and a devaluation × schedule interaction [$F(1,26) = 4.725$, $p < 0.05$]. No main effect of devaluation was present [$F(1,26) = 1.78$, $p = 0.194$] or main effect of ethanol exposure [$F(1,26) = 1.172$, $p = 0.289$]. There was however, a main effect of sex on response rates [$F(1,26) = 7.566$, $p < 0.05$].

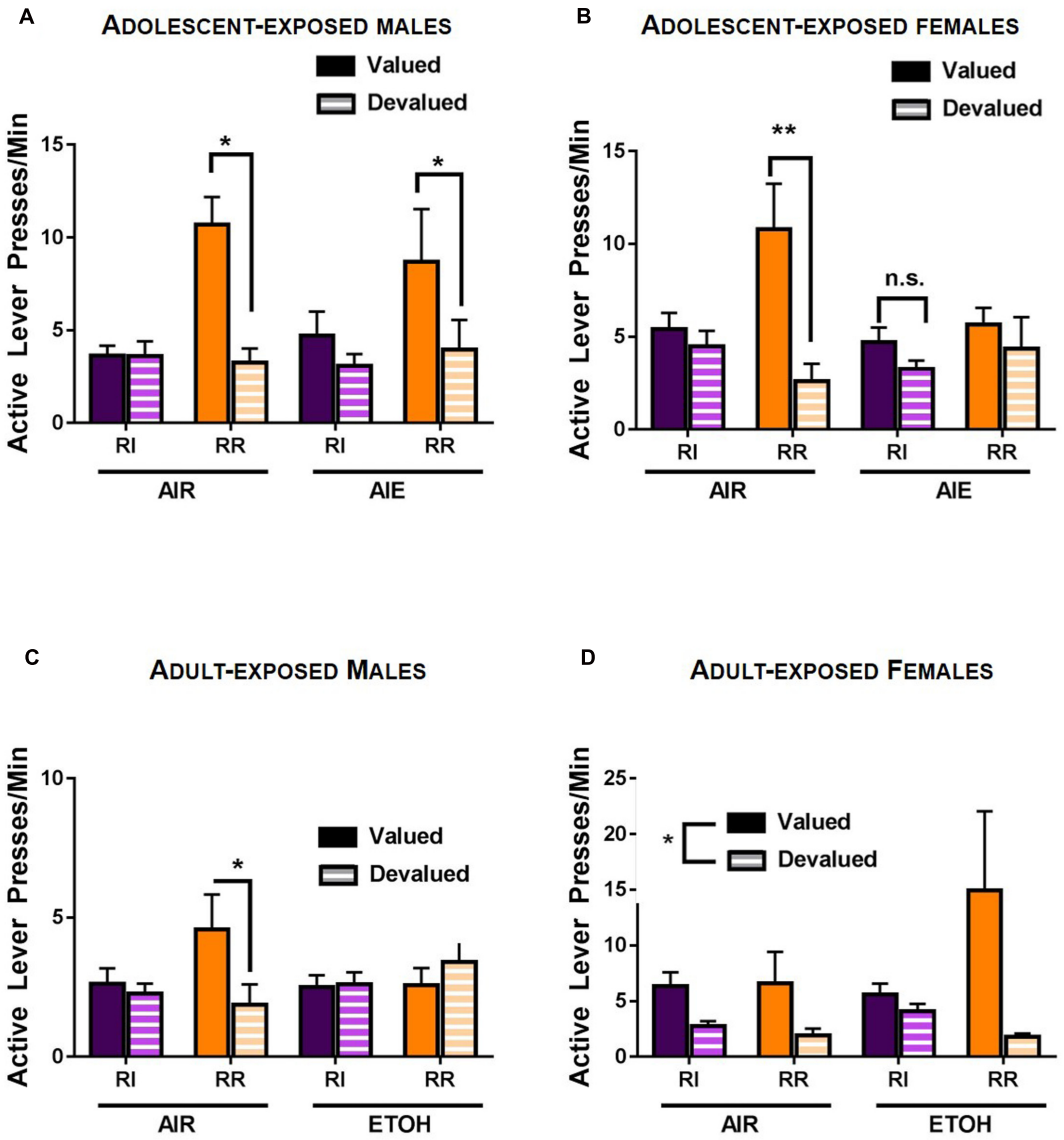


FIGURE 4 | Response strategy selection in an outcome devaluation test in adult rats following ethanol exposure. **(A)** In adult male rats, responding on the RI habit-promoting lever was habitual in both air control and AIE exposed groups, as evidenced by a lack of reduction in response rate when the outcome was devalued by specific satiety. In contrast, responding on the RR lever was goal-directed (sensitive to outcome devaluation) in male rats in both the control (AIR) and ethanol exposed groups. **(B)** AIR exposed female rats showed similar patterns of responding to males. In control female rats, responding on the RI schedule was habitual (i.e., was insensitive to outcome devaluation), while responding on the RR lever was goal-directed and sensitive to outcome devaluation. In contrast, AIE exposed females showed habitual response strategies on both the RI lever and the RR lever; responding on both the RR and RI levers was insensitive to outcome devaluation. **(C)** Air exposed male rats were insensitive to outcome devaluation on the RI lever, consistent with habitual behavior, but were sensitive to outcome devaluation on the RR lever, indicative of goal-directed actions. In contrast, adult ETOH exposed males were insensitive to outcome devaluation on both levers, indicative of habitual response strategies during responding in both action- and habit-promoting conditions. **(D)** In female rats, a main effect of devaluation was present, indicating lower response rates during devaluation in all conditions. This was not mediated by schedule or adult ethanol exposure. *$p < 0.05$.

To deconstruct significant interactions, rmANOVAs were performed separately on response rates for male and female rats. In male rats, this analysis revealed a significant interaction of schedule × devaluation × ethanol exposure [$F(1,14) = 7.349$, $p < 0.05$] and a schedule × ethanol interaction [$F(1,14) = 5.847$, $p < 0.05$]. *Post hoc* analysis indicated no significant effect of devaluation on responding on the RI schedule for ethanol exposed ($p > 0.8$) or air exposed males ($p > 0.4$). In contrast, air exposed control males showed lower rates of responding on the devalued lever in the RR schedule ($p < 0.5$) (**Figure 4C**), indicative of goal-directed behavior. In adult ethanol exposed males, however, response rates did not differ between the valued and devalued conditions on the RR schedule ($p > 0.2$), indicative of habitual responding on the 'action-promoting' lever.

In female rats, rmANOVA indicated a main effect of devaluation [$F(1,12) = 10.295$, $p < 0.01$), and a non-significant trend toward a devaluation x schedule interaction [$F(1,12) = 3.299$, $p = 0.09$) (**Figure 4D**), suggesting that ethanol exposure in adult females did not impact response strategy selection.

DISCUSSION

Our findings indicate that age and sex interact to determine the effects of intermittent, high-dose ethanol exposure on habitual behavior. In particular, we found that intermittent ethanol exposure during adolescence promoted the reliance on habitual response strategies in female rats, while response strategy was not impacted in males. In contrast, exposure to ethanol in adulthood using an identical exposure paradigm was associated with facilitation of habit formation in males but not females. These findings suggest that while intermittent high-dose ethanol exposure altered the ability to flexibly regulate behavior, the effect is dependent upon the age of ethanol exposure as well as sex.

In the adult exposure paradigm, air exposed control males exhibited habitual reward seeking when responding on an interval (habit promoting) schedule, but retained sensitivity to outcome devaluation on a ratio (action promoting) schedule. In males that were exposed to ethanol during adulthood, however, outcome devaluation had no impact on responding on either the RI or the RR schedule, indicating a greater propensity toward habitual response strategies. Female rats showed no effect of adult ethanol exposure on response strategy selection. Given previous reports that adult males develop ethanol seeking habits more rapidly than sucrose seeking habits (Dickinson et al., 2002), and that males form ethanol seeking habits more quickly than females (Barker et al., 2010), these data suggest that in adults, intermittent ethanol exposure may act to promote habitual response strategies in males but not females.

As with investigation of the consequences of adolescent ethanol exposure in females, there is a paucity of data describing sex differences in the impact of intermittent ethanol exposure in adult animals. Current findings suggest that males and females may be differentially sensitive to the both the aversive and rewarding effects of ethanol exposure. For example, while both adult males and females exhibit increases in anxiety following a bout of intoxication, this effect is exaggerated in adult males (Varlinskaya and Spear, 2004). This study also observed protracted elevation in BECs in adult males as compared to adult females, and that this effect was absent in adolescents (Varlinskaya and Spear, 2004). Together with additional literature demonstrating more rapid recovery from early withdrawal in females than in males (Devaud and Chadda, 2001), these findings suggest that adult males may be differentially sensitive to repeated bouts of intoxication and withdrawal than females. Consistent with these sex differences, male rats show persistent elevations in corticosterone following ethanol withdrawal, while corticosterone levels return to baseline in females (Alele and Devaud, 2006). Because chronic corticosterone itself can facilitate reliance on habitual response strategies in males (Gourley et al., 2012; Swanson et al., 2013), it is possible that repeated ethanol intoxication and withdrawal in adult males facilitates habitual behavior via its effects on the glucocorticoid system.

Interestingly, adolescent ethanol exposed male rats did not differ in response strategy selection compared to air exposed controls when tested in adulthood. Both ethanol-exposed and air control males showed habitual response strategies on the interval schedule and goal-directed actions when responding on the ratio schedule. Adolescent males have been shown to be resistant to habitual ethanol seeking relative to adult males (Serlin and Torregrossa, 2014), which is consistent with increased goal-oriented behavior in adolescent males (Doremus-Fitzwater and Spear, 2016). This suggests that ethanol exposure may not act on neurocircuitry to facilitate habit formation and expression in adolescent males in the same way it does in adults (Dickinson et al., 2002; Barker et al., 2010; Corbit et al., 2012). While a number of groups have identified protracted adolescent-like phenotypes following binge-like and intermittent ethanol exposure, our findings were not consistent with this in regard to response strategy selection. We observed that adult males were capable of habit formation following adolescent ethanol exposure – i.e., male rats showed habitual responding under habit-promoting conditions - and thus did not exhibit an adolescent-like resistance to habit formation.

In contrast to our observations in male rats, adolescent ethanol-exposed female rats demonstrated facilitated habitual reward seeking in adulthood. The precise timing of ethanol exposure during adolescence has been shown to have distinct effects on adult behavior (Spear, 2015). In particular, ethanol exposure in early adolescence that encompasses the onset of puberty may have greater effects on adult behavior than ethanol exposure restricted to later adolescence (Spear, 2015). It is possible that the sex differences we observed result from differences in pubertal onset – females mature more rapidly than males and undergo puberty earlier than male rats. Because our ethanol vapor procedure is restricted to early- to mid- adolescence (i.e., is ended at PND 44), this period encompasses the pubertal maturation period of female rats, but may not cover the entire pubertal period of male rats. The timing of drug alcohol exposure relative to puberty may mediate the effect of exposure on neuronal maturation. For example, the effects of amphetamine withdrawal on dopamine receptor expression in the medial prefrontal cortex (mPFC) was dependent upon the timing of drug exposure such that reductions in dopamine D1 receptors in mPFC occurred only in animals exposed to amphetamine prior to pubertal onset (Kang et al., 2016). Recent studies from our lab also suggest that intermittent ethanol exposure during this time period of development in males resulted in profound changes in dopamine neurotransmission in the prelimbic cortex, including reductions in both D1 receptor expression and expression of COMT, which together with the norepinephrine transporter, is the primary mediator of the extracellular levels of dopamine in the PFC (Trantham-Davidson et al., 2017). It is important to note that to date,investigation of dopamine system alterations following

intermittent ethanol exposure in this model is largely limited tomales, and the effects of adolescent ethanol exposure on mPFC dopamine neurotransmission in females is poorly understood. Prefrontal dopamine signaling regulates behavioral flexibility and cognitive control in a number of different tasks including impairments in the ability to detect change in reward value (Winter et al., 2009) and set shifting (Floresco et al., 2006). It has also previously been reported that mPFC dopamine signaling can acutely and bidirectionally regulate habitual reward seeking (Barker et al., 2013). Importantly, changes in dopamine signaling following intermittent ethanol exposure are long lasting. Though findings on the role of PFC dopamine signaling specifically are lacking, chronic changes in dopamine following chronic cocaine (Halbout et al., 2016) or amphetamine (Nelson and Killcross, 2006) are known to promote reliance on habit-like response strategies that are PFC-dependent (Nelson and Killcross, 2013).

In addition to changes in prefrontal dopamine signaling, intermittent ethanol exposure during adolescence has been shown to disrupt GABAergic neurotransmission in the PFC in both male and female adult rats (Centanni et al., 2017; Trantham-Davidson et al., 2017), including impairments in tonic $GABA_A$ currents (Centanni et al., 2017). Normal maturation of the mPFC GABA system has been shown to be required for appropriate goal-directed behaviors in adulthood (Butkovich et al., 2015), suggesting that alterations in $GABA_A$ signaling following adolescent alcohol exposure could contribute to the alterations in response strategy selection in adulthood observed in the present study. However, the absence of sex differences in tonic GABA current in the Centanni et al. (1999) study suggests that dysregulation of GABAergic transmission in the PFC following adolescent ethanol exposure is unlikely to explain the increased reliance on habitual response strategies in female rats. Interestingly, sex-specific effects of adult ethanol exposure on $GABA_A$ receptor subunit expression have been reported (Devaud et al., 1999). Female ethanol withdrawn rats show inceased sensitivity to neuroactive steroids thought to act at.

SUMMARY AND IMPLICATIONS

The ability to toggle between actions and habits is critical to maximize behavioral efficiency. Our findings indicate that intermittent, high-dose ethanol exposure impacts the ability to flexibly regulate behavior in a sex- and age- dependent manner. We observed that high-dose ethanol exposure during adolescence promoted reliance on habitual response strategy in adult female but not male rats. In contrast, this same intermittent, high-dose ethanol exposure paradigm in adults facilitated the use of habits in male but not female rats. Together, these findings point to a need for an increased understanding of sex differences in the impact of drugs and alcohol on the neurocircuits that mediate cognitive control and behavioral flexibility, and how restricted access to drugs and alcohol may have long-lasting effects on the plasticity and function of these circuits.

AUTHOR CONTRIBUTIONS

JB, JO, KB performed the experiments. JB and LC designed the experiments and wrote the manuscript.

ACKNOWLEDGMENTS

We thank the members of the Chandler lab for their assistance with ethanol vapor exposure procedures. This work was supported by NIH grants AA023141 and AA024499 (JB) and AA010761 (LC).

REFERENCES

Adams, C., and Dickinson, A. (1981). "Actions and habits: variations in associative representations during instrumental learning," in *Information Processing in Animals: Memory Mechanisms*, eds N. E. Spear and R. R. Miller (Hillsdale, NJ: Erlbaum).

Alele, P. E., and Devaud, L. L. (2006). Sex differences in steroid modulation of ethanol withdrawal in male and female rats. *J. Pharmacol. Exp. Ther.* 320, 427–436. doi: 10.1124/jpet.106.107896

Barker, J. M., Torregrossa, M. M., Arnold, A. P., and Taylor, J. R. (2010). Dissociation of genetic and hormonal influences on sex differences in alcoholism-related behaviors. *J. Neurosci.* 30, 9140–9144. doi: 10.1523/JNEUROSCI.0548-10.2010

Barker, J. M., Torregrossa, M. M., and Taylor, J. R. (2012). Low prefrontal PSA-NCAM confers risk for alcoholism-related behavior. *Nat. Neurosci.* 15, 1356–1358. doi: 10.1038/nn.3194

Barker, J. M., Torregrossa, M. M., and Taylor, J. R. (2013). Bidirectional modulation of infralimbic dopamine D1 and D2 receptor activity regulates flexible reward seeking. *Front. Neurosci.* 7:126. doi: 10.3389/fnins.2013.00126

Barker, J. M., Zhang, H., Villafane, J. J., Wang, T. L., Torregrossa, M. M., and Taylor, J. R. (2014). Epigenetic and pharmacological regulation of 5HT3 receptors controls compulsive ethanol seeking in mice. *Eur. J. Neurosci.* 39, 999–1008. doi: 10.1111/ejn.12477

Broadwater, M., and Spear, L. P. (2013). Consequences of ethanol exposure on cued and contextual fear conditioning and extinction differ depending on timing of exposure during adolescence or adulthood. *Behav. Brain Res.* 256, 10–19. doi: 10.1016/j.bbr.2013.08.013

Butkovich, L. M., DePoy, L. M., Allen, A. G., Shapiro, L. P., Swanson, A. M., and Gourley, S. L. (2015). Adolescent-onset GABAA α1 silencing regulates reward-related decision making. *Eur. J. Neurosci.* 42, 2114–2121. doi: 10.1111/ejn.12995

Centanni, S. W., Burnett, E. J., Trantham-Davidson, H., and Chandler, L. J. (2017). Loss of δ-GABA A receptor-mediated tonic currents in the adult prelimbic cortex following adolescent alcohol exposure. *Addict. Biol.* 22, 616–628. doi: 10.1111/adb.12353

Corbit, L. H., Nie, H., and Janak, P. H. (2012). Habitual alcohol seeking: time course and the contribution of subregions of the dorsal striatum. *Biol. Psychiatry* 72, 389–395. doi: 10.1016/j.biopsych.2012.02.024

Devaud, L. L., and Chadda, R. (2001). Sex differences in rats in the development of and recovery from ethanol dependence assessed by changes in seizure susceptibility. *Alcohol. Clin. Exp. Res.* 25, 1689–1696. doi: 10.1111/j.1530-0277.2001.tb02176.x

Devaud, L. L., Matthews, D. B., and Morrow, A. L. (1999). Gender impacts behavioral and neurochemical adaptations in ethanol-dependent rats. *Pharmacol. Biochem. Behav.* 64, 841–849. doi: 10.1016/S0091-3057(99)00164-1

Dickinson, A., Wood, N., and Smith, J. W. (2002). Alcohol seeking by rats: action or habit? *Q. J. Exp. Psychol. B* 55, 331–348. doi: 10.1080/02724990244000016

Doremus-Fitzwater, T. L., and Spear, L. P. (2016). Reward-centricity and attenuated aversions: an adolescent phenotype emerging from studies in laboratory animals. *Neurosci. Biobehav. Rev.* 70, 121–134. doi: 10.1016/j.neubiorev.2016.08.015

Flagel, S. B., Akil, H., and Robinson, T. E. (2009). Neuropharmacology individual differences in the attribution of incentive salience to reward-related cues: implications for addiction. *Neuropharmacology* 56, 139–148. doi: 10.1016/j.neuropharm.2008.06.027

Floresco, S. B., Magyar, O., Ghods-Sharifi, S., Vexelman, C., and Tse, M. T. L. (2006). Multiple dopamine receptor subtypes in the medial prefrontal cortex of the rat regulate set-shifting. *Neuropsychopharmacology* 31, 297–309. doi: 10.1038/sj.npp.1300825

Gass, J. T., Glen, W. B., McGonigal, J. T., Trantham-Davidson, H., Lopez, M. F., Randall, P. K., et al. (2014). Adolescent alcohol exposure reduces behavioral flexibility, promotes disinhibition, and increases resistance to extinction of ethanol self-administration in adulthood. *Neuropsychopharmacology* 39, 2570–2583. doi: 10.1038/npp.2014.109

Gourley, S. L., Swanson, A. M., Jacobs, A. M., Howell, J. L., Mo, M., Dileone, R. J., et al. (2012). Action control is mediated by prefrontal BDNF and glucocorticoid receptor binding. *Proc. Natl. Acad. Sci. U.S.A.* 109, 20714–20719. doi: 10.1073/pnas.1208342109

Gremel, C. M., and Costa, R. M. (2013). Orbitofrontal and striatal circuits dynamically encode the shift between goal-directed and habitual actions. *Nat. Commun.* 4:2264. doi: 10.1038/ncomms3264

Halbout, B., Liu, A. T., and Ostlund, S. B. (2016). A closer look at the effects of repeated cocaine exposure on adaptive decision-making under conditions that promote goal-directed control. *Front. Psychiatry* 7:44. doi: 10.3389/fpsyt.2016.00044

Kang, S., Wu, M. M., Galvez, R., and Gulley, J. M. (2016). Timing of amphetamine exposure in relation to puberty onset determines its effects on anhedonia, exploratory behavior, and dopamine D1 receptor expression in young adulthood. *Neuroscience* 339, 72–84. doi: 10.1016/j.neuroscience.2016.09.044

Laviola, G., Macrì, S., Morley-Fletcher, S., and Adriani, W. (2003). Risk-taking behavior in adolescent mice: psychobiological determinants and early epigenetic influence. *Neurosci. Biobehav. Rev.* 27, 19–31. doi: 10.1016/S0149-7634(03)00006-X

Nelson, A., and Killcross, S. (2006). Amphetamine exposure enhances habit formation. *J. Neurosci.* 26, 3805–3812. doi: 10.1523/JNEUROSCI.4305-05.2006

Nelson, A. J. D., and Killcross, S. (2013). Accelerated habit formation following amphetamine exposure is reversed by D1, but enhanced by D2, receptor antagonists. *Front. Neurosci.* 7:76. doi: 10.3389/fnins.2013.00076

Nixon, K., and Crews, F. T. (2002). Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. *J. Neurochem.* 83, 1087–1093. doi: 10.1046/j.1471-4159.2002.01214.x

Quinn, J. J., Hitchcott, P. K., Umeda, E. A., Arnold, A. P., and Taylor, J. R. (2007). Sex chromosome complement regulates habit formation. *Nat. Neurosci.* 10, 1398–1400. doi: 10.1038/nn1994

Saunders, B. T., and Robinson, T. E. (2013). Individual variation in resisting temptation: implications for addiction. *Neurosci. Biobehav. Rev.* 37(9 Pt A), 1955–1975. doi: 10.1016/j.neubiorev.2013.02.008

Serlin, H., and Torregrossa, M. M. (2014). Adolescent rats are resistant to forming ethanol seeking habits. *Dev. Cogn. Neurosci.* 16, 183–190. doi: 10.1016/j.dcn.2014.12.002

Sjoerds, Z., de Wit, S., van den Brink, W., Robbins, T. W., Beekman, A. T. F., Penninx, B. W. J. H., et al. (2013). Behavioral and neuroimaging evidence for overreliance on habit learning in alcohol-dependent patients. *Transl. Psychiatry* 3, e337. doi: 10.1038/tp.2013.107

Spear, L. P. (2015). Adolescent alcohol exposure: are there separable vulnerable periods within adolescence? *Physiol. Behav.* 148, 122–130. doi: 10.1016/j.physbeh.2015.01.027

Swanson, A. M., Shapiro, L. P., Whyte, A. J., and Gourley, S. L. (2013). Glucocorticoid receptor regulation of action selection and prefrontal cortical dendritic spines. *Commun. Integr. Biol.* 6:e26068. doi: 10.4161/cib.26068

Trantham-Davidson, H., Centanni, S. W., Garr, S. C., New, N. N., Mulholland, P. J., Gass, J. T., et al. (2017). Binge-like alcohol exposure during adolescence disrupts dopaminergic neurotransmission in the adult prelimbic cortex. *Neuropsychopharmacology* 42, 1024–1036. doi: 10.1038/npp.2016.190

Treloar, H., Celio, M. A., Lisman, S. A., Miranda, R., and Spear, L. P. (2017). Subjective alcohol responses in a cross-sectional, field-based study of adolescents and young adults: effects of age, drinking level, and dependence/consequences. *Drug Alcohol Depend.* 170, 156–163. doi: 10.1016/j.drugalcdep.2016.11.009

Varlinskaya, E. I., and Spear, L. P. (2004). Acute ethanol withdrawal (hangover) and social behavior in adolescent and adult male and female Sprague-Dawley rats. *Alcohol Clin. Exp. Res.* 28, 40–50. doi: 10.1097/01.ALC.0000108655.51087.DF

Winter, S., Dieckmann, M., and Schwabe, K. (2009). Dopamine in the prefrontal cortex regulates rats behavioral flexibility to changing reward value. *Behav. Brain Res.* 198, 206–213. doi: 10.1016/j.bbr.2008.10.040

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.

The reviewer KS and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 29 September 2017
doi: 10.3389/fnbeh.2017.00176

Heavy Drinking in College Students Is Associated with Accelerated Gray Matter Volumetric Decline over a 2 Year Period

Shashwath A. Meda[1], Alecia D. Dager[1,2], Keith A. Hawkins[2], Howard Tennen[3], Sarah Raskin[4], Rebecca M. Wood[5], Carol S. Austad[5], Carolyn R. Fallahi[5] and Godfrey D. Pearlson[1,2,6]*

[1] Olin Neuropsychiatry Research Center, Hartford HealthCare Corporation, Hartford, CT, United States, [2] Department of Psychiatry, Yale University, New Haven, CT, United States, [3] Department of Community Medicine, University of Connecticut School of Medicine, Farmington, CT, United States, [4] Department of Psychology and Neurosciences, Trinity College, Hartford, CT, United States, [5] Department of Psychology, Central Connecticut State University, New Britain, CT, United States, [6] Department of Neuroscience, Yale University, New Haven, CT, United States

Background: Heavy and/or harmful alcohol use while in college is a perennial and significant public health issue. Despite the plethora of cross-sectional research suggesting deleterious effects of alcohol on the brain, there is a lack of literature investigating the longitudinal effects of alcohol consumption on the adolescent brain. We aim to probe the longitudinal effects of college drinking on gray matter change in students during this crucial neurodevelopmental period.

Methods: Data were derived from the longitudinal Brain and Alcohol Research in College Students (BARCS) study of whom a subset underwent brain MRI scans at two time points 24 months apart. Students were young adults with a mean age at baseline of about 18.5 years. Based on drinking metrics assessed at both baseline and followup, subjects were classified as sustained abstainers/light drinkers ($N = 45$) or sustained heavy drinkers ($N = 84$) based on criteria established in prior literature. Gray matter volumetric change (GMV-c) maps were derived using the longitudinal DARTEL pipeline as implemented in SPM12. GMV-c maps were then subjected to a 1-sample and 2-sample *t*-test in SPM12 to determine within- and between-group GMV-c differences in drinking groups. Supplementary between-group differences were also computed at baseline only.

Results: Within-group analysis revealed significant decline in GMV in both groups across the 2 year followup period. However, tissue loss in the sustained heavy drinking group was more significant, larger per region, and more widespread across regions compared to abstainers/light drinkers. Between-group analysis confirmed the above and showed a greater rate of GMV-c in the heavy drinking group in several brain regions encompassing inferior/medial frontal gyrus, parahippocampus, and anterior cingulate. Supplementary analyses suggest that some of the frontal differences existed at baseline and progressively worsened.

OPEN ACCESS

Edited by:
Mary M. Torregrossa, University of Pittsburgh, United States

Reviewed by:
Dieter J. Meyerhoff, University of California, San Francisco, United States
Zsigmond Tamás Kincses, University of Szeged, Hungary

****Correspondence:***
Shashwath A. Meda
shashwath.meda@hhchealth.org

Received: *28 July 2017*
Accepted: *07 September 2017*
Published: *29 September 2017*

Citation:
Meda SA, Dager AD, Hawkins KA, Tennen H, Raskin S, Wood RM, Austad CS, Fallahi CR and Pearlson GD (2017) Heavy Drinking in College Students Is Associated with Accelerated Gray Matter Volumetric Decline over a 2 Year Period. Front. Behav. Neurosci. 11:176. doi: 10.3389/fnbeh.2017.00176

Conclusion: Sustained heavy drinking while in college was associated with accelerated GMV decline in brain regions involved with executive functioning, emotional regulation, and memory, which are critical to everyday life functioning. Areas of significant GMV decreases also overlapped largely with brain reward and stress systems implicated in addictive behavior.

Keywords: substance use, college, binge, morphometry, cortical

INTRODUCTION

Alcohol is the most widely used intoxicant among adolescents and young adults, with rates of binge drinking and alcohol use disorders peaking between ages 18 and 25 (SAMHSA, 2016), a period commonly referred to as emerging adulthood (Arnett, 2000). Recent national surveys revealed that 39% of 18–25-year-olds reported past-month binge drinking, compared to 25% of adults ages 26 and older (SAMHSA, 2016). This age range also encompasses the final stages of neuromaturation (Gogtay et al., 2004), which may be negatively affected by heavy alcohol use.

Age-related refinements in brain structure and function continue well into emerging adulthood. Region-specific patterns of cortical thinning and gray matter volume reduction have been identified, with higher order association cortices, including prefrontal and temporal regions, maturing last, and varying by sex (Gogtay et al., 2004). Measureable changes in gray matter volume have been observed over a 6-month period in first-year college students, between fall and spring semesters (Bennett and Baird, 2006). These neuromaturational changes are thought to underlie cognitive improvements throughout this age range. In particular, executive functions, subserved by late-developing frontal lobe regions, show dramatic enhancements in late adolescence and emerging adulthood (Casey et al., 2000).

Several neuroimaging investigations have characterized structural brain differences among adolescent drinkers, yet fewer investigations have focused on emerging adults. Extant research has most consistently implicated frontal lobe abnormalities among adolescent drinkers (Silveri et al., 2016). Cross sectional studies have revealed smaller volumes and thinner cortices among adolescent heavy drinkers, particularly in frontal, temporal, and cingulate regions (De Bellis et al., 2005; Medina et al., 2008; Fein et al., 2013; Whelan et al., 2014), with greater alcohol consumption associated with larger volume reductions (De Bellis et al., 2005). A recent voxel-based morphometry study of young adults ages 22–28 identified less gray matter volume among heavy drinkers in anterior cingulate, orbitofrontal, temporal, and insula cortices (Heikkinen et al., 2017). Longitudinal investigations have examined adolescents both before and after the onset of heavy drinking, and described steeper declines in cortical thickness in prefrontal (Luciana et al., 2013; Squeglia et al., 2015) and temporal (Squeglia et al., 2014, 2015) regions in alcohol initiators. One diffusion tensor imaging study ascertained white matter integrity in binge drinkers and controls during late adolescence, and again 3 years later during emerging adulthood. Lower fractional anisotropy was observed at baseline among binge drinkers throughout widespread brain regions (Jacobus et al., 2009; McQueeny et al., 2009), many of which showed additional decline among those who continued binge drinking during the 3-year follow-up period (Jacobus et al., 2013). However, to our knowledge, this is the first longitudinal study to examine trajectories of gray matter volume development in an attempt to shed more light on implications of continued heavy drinking among emerging adult drinkers.

To delineate the influence of ongoing heavy alcohol use on brain gray matter development in emerging adulthood, we collected structural magnetic resonance imaging (MRI) scans at two time-points in a college cohort, at approximately age 18 and again ∼2 years later at age 20. We conducted longitudinal voxel based morphometry (VBM) analyses in individuals who were either abstainers/light drinking controls or heavy drinkers at both time-points (i.e., sustained light use or sustained heavy use). Given that cortical pruning is part of the normal developmental process at this age, we predicted that both groups would show significantly reduced gray matter over time; however, sustained heavy drinkers would show a greater decline in gray matter volume compared to sustained light drinkers, especially in frontal and temporal brain regions.

MATERIALS AND METHODS

Participants

A cohort of first-year students (age range 18–23; mean 18.5 years) was recruited from two local colleges with diverse populations through e-mail, flyers and classroom visits to solicit participation in the Brain and Alcohol Research in College Students (BARCS) study (Ahmadi et al., 2013; Dager et al., 2014; Worhunsky et al., 2016; Meda et al., 2017). The recruitment captured greater than 95% of possible participants. All subjects provided written informed consent to participate in the study. The study was conducted in accordance with the declaration of Helsinki and was approved by institutional review boards at Central Connecticut State University (CCSU), University of Connecticut, Trinity College, Hartford Hospital, and Yale University.

A representative sub-sample of 200 individuals, all free from MRI contraindications, underwent a neuroimaging battery including structural imaging scans at baseline and follow up (24 months apart on average). Participants were divided into two groups at both baseline and 24-month follow-up based on drinking quantity and frequency (also see below section), similar to definitions used in previous studies (Dager et al., 2014; Worhunsky et al., 2016). Heavy drinking was defined as binge drinking (≥4 drinks/occasion for females, ≥5 drinks/occasion for males) ≥13 of the past 26 weeks, or meeting criteria for alcohol abuse. Light drinking included teetotalers or binge drinking <13

of the previous 26 weeks and never meeting criteria for alcohol abuse. The final sample included 139 participants: (i) 55 light drinkers who either abstained or sparingly drank at baseline and follow-up and (ii) 84 heavy drinkers, who drank heavily at baseline and follow-up (Please see **Table 1**). The rest of the participants were excluded from the study due to one of the following reasons (a) not scanned at one of the two time points, (b) scans did not pass quality control at one or both time points (for e.g., excessive motion, bad scan quality, other artifacts etc.) and (c) could not be categorized into one of the above drinking categories at one or both time points and (d) had valid scans at both time points but were neither sustained abstainer/light or heavy drinkers.

Exclusion criteria included current schizophrenia or bipolar disorder, history of seizures or significant head injury, not meeting our criteria for drinking groups (described in the assessments section below), and excessive motion (visual evaluation by imaging expert SAM) during scanning.

Assessments

At both baseline and follow-up, past 6-month alcohol use and abuse diagnosis was assessed using an in-house interview incorporating items from the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA; Bucholz et al., 1994) and the alcohol use module from the Structured Clinical Interview for the DSM-IV (SCID; First et al., 1994). In addition at baseline, we collected a variety of variables related to psychiatric status and family history of alcoholism (FHA). Students were administered a select section of the Barkley Adult ADHD Rating Scale–IV (BAARS-IV) to assess ADHD symptoms (Barkley et al., 2011). Students were asked to self-report whether they had ever been formally diagnosed with ADHD and whether they were currently receiving treatment for the same. We coded individuals as having a history of ADHD if they responded "yes" to both questions. All participants also received the M.I.N.I. structured psychiatric diagnostic assessment (Sheehan et al., 1998) (https://medical-outcomes.com/). We found three prevalent diagnoses including major depressive disorder (MDD), agoraphobia and panic disorder among our college sample, which were binary coded (yes/no) and used for further analyses. FHA was assessed using the Family History Assessment Module (FHAM) (Rice et al., 1995). Students completed the trait sections of the State Trait Anxiety Index (STAI) questionnaire (Spielberger et al., 1983). A total STAI sum score was calculated, normed based on gender and was used for further analysis. Students were also administered the Beck Depression Inventory (BDI) to assess depressive symptoms at study entry (Beck et al., 1961); all items from the inventory were summed to yield a total score. At each scan time point, participants provided urine toxicology samples for negative screening of commonly abused substances, negative alcohol breathalyzer screens, and negative urine pregnancy screens (females).

Image Acquisition and Processing

Magnetic resonance structural brain images were collected on a Siemens Allegra 3T system (Siemens AG, Erlangen, Germany) located at the Olin Neuropsychiatric Research Center in Hartford, CT. Images were collected using a sagittal T1 MPRAGE sequence with the following parameters TR/TE/TI = 2,300/2.74/900 ms, flip angle = 8°, slab thickness = 176 mm, FOV = 176 × 256 mm, matrix = 176 × 256 × 176, voxel size = 1 mm^3, pixel band-width = 190 Hz, scan time = 10:09.

Computation of Longitudinal Gray Matter Volume (GMV) Change

In order to compute gray matter volume change maps, structural images from each time point were subjected to a technique called symmetric diffeomorphic modeling of longitudinal data, implemented in SPM12 (Ashburner and Ridgway, 2012). The following steps were implemented to derive the volumetric rate of change maps for each subject (1) a mid-point image was estimated by optimally mapping the template with each time point image by means of a groupwise-consistent 3D non-linear image registration technique. A Jacobian difference image was then estimated that records the difference for deformation from the mid-point image to the first scan and this to the second scan. This Jacobian difference map was further divided by the time elapsed between the two scans to derive a rate of change map. (2) the mid-point image was then segmented into their respective GM, WM, and CSF constituents. (3) GM images from step (2) were used to create a study-specific template using DARTEL. (4) The Jacobian rate of difference images from step (1) were multiplied by the GM image from step (2) to derive a GMV rate of change map (GMV-c). (5) The images from step (4) were normalized to MNI space and smoothed with a 6 mm FWHM Gaussian kernel. (6) Images derived from step (5) were then subjected to further statistical analyses as described below.

Statistical Analyses

Continuous demographic variables were compared between groups using an independent sample *t*-test in SPSS v21 (https://www.ibm.com/analytics/us/en/technology/spss/). Categorical variables were examined with a chi-square analysis using the same software.

The GMV-c maps were subjected to a 1-sample *t*-test in SPM12 (separately for light and heavy drinkers) to detect significant within-group decreases or expansions over the 2-year follow-up period on a voxel-by-voxel basis across the whole brain. Further, we carried out a 2-sample *t*-test (adjusted for sex) within the general linear model framework to assess GMV-c differences across light and heavy drinking groups (group by time interaction). Given that alcohol may influence brain changes differentially by sex (Squeglia et al., 2014) supplementary analyses were conducted to look for any possible group-by-sex or group-by-sex-by-time interactions. In addition to the above, to assess if groups differed in GMV at baseline, we conducted a follow-up 2-sample *t*-test adjusted for sex and total intracranial volume using only GMV calculated at the first time point. Although the major aim of the paper was to capture and report longitudinal changes, this explicit baseline analysis provides readers a better understanding of what volumetric changes already existed at baseline and how these brain structures change over time. All the above statistical analyses were conducted using permutation based analyses coupled with corrections for whole brain multiple

TABLE 1 | Demographics and clinical characteristics of drinking groups investigated in the study.

Subject demographics	Sustained light users (Total *N* = 55)		Sustained heavy users (Total *N* = 84)		Statistic	
	Mean	*SD*	Mean	*SD*	*t*	*p*-value
Age at Baseline (years)	18.38	0.59	18.48	0.81	0.16	NS
Baseline State-Trait Anxiety Inventory (STAI)	42.46 (*N* = 41)	10.12	54.42 (*N* = 49)	12.32	−0.78	NS
Baseline Beck Depression Inventory (BDI)	4.12 (*N* = 41)	6.27	5.22 (*N* =5 1)	5.56	−0.88	NS
Number of Monthly Drinks (Baseline)	2	5	48	48	−7.18	<0.001
Number of Monthly Drinks (Follow up)	3	5	40	42	−6.54	<0.001
Time Elapsed Between Scans (Months)	24.39	5.02	25.2	5.23	−0.90	NS
Baseline Clinical Characteristics	*N*		*N*		Chi-square	*p*-value
GENDER						
Male	22		49		4.47	0.04
Female	33		35			
CIGARETTE SMOKER						
No	38		49		1.51	NS
Yes	3		1			
Missing	14		34			
FAMILY HISTORY FOR ALCOHOLISM						
Negative	28		34		0.03	NS
Positive	13		17			
Missing	14		33			
ADHD						
No	36		47		3.74	NS
Yes	3		0			
Missing	16		37			
MDD (MINI)						
No	54		78		1.97	NS
Yes	1		6			
AGORAPHOBIA (MINI)						
No	54		78		1.97	NS
Yes	1		6			
PANIC DISORDER (MINI)						
No	40		48		0.65	NS
Yes	1		3			
Missing	14		33			

comparisons using the threshold free cluster enhancement technique (TFCE) as implemented in the TFCE toolbox (http://dbm.neuro.uni-jena.de/tfce/). Resulting maps were thresholded at the $p < 0.05$ family wise error (FWE) whole brain corrected level and displayed using the NeuroElf toolbox (http://neuroelf.net/).

RESULTS

Groups did not differ by age, smoking, FHA or any MINI diagnostic status. Groups also had similar scores on the STAI and BDI. However, groups differed by sex (chi-square = 4.47; $p = 0.03$). Complete description of demographics and clinical characteristics of study participants are provided in **Table 1**.

Within-group analysis revealed significant GMV loss in both drinking groups over time (see **Figure 1**). No significant regional expansion of GMV over time was detected in either group. GMV decline in stable light users was mostly localized to inferior/middle temporal and fusiform gyrus. Gray matter decreases in individuals with sustained heavy alcohol use were lager in magnitude, and included the above regions plus several other brain regions including inferior/middle frontal gyrus (I/MFG), anterior cingulate, insula, thalamus, caudate, and parahippocampal gyri. Longitudinal GMV differences assessed across groups (see **Figure 2**) showed the heavy use group to have a significantly increased rate of gray matter decline, primarily in fronto-striatal regions, including inferior/medial frontal, anterior cingulate, parahippocampus, precentral gyrus,

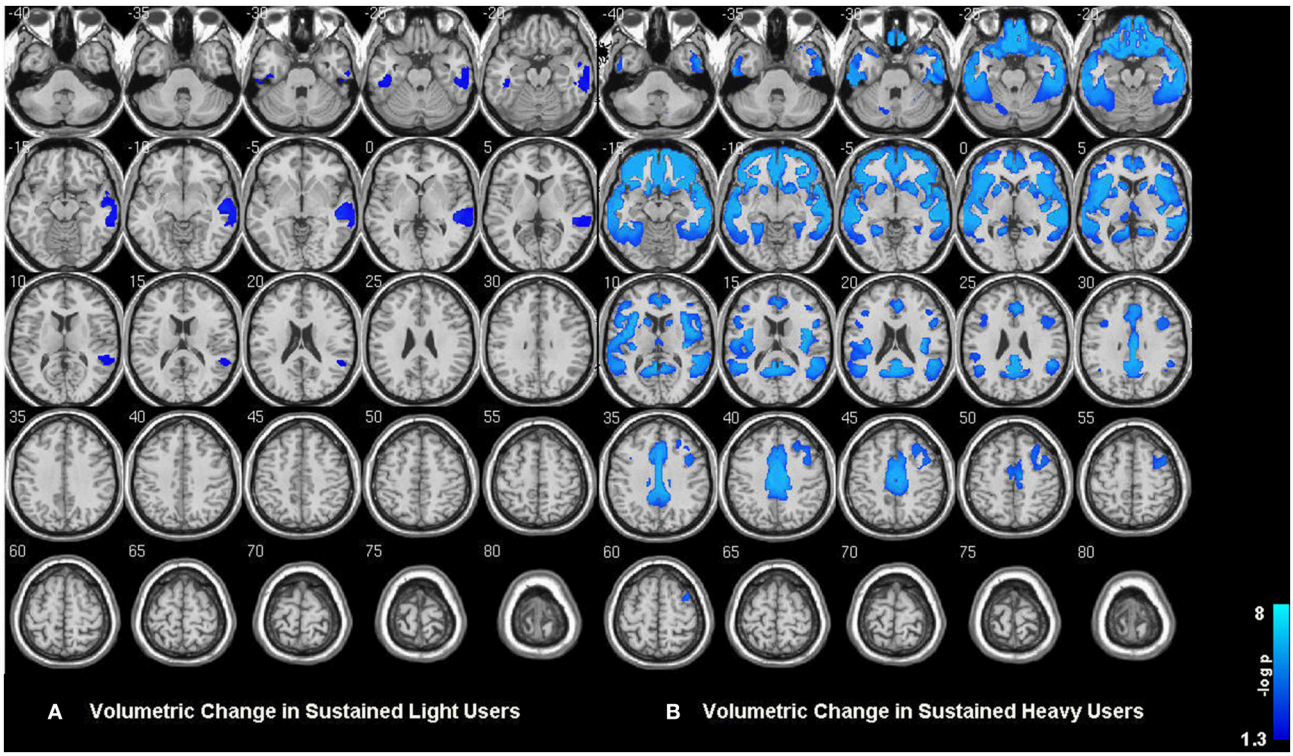


FIGURE 1 | (A) Gray matter volumetric change in sustained abstainers/light and **(B)** sustained heavy users across the two year period. Results are displayed at $p <$ 0.05 FWE corrected across the whole brain. Images are on a negative log *P* scale. Note that both groups only showed significant decline and no expansions.

and insula. A complete description of within-group and between-group results is presented in **Tables 2A,B**. Further, we detected no group-by-sex or group-by-sex-by-time interactions at the whole brain level. Exploratory analysis of between-group baseline differences in GMV revealed significantly decreased GMV in the heavy drinking group in a subset of regions that also showed exaggerated decline across time. Overlap of regions was mainly localized to superior, medial and inferior frontal gyri after correction for multiple comparisons. Regions showing baseline differences are visually represented in **Figure 3**.

DISCUSSION

The present longitudinal study investigated gray matter volume changes over a 2 year period in emerging adult college students who were either sustained light-moderate drinkers or who had continued heavy alcohol use. Consistent with our initial hypotheses, we noted significant brain GMV loss in both groups. However, a direct two group comparison showed a significantly greater rate of loss in students with a sustained pattern of heavy alcohol use over 2 year follow-up. Moreover, this pattern of greater gray matter decline in heavy users occurred largely in regions responsible for emotion, memory, mental flexibility, and decision making, all of which might have a direct impact on student college success (Best et al., 2011).

It is important to recall that cortical pruning and restructuring reflects a normal, beneficial process during adolescence and young adulthood (Gogtay et al., 2004). Although the results noted in abstainers/light drinkers might closely reflect normal developmental processes, the exaggerated GMV-c due to continued heavy drinking would not be fully attributable to such cortical maturation. One possible interpretation could be that alcohol use leads to excess gray matter volume loss similar to that observed in adult alcoholics (Suzuki et al., 2010; Ersche et al., 2013; Sjoerds et al., 2013; Segobin et al., 2014; Droutman et al., 2015). The above hypothesis is supported by animal research that suggests alcohol interferes with neural stem cell proliferation leading to neurodegeneration and changes in brain structure and function (Morris et al., 2010). Animal reports have also shown marked differences in survival of cortical-cells in adolescent animals that were exposed to alcohol compared to controls (Crews et al., 2006; Hansson et al., 2010; Koss et al., 2012). Consistent with the above findings, changes observed in human macro-structural MRI studies such as ours may thus reflect tissue loss or remodeling related to inhibition of cell generation and survival. Preclinical studies also suggest that heavy alcohol intoxication might trigger microglia activation, oxidative stress, and pro-inflammatory changes which in turn leads to neurotoxic degeneration and/or prevents genesis of neurons and glia (Crews and Nixon, 2009; Alfonso-Loeches et al., 2012). Our results are also very consistent with a recent study

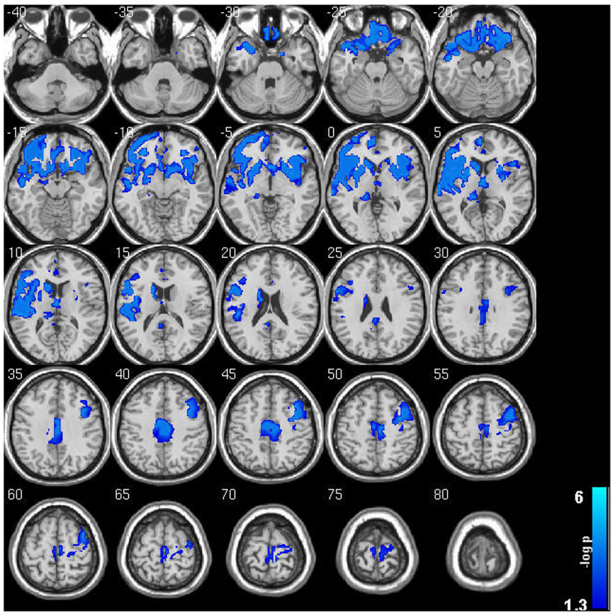


FIGURE 2 | Significant gray matter volumetric change between sustained abstainers/light and sustained heavy user groups across the 2 year period. Note the widespread accelerated GMV decreases in sustained heavy users of alcohol compared to their light user peers. Results are displayed at $p < 0.05$ FWE corrected across the whole brain. Images are on a negative log *P* scale.

that used region of interest based analysis to investigate similar longitudinal gray and white matter changes in adolescents before and after they initiated heavy alcohol use (Squeglia et al., 2015). That study observed a similar pattern of accelerated GMV-c in heavy alcohol initiators along with an attenuated growth of several white matter structures. The current study expands on these findings, demonstrating that continued heavy drinking in emerging adulthood leads to excessive gray matter loss in brain structures relevant to everyday functioning. Also, compared to the above study, we employed a voxel-wise whole brain analysis that should enable us to visualize GMV-c with greater spatial specificity.

Previous studies have shown that disruptions to adolescent brain maturation might adversely impact developmental cognitive and motor performance (Burgaleta et al., 2014). In the current study we noted both baseline decreases and accelerated GMV decline of the parahippocampal gyrus (a region primarily responsible for memory function) in the heavy drinking group, which is consistent with and expands results from cross-sectional reports showing reduced GMV in this medial temporal region for both individuals with alcohol dependence and those with family history of alcohol dependence (Suzuki et al., 2010; Sjoerds et al., 2013), The significant interaction of alcohol use on gray matter volume development as shown here might therefore help explain previous longitudinal studies showing worse attention, working memory, and visuospatial performance in adolescents after initiating alcohol use (Tapert et al., 2002; Hanson et al., 2011).

Interestingly, many of the significantly affected regions overlap with brain systems involved in reward, decision making, emotion and stress, that have been directly linked to addictive behavior (Schoenbaum and Shaham, 2008; Sinha, 2011; Droutman et al., 2015; O'Connor and Kenny, 2015; Silveri et al., 2016). Previous functional studies have shown increased activation to alcohol cues in medial frontal/ACC that underlie attention and motivation (Myrick et al., 2004; Dager et al., 2013). Altered interactions between ACC and parts of the striatum (also implicated in our current study) have shown to be related to motivational shift toward drug-induced cues, thus suggesting potential biological markers of addictive pathology (Myrick et al., 2004; Dager et al., 2013). Another key region identified in our study was inferior frontal gyrus (IFG), which functionally signals drug availability and triggers anticipation of use (Schoenbaum and Shaham, 2008). IFG over-reactivity has also been prospectively linked to escalating drinking in a previous study that used a subset of the current dataset (Worhunsky et al., 2016). It is interesting to note that our supplementary analyses show that some of these frontal differences existed at baseline and seemingly became worse over time. Insula, another region that showed exacerbated GMV loss in our heavy alcohol users, has been shown to play an important role in drug addiction (Droutman et al., 2015). Meta analyses of structural abnormalities in addicted individuals have described significantly reduced insular volume (Ersche et al., 2013). Functional studies have repeatedly observed blunted insular activity in substance-dependent individuals when engaged in decision-making tasks, and have also been shown to predict relapse (Sinha, 2011). The substantial overlap of excess gray matter loss with critical brain systems implicated in addiction might suggest that some of these differences may have been pre-existing. Compulsive drug taking due to poorly modulated decision making as a consequence of inability to learn from negative consequences is a hallmark of addiction. Baseline differences noted in our study were primarily limited to the pre-frontal cortex (PFC) which plays a key role in such decision making processes among other critical executive functions (George and Koob, 2010). Indeed, frontal lobe volumetric differences may be both a pre-existing risk factor as well as consequence of heavy alcohol use in adolescence (Silveri et al., 2016). It is also possible that such volumetric changes might contribute to differential neurochemical transmission sensitivity to alcohol that might in-turn enhance vulnerability to addictive-like behaviors into young adulthood. Given the longitudinal design here, our findings support the view that exacerbated gray matter loss is a consequence of continued heavy use. Although sustained heavy use might have an adverse impact on neurodevelopment, many studies in adolescent and adult alcoholics have demonstrated that certain brain structures and pathways show potential for recovery with alcohol abstinence or curtailment (Gazdzinski et al., 2005; Durazzo et al., 2011, 2016; Dresler et al., 2012; Segobin et al., 2014). Future longitudinal studies should determine the degree of volumetric recovery if drinking is reduced during earlier phases of brain development.

A number of additional factors in college including illicit substance use, stress levels, academic performance, peer pressure

TABLE 2 | Significant regions of GMV decreases for both **(A)** within- and **(B)** between-group analyses.

(Hemisphere) Region	Brodmann area	*x*	*y*	*z*	Max (−log p)	Cluster size (mm^3)
(A) WITHIN-GROUP RESULTS						
Sustained light users						
LH Fusiform Gyrus	20	−45	−30	−24	1.71	170
LH Inferior Temporal Gyrus	20	−60	−27	−30	1.33	10
RH Fusiform Gyrus	20	51	−27	−24	1.76	61
RH Inferior Temporal Gyrus	20	57	−30	−15	1.79	165
RH Middle Temporal Gyrus	21	54	−21	−6	2.13	827
Sustained heavy users						
LH Anterior Cingulate	32	0	39	6	3.70	76.41
LH Caudate	Caudate body	−12	15	9	3.10	45.36
LH Claustrum		−27	24	9	3.70	148.23
LH Fusiform Gyrus	20	−54	−21	−21	3.70	152.82
LH Fusiform Gyrus	37	−39	−45	−15	3.22	118.26
LH Inferior Frontal Gyrus	11	−18	36	−18	3.70	153.09
LH Insula	13	−36	0	15	3.70	99.36
LH Medial Frontal Gyrus	10	−3	54	0	3.70	116.91
LH Middle Frontal Gyrus	46	−36	27	21	1.84	14.58
LH Middle Frontal Gyrus	9	−33	12	30	1.57	11.88
LH Middle Occipital Gyrus	19	−45	−60	−6	2.18	63.45
LH Middle Temporal Gyrus	21	−45	−33	0	3.70	93.96
LH Middle Temporal Gyrus	37	−42	−63	6	1.71	14.85
LH Pyramis	–	−15	−63	−27	1.31	13.5
LH Superior Temporal Gyrus	38	−48	12	−9	3.70	68.04
LH Superior Temporal Gyrus	22	−48	−15	−9	3.70	69.39
LH Superior Temporal Gyrus	39	−42	−51	21	3.22	79.11
LH Superior Temporal Gyrus	41	−54	−30	9	2.49	44.82
LH Thalamus	–	−12	−33	3	1.79	12.69
LH Thalamus	Medial dorsal nucleus	0	−15	9	1.76	13.23
RH Anterior Cingulate	32	12	48	−9	3.70	118.8
RH Anterior Cingulate	25	3	18	−9	3.70	120.69
RH Cingulate Gyrus	24	12	−15	42	3.70	118.8
RH Cingulate Gyrus	31	6	−45	27	3.70	109.89
RH Cingulate Gyrus	32	3	21	36	3.70	57.78
RH Fusiform Gyrus	37	36	−39	−12	2.49	66.96
RH Inferior Frontal Gyrus	47	48	24	−3	3.70	199.8
RH Inferior Frontal Gyrus	45	42	18	9	3.70	88.56
RH Inferior Frontal Gyrus	44	51	12	18	3.10	27
RH Insula	13	42	−18	3	3.70	44.01
RH Medial Frontal Gyrus	25	9	6	−15	3.40	26.19
RH Medial Frontal Gyrus	9	3	33	30	3.40	44.01
RH Middle Frontal Gyrus	10	42	57	−9	3.70	129.87
RH Middle Frontal Gyrus	47	51	42	−6	3.40	44.01
RH Middle Frontal Gyrus	6	39	6	57	2.27	27.81
RH Middle Frontal Gyrus	8	30	24	48	2.22	29.7
RH Middle Frontal Gyrus	46	45	30	18	1.57	11.34
RH Middle Temporal Gyrus	22	69	−30	3	3.70	133.65
RH Middle Temporal Gyrus	21	66	−15	−3	3.70	150.39
RH Parahippocampal Gyrus	30	24	−51	6	3.10	20.52
RH Posterior Cingulate	31	9	−54	24	3.70	150.66
RH Posterior Cingulate	30	18	−54	18	3.22	26.19

(Continued)

TABLE 2 | Continued

(Hemisphere) Region	Brodmann area	*x*	*y*	*z*	Max (−log p)	Cluster size (mm^3)
RH Precentral Gyrus	6	48	−3	9	3.70	67.23
RH Precentral Gyrus	9	39	9	36	2.38	34.02
RH Superior Temporal Gyrus	22	60	3	−6	3.70	156.06
RH Superior Temporal Gyrus	13	51	−45	21	3.70	79.92
(B) BETWEEN–GROUP RESULTS						
LH Anterior Cingulate	24	0	36	6	1.47	8.37
LH Anterior Cingulate	32	−12	39	9	1.45	1.35
LH Caudate	Caudate head	−12	18	−9	3.70	109.35
LH Caudate	Caudate body	−15	12	18	3.70	28.62
LH Cingulate Gyrus	31	−6	−36	33	3.22	48.87
LH Claustrum	–	−27	24	9	3.70	74.52
LH Inferior Frontal Gyrus	11	−18	36	−18	3.70	60.21
LH Inferior Frontal Gyrus	46	−39	39	6	3.70	98.55
LH Inferior Frontal Gyrus	9	−42	9	21	3.70	66.15
LH Insula	13	−39	0	−6	3.70	262.44
LH Lentiform Nucleus	Putamen	−27	−15	3	3.70	18.63
LH Medial Frontal Gyrus	11	0	36	−12	3.70	76.95
LH Medial Frontal Gyrus	10	−3	57	6	3.70	29.16
LH Medial Frontal Gyrus	6	−3	−21	63	2.27	18.63
LH Middle Frontal Gyrus	9	−33	12	30	2.09	1.35
LH Paracentral Lobule	5	−3	−33	57	1.67	6.48
LH Parahippocampal Gyrus	30	−12	−36	6	3.70	26.19
LH Parahippocampal Gyrus	34	−15	−12	−15	3.10	7.56
LH Posterior Cingulate	29	−6	−45	18	3.10	7.83
LH Precuneus	31	−12	−51	27	1.54	1.35
LH Superior Frontal Gyrus	10	−12	69	−6	3.70	33.75
LH Superior Frontal Gyrus	11	−18	48	−15	3.70	38.34
LH Superior Temporal Gyrus	38	−48	12	−9	3.70	43.74
LH Transverse Temporal Gyrus	41	−33	−27	9	3.70	14.31
RH Anterior Cingulate	25	6	18	−3	3.70	39.96
RH Anterior Cingulate	24	3	24	18	1.33	1.08
RH Caudate	Caudate head	18	24	0	3.70	50.22
RH Inferior Frontal Gyrus	47	48	24	−3	3.70	71.01
RH Inferior Frontal Gyrus	13	45	21	6	3.70	26.19
RH Inferior Frontal Gyrus	46	51	45	0	2.09	2.97
RH Inferior Frontal Gyrus	9	51	12	24	1.52	3.51
RH Insula	13	45	6	0	3.70	76.14
RH Medial Frontal Gyrus	6	9	−18	51	3.70	233.28
RH Medial Frontal Gyrus	11	3	57	−18	2.09	2.43
RH Middle Frontal Gyrus	8	39	21	45	3.70	52.65
RH Middle Frontal Gyrus	11	30	39	−9	3.70	75.87
RH Precentral Gyrus	6	36	−9	63	3.22	34.29
RH Precentral Gyrus	4	36	−15	51	2.10	33.21
RH Superior Temporal Gyrus	22	45	−6	−3	3.70	27
RH Thalamus	–	3	−6	6	3.70	39.69

and psychiatric status could have an interactive effect with alcohol consumption (Meda et al., 2017). Although the concurrent use of other commonly abused substances such as marijuana were not analyzed here (due to excessive missing rate ∼40%), prior studies that performed similar analyses to ours noted no significant interactions between alcohol and drug use with gray matter loss patterns (Squeglia et al., 2014). We measured personality and psychiatric metrics such as the STAI, BDI, MDD, and ADHD and found them to be balanced across our diagnostic groups. Also, cigarette

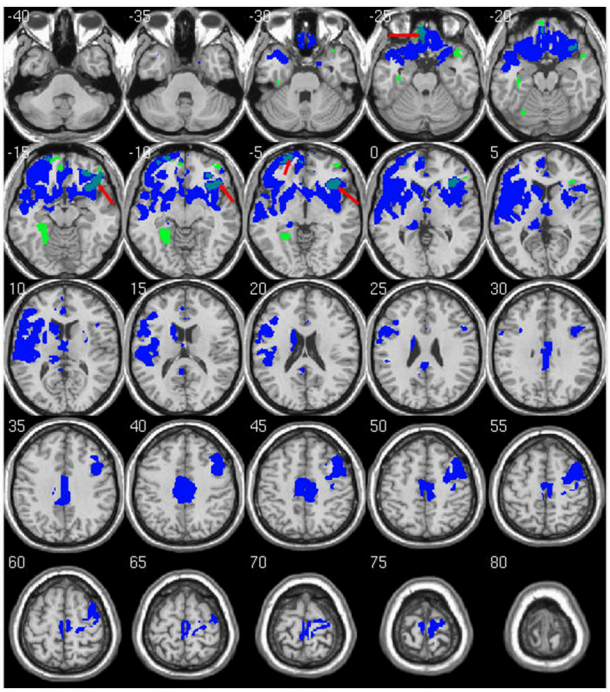


FIGURE 3 | Overlap of significant between-group differences at baseline and longitudinal follow up. Regions marked in green show lower GMV in heavy drinking group compared to abstainers/light at baseline. Regions in blue are the same longitudinal results as shown in **Figure 2**. Overlap between baseline and longitudinal differences are shown in light blue (red arrows). Results are displayed at $p < 0.05$ FWE corrected across the whole brain. Images are on a negative log *P* scale.

smoking, which is often co-morbid with alcohol use and has been associated with gray matter changes (Franklin et al., 2014) was not significantly different between our groups. The proportions of smokers/non-smokers in our sample can be seen in **Table 1**. Given that previous cross-sectional studies have suggested differential alcohol effects exerted across sexes (Squeglia et al., 2014, 2015), we looked at potential group by sex and group by sex by time interactions in our dataset, but found none.

Despite its current strengths, our study had several limitations to consider. We did not have sufficient numbers of individuals who transitioned from heavy to light or light to heavy alcohol use, and were therefore unable to examine gray matter changes associated with other trajectories of alcohol use that are of great interest. We also did not capture longitudinal behavioral data that might help better understand relationships between GMV-c and cognitive functioning, which might be a good avenue for future studies. There may be other important interacting variables, such as stress levels, personality factors, or other substance use, which were not examined here but would be critical areas for future inquiries. We did not scan participants before the onset of drinking, making it difficult to determine whether gray matter volume differences were pre-existing; future studies such as the ongoing ABCD project will help delineate patterns of gray matter development in relation to varying patterns of substance use initiation and escalation.

CONCLUSION

In summary, our study provides important longitudinal evidence regarding excessive gray matter loss in regions related to cognitive control, emotional regulation, and memory that might have both short and long-term implications on student life success. Importantly, regions of abnormal gray matter decline overlapped with those implicated previously in substance abuse, thus providing more evidence toward the theory that initiation and continued alcohol use during young adulthood might confer vulnerability for future, ongoing substance dependence.

AUTHOR CONTRIBUTIONS

SM and AD contributed to design, analysis, and drafting of the manuscript. KH, HT, SR, RW, CA, CF, and GP contributed to the study funding, design, and drafting of the manuscript.

ACKNOWLEDGMENTS

This research was made possible by grant support from the National Institute on Alcohol Abuse and Alcoholism (AA016599 and AA19036, Pearlson).

REFERENCES

Ahmadi, A., Pearlson, G. D., Meda, S. A., Dager, A., Potenza, M. N., Rosen, R., et al. (2013). Influence of alcohol use on neural response to Go/No-Go task in college drinkers. *Neuropsychopharmacology* 38, 2197–2208. doi: 10.1038/npp.2013.119

Alfonso-Loeches, S., Pascual, M., Gomez-Pinedo, U., Pascual-Lucas, M., Renau-Piqueras, J., and Guerri, C. (2012). Toll-like receptor 4 participates in the myelin disruptions associated with chronic alcohol abuse. *Glia* 60, 948–964. doi: 10.1002/glia.22327

Arnett, J. J. (2000). Emerging adulthood. A theory of development from the late teens through the twenties. *Am. Psychol.* 55, 469–480. doi: 10.1037/0003-066X.55.5.469

Ashburner, J., and Ridgway, G. R. (2012). Symmetric diffeomorphic modeling of longitudinal structural MRI. *Front. Neurosci.* 6:197. doi: 10.3389/fnins.2012.00197

Barkley, R. A., Knouse, L. E., and Murphy, K. R. (2011). Correspondence and disparity in the self- and other ratings of current and childhood ADHD symptoms and impairment in adults with ADHD. *Psychol. Assess.* 23, 437–446. doi: 10.1037/a0022172

Beck, A. T., Ward, C. H., Mendelson, M., Mock, J., and Erbaugh, J. (1961). An inventory for measuring depression. *Arch. Gen. Psychiatry* 4, 561–571. doi: 10.1001/archpsyc.1961.01710120031004

Bennett, C. M., and Baird, A. A. (2006). Anatomical changes in the emerging adult brain: a voxel-based morphometry study. *Hum. Brain Mapp.* 27, 766–777. doi: 10.1002/hbm.20218

Best, J. R., Miller, P. H., and Naglieri, J. A. (2011). Relations between executive function and academic achievement from ages 5 to 17 in a large, representative national sample. *Learn. Individ. Differ.* 21, 327–336. doi: 10.1016/j.lindif.2011.01.007

Bucholz, K. K., Cadoret, R., Cloninger, C. R., Dinwiddie, S. H., Hesselbrock, V. M., Nurnberger, J. I. Jr., et al. (1994). A new, semi-structured psychiatric interview for use in genetic linkage studies: a report on the reliability of the SSAGA. *J. Stud. Alcohol* 55, 149–158. doi: 10.15288/jsa.1994.55.149

Burgaleta, M., Johnson, W., Waber, D. P., Colom, R., and Karama, S. (2014). Cognitive ability changes and dynamics of cortical thickness development in healthy children and adolescents. *Neuroimage* 84, 810–819. doi: 10.1016/j.neuroimage.2013.09.038

Casey, B. J., Giedd, J. N., and Thomas, K. M. (2000). Structural and functional brain development and its relation to cognitive development. *Biol. Psychol.* 54, 241–257. doi: 10.1016/S0301-0511(00)00058-2

Crews, F. T., and Nixon, K. (2009). Mechanisms of neurodegeneration and regeneration in alcoholism. *Alcohol Alcohol.* 44, 115–127. doi: 10.1093/alcalc/agn079

Crews, F. T., Mdzinarishvili, A., Kim, D., He, J., and Nixon, K. (2006). Neurogenesis in adolescent brain is potently inhibited by ethanol. *Neuroscience* 137, 437–445. doi: 10.1016/j.neuroscience.2005.08.090

Dager, A. D., Anderson, B. M., Rosen, R., Khadka, S., Sawyer, B., Jiantonio-Kelly, R. E., et al. (2014). Functional magnetic resonance imaging (fMRI) response to alcohol pictures predicts subsequent transition to heavy drinking in college students. *Addiction* 109, 585–595. doi: 10.1111/add.12437

Dager, A. D., Anderson, B. M., Stevens, M. C., Pulido, C., Rosen, R., Jiantonio-Kelly, R. E., et al. (2013). Influence of alcohol use and family history of alcoholism on neural response to alcohol cues in college drinkers. *Alcohol. Clin. Exp. Res.* 37(Suppl. 1), E161–E171. doi: 10.1111/j.1530-0277.2012.01879.x

De Bellis, M. D., Narasimhan, A., Thatcher, D. L., Keshavan, M. S., Soloff, P., and Clark, D. B. (2005). Prefrontal cortex, thalamus, and cerebellar volumes in adolescents and young adults with adolescent-onset alcohol use disorders and comorbid mental disorders. *Alcohol. Clin. Exp. Res.* 29, 1590–1600. doi: 10.1097/01.alc.0000179368.87886.76

Dresler, T., Schecklmann, M., Ernst, L. H., Pohla, C., Warrings, B., Fischer, M., et al. (2012). Recovery of cortical functioning in abstinent alcohol-dependent patients: prefrontal brain oxygenation during verbal fluency at different phases during withdrawal. *World J. Biol. Psychiatry* 13, 135–145. doi: 10.3109/15622975.2011.564654

Droutman, V., Read, S. J., and Bechara, A. (2015). Revisiting the role of the insula in addiction. *Trends Cogn. Sci.* 19, 414–420. doi: 10.1016/j.tics.2015.05.005

Durazzo, T. C., Mon, A., Gazdzinski, S., and Meyerhoff, D. J., (2016). Regional brain volume changes in alcohol-dependent individuals during early abstinence: associations with relapse following treatment. *Addict Biol.* 22, 1416–1425. doi: 10.1111/adb.12420

Durazzo, T. C., Tosun, D., Buckley, S., Gazdzinski, S., Mon, A., Fryer, S. L., et al. (2011). Cortical thickness, surface area, and volume of the brain reward system in alcohol dependence: relationships to relapse and extended abstinence. *Alcohol. Clin. Exp. Res.* 35, 1187–1200. doi: 10.1111/j.1530-0277.2011.01452.x

Ersche, K. D., Williams, G. B., Robbins, T. W., and Bullmore, E. T. (2013). Meta-analysis of structural brain abnormalities associated with stimulant drug dependence and neuroimaging of addiction vulnerability and resilience. *Curr. Opin. Neurobiol.* 23, 615–624. doi: 10.1016/j.conb.2013.02.017

Fein, G., Greenstein, D., Cardenas, V. A., Cuzen, N. L., Fouche, J. P., Ferrett, H., et al. (2013). Cortical and subcortical volumes in adolescents with alcohol dependence but without substance or psychiatric comorbidities. *Psychiatry Res.* 214, 1–8. doi: 10.1016/j.pscychresns.2013.06.001

First, M. B., Frances, A. J., Pincus, H. A., Vettorello, N., and Davis, W. W. (1994). DSM-IV in progress. Changes in substance-related, schizophrenic, and other primarily adult disorders. *Hosp. Community Psychiatry* 45, 18–20.

Franklin, T. R., Wetherill, R. R., Jagannathan, K., Johnson, B., Mumma, J., Hager, N., et al. (2014). The effects of chronic cigarette smoking on gray matter volume: influence of sex. *PLoS ONE* 9:e104102. doi: 10.1371/journal.pone.0104102

Gazdzinski, S., Durazzo, T. C., and Meyerhoff, D. J., (2005). Temporal dynamics and determinants of whole brain tissue volume changes during recovery from alcohol dependence. *Drug Alcohol Depend.* 78, 263–273. doi: 10.1016/j.drugalcdep.2004.11.004

George, O., and Koob, G. F. (2010). Individual differences in prefrontal cortex function and the transition from drug use to drug dependence. *Neurosci. Biobehav. Rev.* 35, 232–247. doi: 10.1016/j.neubiorev.2010.05.002

Gogtay, N., Giedd, J. N., Lusk, L., Hayashi, K. M., Greenstein, D., Vaituzis, A. C., et al. (2004). Dynamic mapping of human cortical development during childhood through early adulthood. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8174–8179. doi: 10.1073/pnas.0402680101

Hanson, K. L., Medina, K. L., Padula, C. B., Tapert, S. F., and Brown, S. A. (2011). Impact of adolescent alcohol and drug use on neuropsychological functioning in young adulthood: 10-year outcomes. *J. Child Adolesc. Subst. Abuse* 20, 135–154. doi: 10.1080/1067828X.2011.555272

Hansson, A. C., Nixon, K., Rimondini, R., Damadzic, R., Sommer, W. H., Eskay, R., et al. (2010). Long-term suppression of forebrain neurogenesis and loss of neuronal progenitor cells following prolonged alcohol dependence in rats. *Int. J. Neuropsychopharmacol.* 13, 583–593. doi: 10.1017/S1461145710000246

Heikkinen, N., Niskanen, E., Kononen, M., Tolmunen, T., Kekkonen, V., Kivimaki, P., et al. (2017). Alcohol consumption during adolescence is associated with reduced grey matter volumes. *Addiction* 112, 604–613. doi: 10.1111/add.13697

Jacobus, J., McQueeny, T., Bava, S., Schweinsburg, B. C., Frank, L. R., Yang, T. T., et al. (2009). White matter integrity in adolescents with histories of marijuana use and binge drinking. *Neurotoxicol. Teratol.* 31, 349–355. doi: 10.1016/j.ntt.2009.07.006

Jacobus, J., Squeglia, L. M., Bava, S., and Tapert, S. F. (2013). White matter characterization of adolescent binge drinking with and without co-occurring marijuana use: a 3-year investigation. *Psychiatry Res.* 214, 374–381. doi: 10.1016/j.pscychresns.2013.07.014

Koss, W. A., Sadowski, R. N., Sherrill, L. K., Gulley, J. M., and Juraska, J. M. (2012). Effects of ethanol during adolescence on the number of neurons and glia in the medial prefrontal cortex and basolateral amygdala of adult male and female rats. *Brain Res.* 1466, 24–32. doi: 10.1016/j.brainres.2012.05.023

Luciana, M., Collins, P. F., Muetzel, R. L., and Lim, K. O. (2013). Effects of alcohol use initiation on brain structure in typically developing adolescents. *Am. J. Drug Alcohol Abuse* 39, 345–355. doi: 10.3109/00952990.2013.837057

McQueeny, T., Schweinsburg, B. C., Schweinsburg, A. D., Jacobus, J., Bava, S., Frank, L. R., et al. (2009). Altered white matter integrity in adolescent binge drinkers. *Alcohol. Clin. Exp. Res.* 33, 1278–1285. doi: 10.1111/j.1530-0277.2009.00953.x

Meda, S. A., Gueorguieva, R. V., Pittman, B., Rosen, R. R., Aslanzadeh, F., Tennen, H., et al. (2017). Longitudinal influence of alcohol and marijuana use on academic performance in college students. *PLoS ONE* 12:e0172213. doi: 10.1371/journal.pone.0172213

Medina, K. L., McQueeny, T., Nagel, B. J., Hanson, K. L., Schweinsburg, A. D., and Tapert, S. F. (2008). Prefrontal cortex volumes in adolescents with alcohol use disorders: unique gender effects. *Alcohol. Clin. Exp. Res.* 32, 386–394. doi: 10.1111/j.1530-0277.2007.00602.x

Morris, S. A., Eaves, D. W., Smith, A. R., and Nixon, K. (2010). Alcohol inhibition of neurogenesis: a mechanism of hippocampal neurodegeneration in an adolescent alcohol abuse model. *Hippocampus* 20, 596–607. doi: 10.1002/hipo.20665

Myrick, H., Anton, R. F., Li, X., Henderson, S., Drobes, D., Voronin, K., et al. (2004). Differential brain activity in alcoholics and social drinkers to alcohol cues: relationship to craving. *Neuropsychopharmacology* 29, 393–402. doi: 10.1038/sj.npp.1300295

O'Connor, R. M., and Kenny, P. J. (2015). Neuroscience: binge drinking and brain stress systems. *Nature* 520, 168–169. doi: 10.1038/520168a

Rice, J. P., Reich, T., Bucholz, K. K., Neuman, R. J., Fishman, R., Rochberg, N., et al. (1995). Comparison of direct interview and family history diagnoses of alcohol dependence. *Alcohol. Clin. Exp. Res.* 19, 1018–1023. doi: 10.1111/j.1530-0277.1995.tb00983.x

SAMHSA (2016). *Key Substance Use and Mental Health Indicators in the United States: Results from the 2015 National Survey on Drug Use and Health (No. HHS*

Publication No. SMA 16-4984). Rockville, MD: Center for Behavioral Health Statistics and Quality.

Schoenbaum, G., and Shaham, Y. (2008). The role of orbitofrontal cortex in drug addiction: a review of preclinical studies. *Biol. Psychiatry* 63, 256–262. doi: 10.1016/j.biopsych.2007.06.003

Segobin, S. H., Chetelat, G., Le Berre, A. P., Lannuzel, C., Boudehent, C., Vabret, F., et al. (2014). Relationship between brain volumetric changes and interim drinking at six months in alcohol-dependent patients. *Alcohol. Clin. Exp. Res.* 38, 739–748. doi: 10.1111/acer.12300

Sheehan, D. V., Lecrubier, Y., Sheehan, K. H., Amorim, P., Janavs, J., Weiller, E., et al. (1998). The Mini-International Neuropsychiatric Interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. *J. Clin. Psychiatry* 59(Suppl. 20), 22–33.

Silveri, M. M., Dager, A. D., Cohen-Gilbert, J. E., and Sneider, J. T. (2016). Neurobiological signatures associated with alcohol and drug use in the human adolescent brain. *Neurosci. Biobehav. Rev.* 70, 244–259. doi: 10.1016/j.neubiorev.2016.06.042

Sinha, R. (2011). New findings on biological factors predicting addiction relapse vulnerability. *Curr. Psychiatry Rep.* 13, 398–405. doi: 10.1007/s11920-011-0224-0

Sjoerds, Z., Van Tol, M. J., Van den Brink, W., Van der Wee, N. J., Van Buchem, M. A., Aleman, A., et al. (2013). Family history of alcohol dependence and gray matter abnormalities in non-alcoholic adults. *World J. Biol. Psychiatry* 14, 565–573. doi: 10.3109/15622975.2011.640942

Spielberger, C. D., Gorssuch, R. L., Lushene, P. R., Vagg, P. R., and Jacobs, G. A. (1983). *Manual for the State-Trait Anxiety Inventory*. Palo Alto, CA: Consulting Psychologists Press, Inc.

Squeglia, L. M., Rinker, D. A., Bartsch, H., Castro, N., Chung, Y., Dale, A. M., et al. (2014). Brain volume reductions in adolescent heavy drinkers. *Dev. Cogn. Neurosci.* 9, 117–125. doi: 10.1016/j.dcn.2014.02.005

Squeglia, L. M., Tapert, S. F., Sullivan, E. V., Jacobus, J., Meloy, M. J., Rohlfing, T., et al. (2015). Brain development in heavy-drinking adolescents. *Am. J. Psychiatry* 172, 531–542. doi: 10.1176/appi.ajp.2015.14101249

Suzuki, Y., Oishi, M., Ogawa, K., and Mizutani, T. (2010). Atrophy of the parahippocampal gyrus and regional cerebral blood flow in the limbic system in chronic alcoholic patients. *Alcohol* 44, 439–445. doi: 10.1016/j.alcohol.2010.05.003

Tapert, S. F., Granholm, E., Leedy, N. G., and Brown, S. A. (2002). Substance use and withdrawal: neuropsychological functioning over 8 years in youth. *J. Int. Neuropsychol. Soc.* 8, 873–883. doi: 10.1017/S1355617702870011

Whelan, R., Watts, R., Orr, C. A., Althoff, R. R., Artiges, E., Banaschewski, T., et al. (2014). Neuropsychosocial profiles of current and future adolescent alcohol misusers. *Nature* 512, 185–189. doi: 10.1038/nature13402

Worhunsky, P. D., Dager, A. D., Meda, S. A., Khadka, S., Stevens, M. C., Austad, C. S., et al. (2016). A preliminary prospective study of an escalation in 'maximum daily drinks', fronto-parietal circuitry and impulsivity-related domains in young adult drinkers. *Neuropsychopharmacology* 41, 1637–1647. doi: 10.1038/npp.2015.332

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: 19 May 2017
doi: 10.3389/fnins.2017.00285

Chronic Ketamine Exposure Causes White Matter Microstructural Abnormalities in Adolescent Cynomolgus Monkeys

Qi Li[1,2,3†], Lin Shi[4,5†], Gang Lu[6], Hong-Luan Yu[7], Fu-Ki Yeung[8], Nai-Kei Wong[9], Lin Sun[10], Kai Liu[8], David Yew[11], Fang Pan[12], De-Feng Wang[8] and Pak C. Sham[1,2,13*]*

[1] Department of Psychiatry, The University of Hong Kong, Hong Kong, Hong Kong, [2] State Key Laboratory for Cognitive and Brain Sciences, The University of Hong Kong, Hong Kong, Hong Kong, [3] The University of Hong Kong Shenzhen Institute of Research and Innovation (HKU-SIRI), The University of Hong Kong, Hong Kong, Hong Kong, [4] Department of Medicine and Therapeutics, Chinese University of Hong Kong, Hong Kong, Hong Kong, [5] Chow Yuk Ho Center of Innovative Technology for Medicine, Chinese University of Hong Kong, Hong Kong, Hong Kong, [6] School of Biomedical Sciences, Chinese University of Hong Kong, Hong Kong, Hong Kong, [7] Department of Psychology, Qilu Hospital of Shandong University, Jinan, China, [8] Research Center for Medical Image Computing, Department of Imaging and Interventional Radiology, Chinese University of Hong Kong, Hong Kong, Hong Kong, [9] Chemical Biology Laboratory for Infectious Diseases, Shenzhen Institute of Hepatology, The Third People's Hospital of Shenzhen, Shenzhen, China, [10] Department of Psychology, Weifang Medical University, Weifang, China, [11] School of Chinese Medicine, Chinese University of Hong Kong, Hong Kong, Hong Kong, [12] Department of Medical Psychology, Shandong University School of Medicine, Jinan, China, [13] Genome Research Centre, The University of Hong Kong, Hong Kong, Hong Kong

OPEN ACCESS

Edited by:
Jacqueline M. Barker, Medical University of South Carolina, United States

Reviewed by:
Philip R. Corlett, Yale University, United States
Marco Grados, Johns Hopkins School of Medicine, United States

***Correspondence:**
De-Feng Wang
dfwang@cuhk.edu.hk
Pak C. Sham
pcsham@hku.hk

[†] These authors have contributed equally to this work.

Specialty section:
This article was submitted to Child and Adolescent Psychiatry, a section of the journal Frontiers in Neuroscience

Received: *10 March 2017*
Accepted: *02 May 2017*
Published: *19 May 2017*

Citation:
Li Q, Shi L, Lu G, Yu H-L, Yeung F-K, Wong N-K, Sun L, Liu K, Yew D, Pan F, Wang D-F and Sham PC (2017) Chronic Ketamine Exposure Causes White Matter Microstructural Abnormalities in Adolescent Cynomolgus Monkeys. Front. Neurosci. 11:285. doi: 10.3389/fnins.2017.00285

Acute and repeated exposures to ketamine mimic aspects of positive, negative, and cognitive symptoms of schizophrenia in humans. Recent studies by our group and others have shown that chronicity of ketamine use may be a key element for establishing a more valid model of cognitive symptoms of schizophrenia. However, current understanding on the long-term consequences of ketamine exposure on brain circuits has remained incomplete, particularly with regard to microstructural changes of white matter tracts that underpin the neuropathology of schizophrenia. Thus, the present study aimed to expand on previous investigations by examining causal effects of repeated ketamine exposure on white matter integrity in a non-human primate model. Ketamine or saline (control) was administered intravenously for 3 months to male adolescent cynomolgus monkeys ($n = 5$/group). Diffusion tensor imaging (DTI) experiments were performed and tract-based spatial statistics (TBSS) was used for data analysis. Fractional anisotropy (FA) was quantified across the whole brain. Profoundly reduced FA on the right side of sagittal striatum, posterior thalamic radiation (PTR), retrolenticular limb of the internal capsule (RLIC) and superior longitudinal fasciculus (SLF), and on the left side of PTR, middle temporal gyrus and inferior frontal gyrus were observed in the ketamine group compared to controls. Diminished white matter integrity found in either fronto-thalamo-temporal or striato-thalamic connections with tracts including the SLF, PTR, and RLIC lends support to similar findings from DTI studies on schizophrenia in humans. This study suggests that chronic ketamine exposure is a useful pharmacological paradigm that might provide translational insights into the pathophysiology and treatment of schizophrenia.

Keywords: ketamine, diffusion tensor imaging (DTI), thalamus, frontal gyrus, cynomologus monkeys

INTRODUCTION

Acute and repeated exposures to ketamine, an *N*-methyl-*D*-aspartate (NMDA) receptor antagonist, can be used to model aspects of positive, negative, and cognitive symptoms of schizophrenia in humans (Krystal et al., 1994; Newcomer et al., 1999; Dawson et al., 2013; Stone et al., 2014). A growing body of neuroimaging studies have demonstrated that acute administration of ketamine induces symptoms in healthy individuals comparable to an acute psychotic state, which include delusions (Abel et al., 2003a; Corlett et al., 2013), dissociative sensation (Deakin et al., 2008; De Simoni et al., 2013) and emotion blunting (Abel et al., 2003b; Daumann et al., 2008). However, a number of studies have indicated that acute ketamine administration does not significantly impair cognitive functions such as working memory, verbal fluency, and attention (Honey et al., 2004, 2005; Fu et al., 2005; Daumann et al., 2008). Furthermore, acute ketamine exposure has yielded inconsistent findings on resting-state functional connectivity particularly prefrontal cortex functional connectivity with subcortical brain regions (Scheidegger et al., 2012; Driesen et al., 2013), and these regions play an important role in executive functions (Bonelli and Cummings, 2007). Recently, it has been postulated that the most-reported short-term psychological effects of ketamine dependence were "floating or circling" sensation, while the long-term effects of ketamine dependence were memory impairment, personality changes, and slowed reactions (Curran and Morgan, 2000; Morgan et al., 2004; Fan et al., 2015). Thus, chronic ketamine use may provide a more valid model of cognitive symptoms of schizophrenia. Improved mechanistic characterization of the effects of long-term ketamine exposure on brain circuits is likewise crucial to a better understanding of the etiology of cognitive symptoms of schizophrenia.

Moreover, compelling phenotypic similarity between chronic ketamine use and schizophrenia exists in functional neural activity or white matter alterations, particularly, in light of disconnections involving the prefrontal cortex. In a recent functional magnetic resonance imaging (*f*MRI) study, it was reported that effects of chronic ketamine use on resting-state functional connectivity are coupled to increased activity in the frontal gyrus and decreased activity in the anterior cingulate cortex (ACC; Liao et al., 2012). Our previous study has also demonstrated that repeated exposure to ketamine in non-human primates reduced functional neural activity in the midbrain, posterior cingulate cortex (PCC), and visual cortex, but increased activity in the striatum (Yu et al., 2012). Diffusion tensor imaging (DTI) is an MRI-based neuroimaging technique that provides information about white matter microstructure *in vivo*. Damages in frontal white matter and corpus callosum have been reported in human cocaine addicts (Moeller et al., 2007; Wang et al., 2013). UP until now, only two clinical DTI findings have reported that chronic ketamine exposure disrupts white matter integrity in the frontal cortex (Liao et al., 2010; Edward Roberts et al., 2014). Liao et al. also reported reduced bilateral frontal gray matter volume in chronic ketamine users (Liao et al., 2011). However, exposure to other psychoactive substances of patients with ketamine dependence may confound interpretation of these results. In view of a paucity of knowledge about the long-term consequences of ketamine administration in brain structures, along with ethical concerns and technical difficulties in addressing this issue in humans, investigation on chronic ketamine exposure in preclinical animal models is clearly warranted.

Adolescents are socially vulnerable to drugs of abuse and display susceptibility to the development of drug dependence (Anthony and Petronis, 1995; Chambers et al., 2003). Despite the high prevalence of ketamine use among adolescents and young adults in Hong Kong and internationally (Lankenau and Clatts, 2004; Tang et al., 2015), disproportionately little is known about its impact on the developing brain. Again, it is particularly challenging to evaluate the drug effects on this age group in clinic. Non-human primates represent an excellent animal model because they share high similarity with humans in the pharmacokinetics and metabolism of several drugs and more importantly their prefrontal cortex is evolutionarily closely aligned with human counterparts (Preuss, 1995; Innocenti et al., 2016). Given the well-established role of prefrontal cortex in cognitive symptoms of psychosis, the aim of the present study was to expand on previous DTI investigations by examining potential causal effects of repeated ketamine exposure on white matter integrity and connectivity profiles between prefrontal cortex and subcortical brain regions in an adolescent non-human primate model. We predicted that chronic ketamine exposure perturbs the integrity of prefrontal cortex and its connectivity with subcortical regions.

MATERIALS AND METHODS

Animals and Ketamine Administration

Male adolescent cynomolgus monkeys (*Macaca fascicularis*) were purchased from Yunnan Laboratory Primate Inc. and all experiments were conducted in full compliance with license from the Ethics Committee of Shandong University. The animals were bred from a colony of natural-habitat-reared *M. fascicularis* and were kept at Hongli Animal Center (approval ID for use of non-human primates in this study: SYXK, 20050041) in a room maintained at temperature of 25°C with a 12:12 h light-dark cycle. Monkeys were individually housed in stainless steel cages (90 × 90 × 70 cm, SCXK Su 2003-0006) with water available *ad libitum* and were allowed to have visual and auditory contact with each other. They were fed twice daily with whole grains diet (Military Medical Animal Center of the Chinese Liberation Army, SCXK), supplemented with fresh fruits each day. Rearing procedures had been reviewed and approved by the Animal Care Committee of Shandong University.

Ten monkeys were randomly divided into two groups: Ketamine (1 mg/kg) or saline (control) was administered intravenously for 3 months to male adolescent *M. fascicularis* at 4.12 ± 0.65 (mean ± standard deviation) years old (ketamine group = 5, control group = 5). Based on studies by our group and others (Stoet and Snyder, 2006; Yu et al., 2012; Sun et al., 2014), ketamine dose of 1 mg/kg in an injection volume of 1 mL in saline was chosen and freshly prepared on the day of injection. Ketamine was given daily intravenously via arm vein under mild physical constraints for 13 weeks (i.e., ∼3 months), while

control monkeys were given sterile saline 1 mL. Ketamine was not given to the monkeys 24 h before the day of DTI scanning. No other pharmacological agents were administered to monkeys throughout the whole experiments. Animal body weights were recorded monthly to monitor the monkeys' well-being.

Diffusion Tensor Imaging (DTI)

DTI experiments were performed on a 3-T Signa Magnetic Resonance System (GE Company). Briefly, monkeys were anesthetized by intramuscular injection with ketamine (10 mg/kg) and xylazine hydrochloride (Sumianxin, 0.1 mL/kg; China Institute of Military Veterinary, Academy of Military Medical Sciences, Changchun, China; Feng et al., 2013). Anesthetized animals were immediately transferred to the MRI room. Anesthesia was sustained throughout the whole scanning period with no extra anesthetic doses being required. The monkeys were kept in place with sponges and sand bags and were oriented in a head-forward sphinx position. Respiration rate and body temperature were continuously monitored during the scan. DTI scanning was performed by using an EP/S sequence with the following parameters: repetition time = 6 s; echo time = 89.8 ms; field of view = 14 cm; flip angle = 90°; matrix = 256 × 256; slice thickness = 2.6 mm. Diffusion weighted imaging (b = 1,000 s/mm^2) was performed in 25 non-collinear directions with 1 non-diffusion weighted image.

Data Analysis

Fractional anisotropy (FA) was derived from diffusion data which quantifies how strongly directional a local tract structure is. In order to localize brain changes, group comparison of FA-value was performed between control group and ketamine group ($p < 0.005$, uncorrected). Tract-based spatial statistics (TBSS; Smith et al., 2006) part of FSL (Smith et al., 2004) was used in data processing. First, the raw diffusion data were preprocessed and corrected to eliminate the effects of head movement and eddy currents by using FMRIB Diffusion Toolbox (FDT). Then, a binary brain mask was created by brain-extracting the no diffusion weighting (b = 0) image by using Brain Extraction Tool (BET; Smith, 2002) and mean FA images were created by fitting a tensor model to the corrected diffusion data with its brain mask. Before group comparison, all FA images were aligned onto the MNI152 standard space by non-linear registration and resliced into 1 × 1 × 1 mm^3 resolution by TBSS software for later accurate cluster quantification. A mean FA skeleton was created such that it most represents the centers of all tracts common to the group. The voxel-wise group statistical comparison was performed to show which voxels on the mean FA skeleton mask had significant difference between control group and ketamine group. The analysis statistics was based on TBSS with threshold-free cluster enhancement (TFCE) algorithm. TFCE method was adopted with a non-parametric approach of permutation test of 500 permutations (Smith and Nichols, 2009). Results were generated based on significant threshold at uncorrected one-tailed TFCE $p < 0.005$.The quantification of voxel size within a cluster was then performed by using Xjview toolbox (http://www.alivelearn.net/xjview). To locate the actual anatomical regions of those significant clusters in monkey, a diffusion tensor based white matter brain atlas for rhesus macaques was applied (Adluru et al., 2012) onto the results as anatomical underlay.

RESULTS

Regions of Significant Difference in Fractional Anisotropy (FA) between Groups

Group comparison between chronic ketamine administration group and controls revealed seven different anatomical regions, where the ketamine group had FA-values significantly lower than that of the control group. The anatomic location and size of these clusters with peak *t*-values for ketamine group and controls are as shown in **Table 1**. In this table, statistical analysis found significant FA changes ($p < 0.005$) between ketamine group and controls in the right side of sagittal striatum (SS; $t = 4.3829$), posterior thalamic radiation (PTR, right side, $t = 3.9214$; left side, $t = 7.1843$), middle temporal gyrus white matter (MTG-WM, left side, $t = 4.3206$), inferior frontal gyrus WM (IFG-WM, left side, $t = 3.8225$), retrolenticular limb of the internal capsule (RLIC, right side, $t = 2.5386$), and superior longitudinal fasciculus (SLF, right side, $t = 4.9845$).

Figure 1 showed spatial distribution of the brain regions indicating a reduction of FA ($p < 0.005$, TFCE-uncorrected) in right SS in the ketamine administration group when compared with controls. In addition, the significant regions were located in the posterior region of the thalamic radiation. Both right and left PTR showed reduced FA ($p < 0.005$) in the ketamine administration group (**Figures 2**, **3**). The RLIC comes from the thalamus and more posteriorly this becomes the optical radiation (Larry et al., 2012). **Figure 6** indicated a reduction of FA ($p < 0.005$) in the right RLIC in the ketamine group compared with controls. SLF, the largest association bundles, connects to the cortex of the frontal, parietal, occipital and temporal lobes (Larry et al., 2012). We found reduced FA in the right SLF in ketamine administration group ($p < 0.005$; **Figure 7**). Consistent with a ground-breaking finding reported by Liao et al. (2010), the present study showed decreased FA ($p < 0.005$) in left side of IFG (**Figure 5**) and MTG (**Figure 4**) in chronic ketamine administration group compared with controls. Moreover, our previous functional image study on the same model showed hypofunctions in the PCC and visual cortex (Yu et al., 2012).

There were no cluster areas where the ketamine group had FA-values higher than that of controls.

DISCUSSION

The present study provides direct experimental evidence that chronic ketamine exposure significantly disrupts white matter microstructures across a number of critical brain circuits. We found that repeated ketamine exposure elicits bidirectional reduced FA of PTR, left side of middle temporal gyrus WM (MTG-WM) and inferior frontal gyrus WM (IFG-WM), and also reduced FA in the right side of sagittal striatum (SS), RLIC, and SLF. More importantly, reduced integrity of fronto-thalamo-temporal or striato-thalamic white matter connectivity observed in this study is consistent with, and extend the previous clinical findings in chronic ketamine users.

TABLE 1 | Regions with significant FA differences between chronic ketamine exposure group and controls.

Region of significant clusters	Hemisphere	Cluster size (voxels)	Peak coordinates [*x*, *y*, *z*]	Peak *t*-value
Sagittal striatum (SS)	Right	7	[52, 27, 33]	4.3829
Posterior thalamic radiation (PTR)	Right	27	[54, 23, 35]	3.9214
Posterior thalamic radiation (PTR)	Left	12	[20, 27, 35]	7.1843
Middle temporal gyrus WM (MTG-WM)	Left	4	[15, 37, 37]	4.3206
Inferior frontal gyrus WM (IFG-WM)	Left	3	[23, 61, 43]	3.8225
Retrolenticular limb of the internal capsule (RLIC)	Right	4	[52, 38, 38]	2.5386
Superior longitudinal fasciculus (SLF)	Right	21	[54, 27, 42]	4.9845

x, y, z are co-ordinates based on the reference DTI monkey brain template FA map (Adluru et al., 2012) with its atlas (http://www.nitrc.org/projects/rmdtitemplate/). z indicates the vertical distance from the lowest axial plane of reference image in mm, x refers to sagittal plane, y refers to coronal plane. The uncorrected p-value of all the voxels in the cluster are $p < 0.005$. WM, white matter.

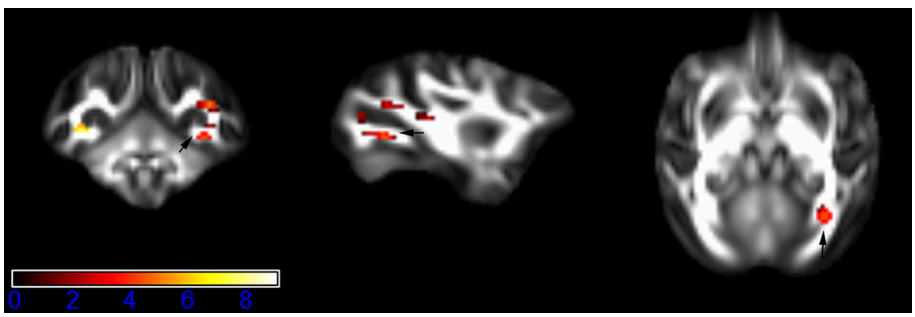


FIGURE 1 | Areas in sagittal striatum (SS, right side) with reduced fractional anisotropy values in the chronic ketamine exposure group compared to controls at $p < 0.005$ (in red; uncorrected). The anatomical underlay is DTI-based monkey brain image template (UWRMAC-DTI271) FA map with white matter atlas. Figure from left to right side is coronal view, sagittal view, and axial view of the images, respectively. The color bar represents the *t*-value.

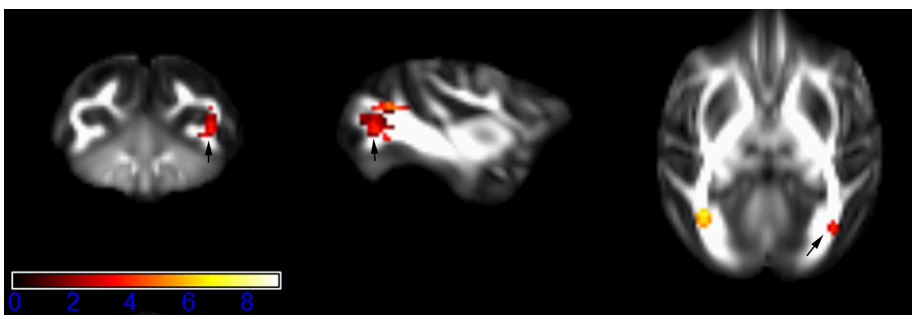


FIGURE 2 | Areas in posterior thalamic radiation (PTR, right side) with reduced fractional anisotropy values in the chronic ketamine exposure group compared to controls at $p < 0.005$ (in red; uncorrected). The anatomical underlay is DTI-based monkey brain image template (UWRMAC-DTI271) FA map with white matter atlas. Figure from left to right side is coronal view, sagittal view, and axial view of the images, respectively. The color bar represents the *t*-value.

Our current DTI findings of abnormality in the IFG-WM and MTG-WM with chronic ketamine exposure in non-human primate are largely consistent with white matter tract patterns observed in previous clinical imaging studies of schizophrenia in humans (Thompson et al., 2001; Kyriakopoulos et al., 2008; Ellison-Wright and Bullmore, 2009). In a comprehensive meta-analysis of DTI studies on schizophrenia, Ellison-Wright et al. (Ellison-Wright and Bullmore, 2009) summarized two locations of FA reductions in the deep white matter of the left frontal and temporal lobes. Disruption of fronto-temporal white matter network may contribute to cognitive deficits in schizophrenia (Kubicki et al., 2007). Even though abnormalities in the white matter have not been found consistently in schizophrenia patients in methodologically varied studies, the frontal white matter seems to be commonly affected. Recently, Zalesky et al. (2011) showed that a fronto-parietal/occipital network may represent the key macro-circuit affected in schizophrenia, while an aberrant network structure of bilateral inferior frontal cortex and temporal has been reported in schizophrenia patients (van den Heuvel et al., 2010). These findings suggest that

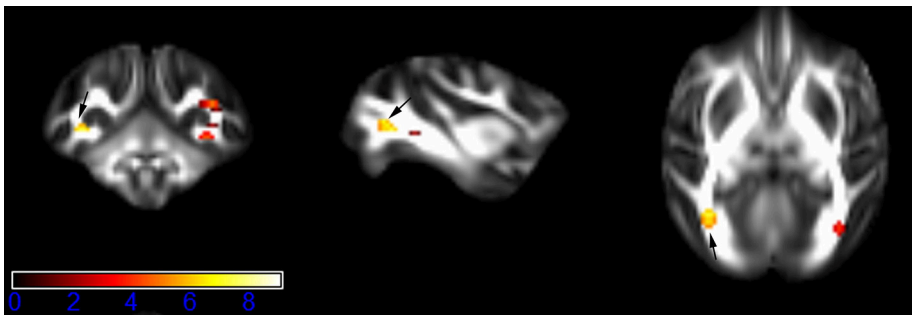


FIGURE 3 | Areas in posterior thalamic radiation (PTR, left side) with reduced fractional anisotropy values in the chronic ketamine exposure group compared to controls at $p < 0.005$ (in red; uncorrected). The anatomical underlay is DTI-based monkey brain image template (UWRMAC-DTI271) FA map with white matter atlas. Figure from left to right side is coronal view, sagittal view, and axial view of the images, respectively. The color bar represents the *t*-value.

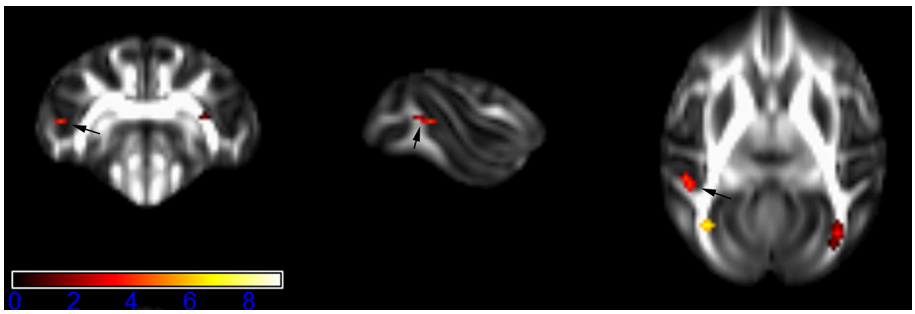


FIGURE 4 | Areas in middle temporal gyrus WM (MTG-WM, left side) with reduced fractional anisotropy values in the chronic ketamine exposure group compared to controls at $p < 0.005$ (in red; uncorrected). The anatomical underlay is DTI-based monkey brain image template (UWRMAC-DTI271) FA map with white matter atlas. Figure from left to right side is coronal view, sagittal view, and axial view of the images, respectively. The color bar represents the *t*-value.

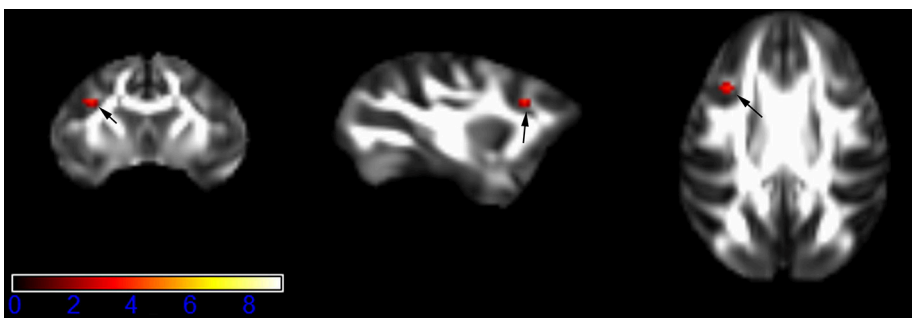


FIGURE 5 | Areas in inferior frontal gyrus WM (IFG-WM, left side) with reduced fractional anisotropy values in the chronic ketamine exposure group compared to controls at $p < 0.005$ (in red; uncorrected). The anatomical underlay is DTI-based monkey brain image template (UWRMAC-DTI271) FA map with white matter atlas. Figure from left to right side is coronal view, sagittal view, and axial view of the images, respectively. The color bar represents the *t*-value.

schizophrenia patients have a less organized brain networks with a reduced central role for the key frontal hub, which results in limited integration of information between brain regions.

Consistent with the view of "hypofrontality" in schizophrenia (Wolkin et al., 1992), a growing number of brain imaging studies have shown that the frontal white matter abnormalities may be as a fundamental change in patients with chronic drug dependence (Nestler, 2005; Wang et al., 2013). Animal studies by our group and others have shown that apoptosis of neuronal cells in the frontal cortex is induced by chronic exposure to ketamine (Zou et al., 2009; Yeung et al., 2010; Sun et al., 2014). To date, however, there have been only two clinical studies examining white matter integrity in chronic ketamine users (Liao et al., 2010; Edward Roberts et al., 2014). Liao et al. (2010) first reported that white matter changes with reduced FA in the bilateral frontal and left temporo-parietal cortices are

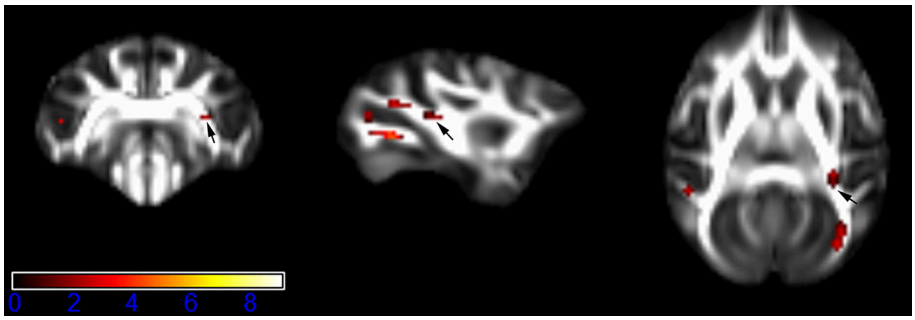


FIGURE 6 | Areas in retrolenticular limb of the internal capsule (RLIC, right side) with reduced fractional anisotropy values in the chronic ketamine exposure group compared to controls at $p < 0.005$ (in red; uncorrected). The anatomical underlay is DTI-based monkey brain image template (UWRMAC-DTI271) FA map with white matter atlas. Figure from left to right side is coronal view, sagittal view, and axial view of the images, respectively. The color bar represents the *t*-value.

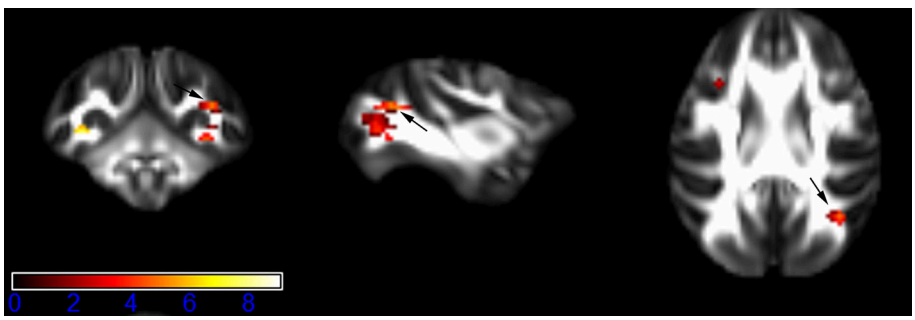


FIGURE 7 | Areas in superior longitudinal fasciculus (SLF, right side) with reduced fractional anisotropy values in the chronic ketamine exposure group compared to controls at $p < 0.005$ (in red; uncorrected). The anatomical underlay is DTI-based monkey brain image template (UWRMAC-DTI271) FA map with white matter atlas. Figure from left to right side is coronal view, sagittal view, and axial view of the images, respectively. The color bar represents the *t*-value.

associated with chronic ketamine use. Later, Edward Roberts et al. (2014) partially replicated the findings reported by Liao et al., which revealed a reduction in axial diffusivity in the right side of prefrontal white matter in chronic ketamine users. They further observed that the connectivity between caudate nucleus and lateral prefrontal cortex pathway was positively associated with dissociative experiences in ketamine users (Edward Roberts et al., 2014). Our findings are also in partial agreement with those studies, as the brain areas of abnormality observed in this study were also located in the left side of frontal lobe (IFG-WM) and temporal lobe (MTG-WM). The absence of an effect of ketamine on right side of frontal lobe in this study may be because a shorter duration of ketamine exposure or the use of only male animals in our experiment. A second consideration is that all the subjects in our study started to receive ketamine during adolescence, which is a stage of development known to involve brain plasticity (Blakemore and Choudhury, 2006). In addition, frontal lobe deficits may well be associated with the ketamine-related memory defects and cognitive symptoms in schizophrenia patients. Our research group have demonstrated that chronic exposure to ketamine impaired working memory in mice, which was correlated with dysfunction of GABA signaling system in the prefrontal cortex (Tan et al., 2011). However, further investigation including studies on working memory symptoms combined with neuroimaging is needed to better understand the neuropathological roles of the frontal lobe in chronic ketamine users.

Both IFG-WM and MTG-WM lie laterally to the SLF (Adluru et al., 2012), the major white matter connection between the prefrontal and parietal/temporal cortices, which are functionally related to verbal working memory performance (Hazlett et al., 2008; Karlsgodt et al., 2008). Edward Roberts et al. (2014) reported reduced axial diffusivity in the right side of SLF in ketamine users. In addition, recent evidence has demonstrated that activities in the right IFG network connected by SLF, especially in the right hemisphere, play prominent roles in corporeal awareness during illusion (Amemiya and Naito, 2016). Patients with first-episode paranoid schizophrenia exhibited reduced FA in the right SLF and right internal capsule (Guo et al., 2012). In line with these findings, decreased FA in the right SLF in our study may implicate fronto-parietal white matter disconnectivity in symptoms associated with chronic ketamine exposure in monkeys.

White matter FA in bilateral PTR, a region encompassing fiber pathways that connects the caudal parts of thalamus with the occipital and parietal lobes, was reduced in monkeys chronically

exposed to ketamine. This association tract is of interest because it plays a key role connecting visual and motor processes, and is an integral part of neural network regulating cognitive performance (Cremers et al., 2016). Bilateral FA reduction in the PTR has been reported in patients with schizophrenia (Peters et al., 2010; Melonakos et al., 2011; Melicher et al., 2015). However, Edward Roberts et al. (2014) found impairment only on the right side of PTR in chronic ketamine users. Our results encourage further investigation into the role of this projection in ketamine use.

The sagittal striatum, which includes a large part of both the inferior longitudinal fasciculus and inferior fronto-occipital fasciculus, projects to the frontal and limbic cortices (Adluru et al., 2012). Striatum and its cortical connections are critical in the pathogenesis of the complex cognitive symptoms of schizophrenia (Simpson et al., 2010). There is corroborative evidence from animal models and clinical investigations that sensorimotor gating function is modulated by cortico-striato-thalamic circuitry (Hazlett et al., 2008; Li et al., 2009, 2010). In the present study, compromised white matter tract integrity seen on right side of striato-thalamic connections, specifically tracts including RLIC and PTR, supports white matter alterations in the brain of chronic ketamine users as reported by Edward Roberts et al. (2014). These white matter alterations in the brain may also contribute to the dissociative experiences reported by chronic ketamine users (Edward Roberts et al., 2014). Moreover, reduced FA in RLIC was reported to be associated with negative symptoms in patients with schizophrenia (Arnedo et al., 2015).

CONCLUSION

The results of the present study confirm chronic ketamine use during adolescence causes brain damage in areas known to be involved in neurodevelopment during adolescence, in particular, fronto-thalamo-temporal white matter connection (Alcauter et al., 2014; Xiao et al., 2016). Based on clinical findings on chronic ketamine exposure, our study has systematically surveyed major brain regions posited to have reductions in white matter integrity. More importantly, the current observations of reduced white matter in eitherfronto-thalamo-temporal or striato-thalamic networks involving white matter tracts including the SLF, PTR, and RLIC echo findings in human DTI studies on schizophrenia. These results furnish a rational basis for studying chronic ketamine exposure of adolescent brain as a pharmacological paradigm and may yield translational insights into the pathophysiology and treatment of schizophrenia.

LIMITATIONS

Firstly, we acknowledge that the sample size of the current study was modest. Our findings are nonetheless promising, being the first direct evidence for establishing the casual effects of chronic ketamine exposure on microstructure integrity of white matter tracts in adolescent non-human primates. Secondly, we examined only male adolescent monkeys in this study. A decision to dedicate finite experimental resources to males was made because male subjects are generally more vulnerable to drug addiction (DePoy et al., 2016), and there is evidence that males experiencing neurodevelopmental perturbations associated with schizophrenia were inferior to females in memory performance (Goldstein et al., 1998).

AUTHOR CONTRIBUTIONS

QL, LS (2nd author), GL, DY, FP, DW, and PS contributed to design, acquisition, analysis, and interpretation of data for this work. QL wrote the draft for the manuscript. GL, HY, LS (7th author), and FP performed the experiments. LS (2nd author), FY, KL, QL, and DW prepared all figures and image analysis. NW, GL, HY, LS (2nd author), and PS revised and edited the manuscript. PS approved the final draft of the manuscript. All authors reviewed the manuscript for intellectual content and approved submission.

FUNDING

Research reported in this publication was supported by a National Natural Science Foundation of China (NSFC) grant (NO. 81300987).

REFERENCES

Abel, K. M., Allin, M. P., Kucharska-Pietura, K., Andrew, C., Williams, S., David, A. S., et al. (2003a). Ketamine and fMRI BOLD signal: distinguishing between effects mediated by change in blood flow versus change in cognitive state. *Hum. Brain Mapp.* 18, 135–145. doi: 10.1002/hbm.10064

Abel, K. M., Allin, M. P., Kucharska-Pietura, K., David, A., Andrew, C., Williams, S., et al. (2003b). Ketamine alters neural processing of facial emotion recognition in healthy men: an fMRI study. *Neuroreport* 14, 387–391. doi: 10.1097/00001756-200303030-00018

Adluru, N., Zhang, H., Fox, A. S., Shelton, S. E., Ennis, C. M., Bartosic, A. M., et al. (2012). A diffusion tensor brain template for rhesus macaques. *Neuroimage* 59, 306–318. doi: 10.1016/j.neuroimage.2011.07.029

Alcauter, S., Lin, W., Smith, J. K., Short, S. J., Goldman, B. D., Reznick, J. S., et al. (2014). Development of thalamocortical connectivity during infancy and its cognitive correlations. *J. Neurosci.* 34, 9067–9075. doi: 10.1523/JNEUROSCI.0796-14.2014

Amemiya, K., and Naito, E. (2016). Importance of human right inferior frontoparietal network connected by inferior branch of superior longitudinal fasciculus tract in corporeal awareness of kinesthetic illusory movement. *Cortex* 78, 15–30. doi: 10.1016/j.cortex.2016.01.017

Anthony, J. C., and Petronis, K. R. (1995). Early-onset drug use and risk of later drug problems. *Drug Alcohol Depend.* 40, 9–15. doi: 10.1016/0376-8716(95)01194-3

Arnedo, J., Mamah, D., Baranger, D. A., Harms, M. P., Barch, D. M., Svrakic, D. M., et al. (2015). Decomposition of brain diffusion imaging data uncovers latent schizophrenias with distinct patterns of white matter anisotropy. *Neuroimage* 120, 43–54. doi: 10.1016/j.neuroimage.2015.06.083

Blakemore, S. J., and Choudhury, S. (2006). Development of the adolescent brain: implications for executive function and social cognition. *J. Child Psychol. Psychiatry* 47, 296–312. doi: 10.1111/j.1469-7610.2006.01611.x

Bonelli, R. M., and Cummings, J. L. (2007). Frontal-subcortical circuitry and behavior. *Dialogues Clin. Neurosci.* 9, 141–151.

Chambers, R. A., Taylor, J. R., and Potenza, M. N. (2003). Developmental neurocircuitry of motivation in adolescence: a critical period of addiction vulnerability. *Am. J. Psychiatry* 160, 1041–1052. doi: 10.1176/appi.ajp.160.6.1041

Corlett, P. R., Cambridge, V., Gardner, J. M., Piggot, J. S., Turner, D. C., Everitt, J. C., et al. (2013). Ketamine effects on memory reconsolidation favor a learning model of delusions. *PLoS ONE* 8:e65088. doi: 10.1371/journal.pone.0065088

Cremers, L. G. M., de Groot, M., Hofman, A., Krestin, G. P., van der Lugt, A., Niessen, W. J., et al. (2016). Altered tract-specific white matter microstructure is related to poorer cognitive performance: the Rotterdam Study. *Neurobiol. Aging* 39, 108–117. doi: 10.1016/j.neurobiolaging.2015.11.021

Curran, H. V., and Morgan, C. (2000). Cognitive, dissociative and psychotogenic effects of ketamine in recreational users on the night of drug use and 3 days later. *Addiction* 95, 575–590. doi: 10.1046/j.1360-0443.2000.9545759.x

Daumann, J., Heekeren, K., Neukirch, A., Thiel, C. M., Moller-Hartmann, W., and Gouzoulis-Mayfrank, E. (2008). Pharmacological modulation of the neural basis underlying inhibition of return (IOR) in the human 5-HT2A agonist and NMDA antagonist model of psychosis. *Psychopharmacology* 200, 573–583. doi: 10.1007/s00213-008-1237-1

Dawson, N., Morris, B. J., and Pratt, J. A. (2013). Subanaesthetic ketamine treatment alters prefrontal cortex connectivity with thalamus and ascending subcortical systems. *Schizophr. Bull.* 39, 366–377. doi: 10.1093/schbul/sbr144

De Simoni, S., Schwarz, A. J., O'Daly, O. G., Marquand, A. F., Brittain, C., Gonzales, C., et al. (2013). Test-retest reliability of the BOLD pharmacological MRI response to ketamine in healthy volunteers. *Neuroimage* 64, 75–90. doi: 10.1016/j.neuroimage.2012.09.037

Deakin, J. F., Lees, J., McKie, S., Hallak, J. E., Williams, S. R., and Dursun, S. M. (2008). Glutamate and the neural basis of the subjective effects of ketamine: a pharmaco-magnetic resonance imaging study. *Arch. Gen. Psychiatry* 65, 154–164. doi: 10.1001/archgenpsychiatry.2007.37

DePoy, L. M., Allen, A. G., and Gourley, S. L. (2016). Adolescent cocaine self-administration induces habit behavior in adulthood: sex differences and structural consequences. *Transl. Psychiatry* 6:e875. doi: 10.1038/tp.2016.150

Driesen, N. R., McCarthy, G., Bhagwagar, Z., Bloch, M., Calhoun, V., D'Souza, D. C., et al. (2013). Relationship of resting brain hyperconnectivity and schizophrenia-like symptoms produced by the NMDA receptor antagonist ketamine in humans. *Mol. Psychiatry* 18, 1199–1204. doi: 10.1038/mp.2012.194

Edward Roberts, R., Curran, H. V., Friston, K. J., and Morgan, C. J. (2014). Abnormalities in white matter microstructure associated with chronic ketamine use. *Neuropsychopharmacology* 39, 329–338. doi: 10.1038/npp.2013.195

Ellison-Wright, I., and Bullmore, E. (2009). Meta-analysis of diffusion tensor imaging studies in schizophrenia. *Schizophr. Res.* 108, 3–10. doi: 10.1016/j.schres.2008.11.021

Fan, N., Xu, K., Ning, Y., Wang, D., Ke, X., Ding, Y., et al. (2015). Development of a checklist of short-term and long-term psychological symptoms associated with ketamine use. *Shanghai Arch. Psychiatry* 27, 186–194. doi: 10.11919/j.issn.1002-0829.214158

Feng, Y., Chai, J., Chu, W., Ma, L., Zhang, P., and Duan, H. (2013). Combination of ketamine and xylazine exacerbates cardiac dysfunction in severely scalded rats during the shock stage. *Exp. Ther. Med.* 6, 641–648. doi: 10.3892/etm.2013.1213

Fu, C. H., Abel, K. M., Allin, M. P., Gasston, D., Costafreda, S. G., Suckling, J., et al. (2005). Effects of ketamine on prefrontal and striatal regions in an overt verbal fluency task: a functional magnetic resonance imaging study. *Psychopharmacology* 183, 92–102. doi: 10.1007/s00213-005-0154-9

Goldstein, J. M., Seidman, L. J., Goodman, J. M., Koren, D., Lee, H., Weintraub, S., et al. (1998). Are there sex differences in neuropsychological functions among patients with schizophrenia? *Am. J. Psychiatry* 155, 1358–1364. doi: 10.1176/ajp.155.10.1358

Guo, W., Liu, F., Liu, Z., Gao, K., Xiao, C., Chen, H., et al. (2012). Right lateralized white matter abnormalities in first-episode, drug-naive paranoid schizophrenia. *Neurosci. Lett.* 531, 5–9. doi: 10.1016/j.neulet.2012.09.033

Hazlett, E. A., Buchsbaum, M. S., Zhang, J., Newmark, R. E., Glanton, C. F., Zelmanova, Y., et al. (2008). Frontal-striatal-thalamic mediodorsal nucleus dysfunction in schizophrenia-spectrum patients during sensorimotor gating. *Neuroimage* 42, 1164–1177. doi: 10.1016/j.neuroimage.2008.05.039

Honey, G. D., Honey, R. A., O'Loughlin, C., Sharar, S. R., Kumaran, D., Suckling, J., et al. (2005). Ketamine disrupts frontal and hippocampal contribution to encoding and retrieval of episodic memory: an fMRI study. *Cereb. Cortex* 15, 749–759. doi: 10.1093/cercor/bhh176

Honey, R. A., Honey, G. D., O'Loughlin, C., Sharar, S. R., Kumaran, D., Bullmore, E. T., et al. (2004). Acute ketamine administration alters the brain responses to executive demands in a verbal working memory task: an FMRI study. *Neuropsychopharmacology* 29, 1203–1214. doi: 10.1038/sj.npp.1300438

Innocenti, G. M., Dyrby, T. B., Andersen, K. W., Rouiller, E. M., and Caminiti, R. (2016). The crossed projection to the striatum in two species of monkey and in humans: behavioral and evolutionary significance. *Cereb. Cortex.* doi: 10.1093/cercor/bhw161. [Epub ahead of print].

Karlsgodt, K. H., van Erp, T. G., Poldrack, R. A., Bearden, C. E., Nuechterlein, K. H., and Cannon, T. D. (2008). Diffusion tensor imaging of the superior longitudinal fasciculus and working memory in recent-onset schizophrenia. *Biol. Psychiatry* 63, 512–518. doi: 10.1016/j.biopsych.2007.06.017

Krystal, J. H., Karper, L. P., Seibyl, J. P., Freeman, G. K., Delaney, R., Bremner, J. D., et al. (1994). Subanesthetic effects of the noncompetitive NMDA antagonist, ketamine, in humans. Psychotomimetic, perceptual, cognitive, and neuroendocrine responses. *Arch. Gen. Psychiatry* 51, 199–214. doi: 10.1001/archpsyc.1994.03950030035004

Kubicki, M., McCarley, R., Westin, C. F., Park, H. J., Maier, S., Kikinis, R., et al. (2007). A review of diffusion tensor imaging studies in schizophrenia. *J. Psychiatr. Res.* 41, 15–30. doi: 10.1016/j.jpsychires.2005.05.005

Kyriakopoulos, M., Bargiotas, T., Barker, G. J., and Frangou, S. (2008). Diffusion tensor imaging in schizophrenia. *Eur. Psychiatry* 23, 255–273. doi: 10.1016/j.eurpsy.2007.12.004

Lankenau, S. E., and Clatts, M. C. (2004). Drug injection practices among high-risk youths: the first shot of ketamine. *J. Urban Health* 81, 232–248. doi: 10.1093/jurban/jth110

Larry, R. S., Berg, D., Bloom, F. E., Sascha, D. L., Anirvan, G., and Nicholas, C. S. (2012). *Fundamental Neuroscience, 4th Edn*. Waltham, MA: Elsevier.

Li, Q., Cheung, C., Wei, R., Cheung, V., Hui, E. S., You, Y., et al. (2010). Voxel-based analysis of postnatal white matter microstructure in mice exposed to immune challenge in early or late pregnancy. *Neuroimage* 52, 1–8. doi: 10.1016/j.neuroimage.2010.04.015

Li, Q., Cheung, C., Wei, R., Hui, E. S., Feldon, J., Meyer, U., et al. (2009). Prenatal immune challenge is an environmental risk factor for brain and behavior change relevant to schizophrenia: evidence from MRI in a mouse model. *PLoS ONE* 4:e6354. doi: 10.1371/journal.pone.0006354

Liao, Y., Tang, J., Corlett, P. R., Wang, X., Yang, M., Chen, H., et al. (2011). Reduced dorsal prefrontal gray matter after chronic ketamine use. *Biol. Psychiatry* 69, 42–48. doi: 10.1016/j.biopsych.2010.08.030

Liao, Y., Tang, J., Fornito, A., Liu, T., Chen, X., Chen, H., et al. (2012). Alterations in regional homogeneity of resting-state brain activity in ketamine addicts. *Neurosci. Lett.* 522, 36–40. doi: 10.1016/j.neulet.2012.06.009

Liao, Y., Tang, J., Ma, M., Wu, Z., Yang, M., Wang, X., et al. (2010). Frontal white matter abnormalities following chronic ketamine use: a diffusion tensor imaging study. *Brain* 133(Pt 7), 2115–2122. doi: 10.1093/brain/awq131

Melicher, T., Horacek, J., Hlinka, J., Spaniel, F., Tintera, J., Ibrahim, I., et al. (2015). White matter changes in first episode psychosis and their relation to the size of sample studied: a DTI study. *Schizophr. Res.* 162, 22–28. doi: 10.1016/j.schres.2015.01.029

Melonakos, E. D., Shenton, M. E., Rathi, Y., Terry, D. P., Bouix, S., and Kubicki, M. (2011). Voxel-based morphometry (VBM) studies in schizophrenia-can white matter changes be reliably detected with VBM? *Psychiatry Res.* 193, 65–70. doi: 10.1016/j.pscychresns.2011.01.009

Moeller, F. G., Hasan, K. M., Steinberg, J. L., Kramer, L. A., Valdes, I., Lai, L. Y., et al. (2007). Diffusion tensor imaging eigenvalues: preliminary evidence for altered myelin in cocaine dependence. *Psychiatry Res.* 154, 253–258. doi: 10.1016/j.pscychresns.2006.11.004

Morgan, C. J., Monaghan, L., and Curran, H. V. (2004). Beyond the K-hole: a 3-year longitudinal investigation of the cognitive and subjective effects of ketamine in recreational users who have substantially reduced their use of the drug. *Addiction* 99, 1450–1461. doi: 10.1111/j.1360-0443.2004.00879.x

Nestler, E. J. (2005). Is there a common molecular pathway for addiction? *Nat. Neurosci.* 8, 1445–1449. doi: 10.1038/nn1578

Newcomer, J. W., Farber, N. B., Jevtovic-Todorovic, V., Selke, G., Melson, A. K., Hershey, T., et al. (1999). Ketamine-induced NMDA receptor hypofunction as

a model of memory impairment and psychosis. *Neuropsychopharmacology* 20, 106–118. doi: 10.1016/S0893-133X(98)00067-0

Peters, B. D., Blaas, J., and de Haan, L. (2010). Diffusion tensor imaging in the early phase of schizophrenia: what have we learned? *J. Psychiatr. Res.* 44, 993–1004. doi: 10.1016/j.jpsychires.2010.05.003

Preuss, T. M. (1995). Do rats have prefrontal cortex? The rose-woolsey-akert program reconsidered. *J. Cogn. Neurosci.* 7, 1–24. doi: 10.1162/jocn.1995.7.1.1

Scheidegger, M., Walter, M., Lehmann, M., Metzger, C., Grimm, S., Boeker, H., et al. (2012). Ketamine decreases resting state functional network connectivity in healthy subjects: implications for antidepressant drug action. *PLoS ONE* 7:e44799. doi: 10.1371/journal.pone.0044799

Simpson, E. H., Kellendonk, C., and Kandel, E. (2010). A possible role for the striatum in the pathogenesis of the cognitive symptoms of schizophrenia. *Neuron* 65, 585–596. doi: 10.1016/j.neuron.2010.02.014

Smith, S. M. (2002). Fast robust automated brain extraction. *Hum. Brain Mapp.* 17, 143–155. doi: 10.1002/hbm.10062

Smith, S. M., and Nichols, T. E. (2009). Threshold-free cluster enhancement: addressing problems of smoothing, threshold dependence and localisation in cluster inference. *Neuroimage* 44, 83–98. doi: 10.1016/j.neuroimage.2008.03.061

Smith, S. M., Jenkinson, M., Johansen-Berg, H., Rueckert, D., Nichols, T. E., Mackay, C. E., et al. (2006). Tract-based spatial statistics: voxelwise analysis of multi-subject diffusion data. *Neuroimage* 31, 1487–1505. doi: 10.1016/j.neuroimage.2006.02.024

Smith, S. M., Jenkinson, M., Woolrich, M. W., Beckmann, C. F., Behrens, T. E., Johansen-Berg, H., et al. (2004). Advances in functional and structural MR image analysis and implementation as FSL. *Neuroimage* 23(Suppl. 1), S208–S219. doi: 10.1016/j.neuroimage.2004.07.051

Stoet, G., and Snyder, L. H. (2006). Effects of the NMDA antagonist ketamine on task-switching performance: evidence for specific impairments of executive control. *Neuropsychopharmacology* 31, 1675–1681. doi: 10.1038/sj.npp.1300930

Stone, J. M., Pepper, F., Fam, J., Furby, H., Hughes, E., Morgan, C., et al. (2014). Glutamate, N-acetyl aspartate and psychotic symptoms in chronic ketamine users. *Psychopharmacology* 231, 2107–2116. doi: 10.1007/s00213-013-3354-8

Sun, L., Li, Q., Li, Q., Zhang, Y., Liu, D., Jiang, H., et al. (2014). Chronic ketamine exposure induces permanent impairment of brain functions in adolescent cynomolgus monkeys. *Addict. Biol.* 19, 185–194. doi: 10.1111/adb.12004

Tan, S., Rudd, J. A., and Yew, D. T. (2011). Gene expression changes in GABA(A) receptors and cognition following chronic ketamine administration in mice. *PLoS ONE* 6:e21328. doi: 10.1371/journal.pone.0021328

Tang, M., Ching, C. K., Tse, M. L., Ng, C., Lee, C., Chong, Y. K., et al. (2015). Surveillance of emerging drugs of abuse in Hong Kong: validation of an analytical tool. *Hong Kong Med. J.* 21, 114–123. doi: 10.12809/hkmj144398

Thompson, P. M., Vidal, C., Giedd, J. N., Gochman, P., Blumenthal, J., Nicolson, R., et al. (2001). Mapping adolescent brain change reveals dynamic wave of accelerated gray matter loss in very early-onset schizophrenia. *Proc. Natl. Acad. Sci. U.S.A.* 98, 11650–11655. doi: 10.1073/pnas.201243998

van den Heuvel, M. P., Mandl, R. C., Stam, C. J., Kahn, R. S., and Hulshoff Pol, H. E. (2010). Aberrant frontal and temporal complex network structure in schizophrenia: a graph theoretical analysis. *J. Neurosci.* 30, 15915–15926. doi: 10.1523/JNEUROSCI.2874-10.2010

Wang, C., Zheng, D., Xu, J., Lam, W., and Yew, D. T. (2013). Brain damages in ketamine addicts as revealed by magnetic resonance imaging. *Front. Neuroanat.* 7:23. doi: 10.3389/fnana.2013.00023

Wolkin, A., Sanfilipo, M., Wolf, A. P., Angrist, B., Brodie, J. D., and Rotrosen, J. (1992). Negative symptoms and hypofrontality in chronic schizophrenia. *Arch. Gen. Psychiatry* 49, 959–965. doi: 10.1001/archpsyc.1992.01820120047007

Xiao, Y., Friederici, A. D., Margulies, D. S., and Brauer, J. (2016). Development of a selective left-hemispheric fronto-temporal network for processing syntactic complexity in language comprehension. *Neuropsychologia* 83, 274–282. doi: 10.1016/j.neuropsychologia.2015.09.003

Yeung, L. Y., Wai, M. S., Fan, M., Mak, Y. T., Lam, W. P., Li, Z., et al. (2010). Hyperphosphorylated tau in the brains of mice and monkeys with long-term administration of ketamine. *Toxicol. Lett.* 193, 189–193. doi: 10.1016/j.toxlet.2010.01.008

Yu, H., Li, Q., Wang, D., Shi, L., Lu, G., Sun, L., et al. (2012). Mapping the central effects of chronic ketamine administration in an adolescent primate model by functional magnetic resonance imaging (fMRI). *Neurotoxicology* 33, 70–77. doi: 10.1016/j.neuro.2011.11.001

Zalesky, A., Fornito, A., Seal, M. L., Cocchi, L., Westin, C. F., Bullmore, E. T., et al. (2011). Disrupted axonal fiber connectivity in schizophrenia. *Biol. Psychiatry* 69, 80–89. doi: 10.1016/j.biopsych.2010.08.022

Zou, X., Patterson, T. A., Sadovova, N., Twaddle, N. C., Doerge, D. R., Zhang, X., et al. (2009). Potential neurotoxicity of ketamine in the developing rat brain. *Toxicol. Sci.* 108, 149–158. doi: 10.1093/toxsci/kfn270

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: 14 August 2017
doi: 10.3389/fnbeh.2017.00151

Persistent Decreases in Adult Subventricular and Hippocampal Neurogenesis Following Adolescent Intermittent Ethanol Exposure

*Wen Liu and Fulton T. Crews **

Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States

Neurogenesis in hippocampal dentate gyrus (DG) and subventricular zone (SVZ) matures during adolescence to adult levels. Binge drinking is prevalent in adolescent humans, and could alter brain neurogenesis and maturation in a manner that persists into adulthood. To determine the impact of adolescent binge drinking on adult neurogenesis, Wistar rats received adolescent intermittent ethanol (AIE) exposure (5.0 g/kg/day, i.g., 2 days on/2 days off from postnatal day, P25–P54) and sacrificed on P57 or P95. Neural progenitor cell proliferation, differentiation, survival and maturation using immunohistochemistry was determined in the DG and SVZ. We found that AIE exposure decreased neurogenesis in both brain regions in adulthood (P95). In the DG at P57, AIE exposure resulted in a significant reduction of SOX2+, Tbr2+, Prox1+ and parvalbumin (PV)+IR expression, and at P95 decreased DCX+ and PV+IR expression. AIE exposure also reduced the expression of two cell proliferation markers (Ki67+ and BrdU+IR with 300 mg/kg, 2 h) at P95. The immune signaling molecule β-2 microglobulin+ and the cell death marker activated caspase-3+IR were significantly increased in the DG by AIE exposure. In the SVZ, AIE exposure decreased SOX2+, Mash1+, DCX+ and Dlx2+IR expression at P95, but not at P57. Thus, in adulthood both brain regions have reduced neurogenesis following AIE exposure. To assess progenitor cell survival and maturation, rats were treated with BrdU (150 mg/kg/day, 14 days) to label proliferating cells and were sacrificed weeks later on P95. In the hippocampus DG, AIE exposure increased survival BrdU+ cells which differentiated into Iba1+ microglia. In contrast, SVZ had decreased BrdU+ cells similar to decreased DCX+ neurogenesis. These data indicate that AIE exposure causes a lasting decrease in both adult hippocampal DG and forebrain SVZ neurogenesis with brain regional differences in the AIE response that persist into adulthood.

Keywords: adolescent intermittent ethanol, neurogenesis, hippocampal dentate gyrus, subventricular zone, rat

OPEN ACCESS

Edited by:
Mary M. Torregrossa, University of Pittsburgh, United States

Reviewed by:
S. Alex Marshall, High Point University, United States
Christian P. Müller, University of Erlangen-Nuremberg, Germany

***Correspondence:**
Fulton T. Crews
ftcrews@med.unc.edu

Received: *21 June 2017*
Accepted: *28 July 2017*
Published: *14 August 2017*

Citation:
Liu W and Crews FT (2017) Persistent Decreases in Adult Subventricular and Hippocampal Neurogenesis Following Adolescent Intermittent Ethanol Exposure. Front. Behav. Neurosci. 11:151. doi: 10.3389/fnbeh.2017.00151

Abbreviations: AIE, adolescent intermittent ethanol; ANOVA, analysis of variance; BEC, blood ethanol concentration; DCX, doublecortin; DG, dentate gyrus; HIP, hippocampus; i.g., intragastric; i.p., intraperitoneal; NPCs, neuroprogenitor cells; PBS, phosphate-buffered saline; PV, parvalbumin; Prox1, prospero homeobox protein 1; RMS, rostral migratory stream; Sox2, sex determining regions Y-box 2; SVZ, subventricular zone; Tbr2, T-box brain protein 2; TLR, Toll-like receptor.

INTRODUCTION

Adult neurogenesis involves the formation of new brain neurons that alter neurocircuits and contribute to neuroplasticity. Adult brain neurons are generated from neural stem cells and progenitor cells in two specific neurogenic niches of the brain: the hippocampal dentate gyrus (DG) subgranular zone and the lateral ventricle subventricular zone (SVZ; Eriksson et al., 1998; Spalding et al., 2013; Ernst et al., 2014). Neurons born in the hippocampal DG differentiate and integrate into existing networks as granule cells, and neurons born in the SVZ migrate to the olfactory bulbs where they become periglomerular neurons. Neurogenesis is a complex developmental process that includes neuroprogenitor cell (NPC) proliferation, migration, differentiation and survival. Emerging new findings have found that the progenitors and developmental milestones of neurogenesis in the DG and SVZ are different (Kempermann et al., 2004, 2015; Abrous et al., 2005; Ming and Song, 2005, 2011; Lledo et al., 2006; Song et al., 2016). In the DG, six stages identified by morphology and expression of markers have been defined (Kempermann et al., 2015); whereas in the SVZ progenitor stages of differentiation have been divided into five phases of adult neurogenesis (Ming and Song, 2011). In the DG, markers of NPCs that distinguish stages of neurogenesis, including sex determining regions Y-box 2 (SOX2) which is expressed in non-radial and horizontal type-1 cells, and Tbr2, a T-box transcription factor expressed by type-2a and -2b intermediate NPCs that persist to maturation into neuroblasts (type-3). Immature/mature neuron markers include doublecortin (DCX), a microtubule-associated protein expressed in migrating neuroblasts but lost after maturation to mature granule cells in DG (Brown et al., 2003; Rao and Shetty, 2004; Couillard-Despres et al., 2005), prospero homeobox protein 1 (Prox1), which is expressed in the neuroblast, immature and mature neurons (Lavado and Oliver, 2007; Lavado et al., 2010; Hodge and Hevner, 2011), and parvalbumin (PV), which labels mature γ-aminobutyric acid (GABAergic) interneurons of the nervous system. Adult SVZ neurogenesis involves different NPCs and development (Ming and Song, 2005, 2011), briefly, radial glia-like cells (type B) in the SVZ have SOX2, Mash1 is expressed in amplifying NPC (type C), and formation of neuroblasts. Dlx2 and DCX are markers of SVZ neuroblasts and immature neurons, and mature interneurons are expressed by NeuN. These NPC maturation and markers studies provide opportunities to determine sensitivity of various NPC progenitor stages in both DG and SVZ to environment, drugs and/or other factors.

New emerging evidence has suggested that altered neurogenesis and gliogenesis may contribute to drug taking and drug seeking (Mandyam and Koob, 2012). Further, studies suggest that drug-induced loss of neurogenesis contributes to addiction and recovery from a variety of drugs, including alcohol (Mandyam and Koob, 2012; Staples and Mandyam, 2016). Previous studies have found that chronic ethanol treatment of adult rats reduces NPC proliferation in the DG (Nixon and Crews, 2002; Herrera et al., 2003; He et al., 2005) and SVZ (Crews et al., 2004; Hansson et al., 2010; Campbell et al., 2014). A binge model of alcohol dependence found reduced adult hippocampal DG neurogenesis following ethanol treatment (Nixon and Crews, 2004; Hansson et al., 2010). Interestingly, abstinence after adult binge ethanol treatment results in bursts of NPC proliferation over the first week of abstinence that restore DG neurogenesis after about 30 days of abstinence (Nixon and Crews, 2004; Nixon et al., 2008). Further, stress and chronic ethanol self-administration by adult mice reduce hippocampal neurogenesis and induce depression-like behavior that are reversed by the antidepressant fluoxetine (Stevenson et al., 2009). Although hippocampal DG neurogenesis has been linked to drug dependence, SVZ neurogenesis has not been extensively studied. One study using a rat 7-week ethanol treatment model of chronic relapsing alcohol dependence that increases ethanol self-administration results in a loss of both DG and SVZ neurogenesis (Hansson et al., 2010). Interestingly, in this study DG neurogenesis recovered over weeks of abstinence similar to the binge studies noted above whereas SVZ neurogenesis was decreased after 21 days of abstinence following ethanol treatment (Hansson et al., 2010). Thus, neurogenesis in DG and SVZ differ in NPC markers, maturation and inhibition by ethanol.

Adolescence is a critical developmental stage during which brain networks that regulate cognition and emotion mature (Dahl, 2004; Crews et al., 2016). Binge drinking is also common in human adolescents (Wechsler et al., 1995; O'Malley et al., 1998). Human and animal studies suggest that alcohol exposure during adolescence alters brain development causing unique long lasting changes in adulthood (Spear, 2000). Previous studies have found hippocampal neurogenesis is greater in adolescents than adults (He and Crews, 2007) and adolescent rat hippocampal DG neurogenesis is more sensitive to ethanol inhibition than adult DG neurogenesis (Crews et al., 2006). More recent studies using adolescent intermittent ethanol (AIE) exposure have found reduced adult neurogenesis using ethanol vapor (Ehlers et al., 2013a), intragastric (i.g) ethanol exposure (Ehlers et al., 2013a; Broadwater et al., 2014; Vetreno and Crews, 2015), intraperitoneal (i.p) exposure (Sakharkar et al., 2016) or ethanol self-administration (Briones and Woods, 2013). Broadwater et al. (2014) directly compared adolescent and adulthood binge ethanol exposure on adult neurogenesis and found AIE persistently reduced adult DG neurogenesis whereas identical adult treatment only transiently reduced DG neurogenesis suggesting ethanol exposure during adolescence, but not adulthood, markedly reduced DCX expression in the hippocampal DG 4 weeks after the final ethanol exposure. Although acute adolescent binge ethanol exposure has been found to reduce both DG and SVZ neurogenesis (Crews et al., 2006), to our knowledge the impact of AIE on proliferation and maturation of NPC in SVZ neurogenesis has not been determined. This study reports for the first time the impact of AIE exposure, a model of underage binge drinking, on multiple markers across the stages of neurogenesis in both the hippocampal DG and forebrain SVZ in adulthood. We report here that AIE causes a persistent loss of both SVZ and DG neurogenesis, and that the AIE-induced changes in markers of NPC stages and cell death suggest that the mechanisms of the AIE-induced loss of new neuron

formation in DG and SVZ is different for each brain region.

MATERIALS AND METHODS

Animals and AIE Exposure

Timed-pregnant Wistar rats ($n = 10$), young mothers at the same age, were ordered from Harlan Laboratories, Inc. (Indianapolis, IN, USA) under a protocol approved by the Institutional Animal Care and Use Committees at the University of North Carolina. All animals were maintained at 22°C under a 12:12-h light/dark cycles with free access to food and water. Timed-pregnant dams at embryonic day 17 (E17), were allowed to acclimate to our vivarium. On the day following birth (postnatal day 1, P1), litters were culled to 10 pups. On weaning at P21, male offspring were pair-housed with a same-gender, same-age non-littermate and body weight match assigned to two experimental groups, including control and AIE group. This study was done in all males. The AIE group was exposed intermittently (e.g., 2 days on, 2 days off) with ethanol (5 g/kg, 25% ethanol w/v, i.g., 10 ml/kg) during adolescence (P25–P54); and the control group was administered with the same volume of water as described previously (Liu and Crews, 2015). Body weight was measured every 4 days. All control and AIE rats grew normally from P25 (control: 44.0 ± 1.3 g; AIE: 45.9 ± 1.3 g) to P54 (control: 262.7 ± 3.9 g; AIE: 258.4 ± 4.8 g) during AIE exposure. As expected, there was a significant main difference of age with body weight [$F_{(1,384)} = 1298.25$, $p < 0.001$], and no significant effect on group [$F_{(7,384)} = 0.07$, $p = 0.786$], age × group interaction [$F_{(7,384)} = 0.60$, $p = 0.753$]. From P57 to P95 after final ethanol exposure, the body weight of all animals also revealed a significant main difference of age [$F_{(9,320)} = 118.28$, $p < 0.001$], and but no group effect [$F_{(1,320)} = 0.01$, $p = 0.932$] or age × group interaction [$F_{(9,320)} = 0.26$, $p = 0.985$]. All rats appeared normal throughout the experiment consistent with AIE binge exposure not causing observable changes in health. During AIE exposure, tail blood samples were collected and measured twice 1 h after ethanol exposure (5 g/kg i.g.) at P38 and 54. Blood ethanol concentrations (O'Malley et al., 1998) were measured using a GM7 Analyzer (Analox, London, UK). Their average values were 152.17 ± 11.09 at P38 and 211.86 ± 19.26 mg/dl at P54. Statistics showed that the BECs had a significant difference between at postnatal day 38 and 54 [$F_{(1,50)} = 7.21$, $p < 0.01$]. At P54, animals of both control and AIE group were randomly reassigned to three groups respectively with body weight match after the last ethanol exposure. BrdU (5′-bromo-2-deoxyuridine, Sigma; St. Louis, MO, USA) is incorporated into DNA during cell division. To assess proliferation, control and AIE groups were sacrificed on P57, 2 h after BrdU injection ($n = 8$/each group, 300 mg/kg, dissolved in 0.9% saline, i.p.). A second group of control and AIE rats were sacrificed on P95 2 h after BrdU injection($n = 8$/each group, 300 mg/kg i.p.) to assess persistent changes in progenitor proliferation. For progenitor cell survival and differentiation study, a third group of control ($n = 8$) and AIE ($n = 10$) were administered BrdU 150 mg/kg daily starting at P54 for 14 days, and were allowed to survive 4 weeks after BrdU treatment, and sacrificed on P95 (See Supplementary Figure S1 for additional details).

Animal Tissue Collection, Preparation and Immunohistochemistry

Rats were deeply anesthetized with an overdose of sodium pentobarbital, and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH7.4) followed by 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4). Brains were removed, and post-fixed for 24 h in 4% paraformaldehyde at 4°C. Coronal sections were obtained at a thickness of 40 μm in 1:12 series after cryoprotection with 20% and 30% sucrose, gradually. Every 12th section was used for each of the following antigens.

For BrdU staining, DNA denaturation was performed by incubating the section in 2 N HCl for 30 min 37°C (Kuhn et al., 1996). The sections were then incubated with 0.6% hydrogen peroxide (H_2O_2) for 30 min to remove endogenous peroxidase activity. Anti-mouse BrdU (Chemicon, MAB3424, Temecula, CA, USA) was used at a dilution of 1:2000 and incubated overnight at 4°C, and sections were incubated with biotinylated horse anti-mouse secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h. Then avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories) was added for 1 h at room temperature. Finally, the BrdU-positive cells were visualized using DAB (nickel-enhanced diaminobenzidine). For all other antigens, sections were incubated in 0.6% H_2O_2 for 30 min, and blocked in 3% goat serum (0.1% Triton X-100) for 1 h at room temperature. In the current studies, all other primary antibodies were used with different dilutions (**Table 1**) overnight at 4°C. At the second day, sections were rinsed in PBS, and incubated with biotinylated secondary goat anti-rabbit or anti-mouse or anti-chicken antibody (1:200, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Subsequently, the positive cells were visualized using DAB.

Double fluorescence staining for BrdU and Ki67 was done using a mix of mouse anti-BrdU (1:2000, MAB3424, Chemicom, Temecula, CA, USA) and rabbit anti-Ki67 (1:400, ab66155, Abcam, Inc., Cambridge, MA, USA). Then, sections were incubated in the dark for 1 h with following secondary antibodies (1:200, a mix of goat anti-mouse 594 and anti-rabbit 488, Alexa Fluors, Molecular Probes Eugene, OR, USA). For mouse anti-BrdU (1:2000, MAB3424, Chemicon) and chicken anti-Tbr2 (1:800, S-C123463/31819, Lifespan bioscience Inc., Seattle, WA, USA), the secondary antibodies were used with a mix of goat anti-mouse 488 and anti-chicken 594 (1:200, Alexa Fluors, Molecular Probes Eugene, OR, USA). For mouse anti-BrdU (1:2000, MAB3424, Chemicom) and rabbit anti-Iba1 (1:500, 019-19741, Wako/Fisher, Cambridge, MA, USA), the secondary antibodies were used with a mix of goat anti-mouse 488 and anti-rabbit 594 (1:200, Alexa Fluors, Molecular Probes Eugene, OR, USA). For mouse anti-BrdU (1:2000, MAB3424, Chemicom) and anti-rabbit NeuN (1:1000, EMB Millipore, ABN78, Billerica, MA, USA), sections were incubated in the dark for 1 h with following secondary antibodies (1:200, a mix of goat anti-mouse 594 and anti-rabbit 488 Alexa Fluors,

TABLE 1 | Primary antibodies in the present study.

Antibodies	Isotype	Source/Purification	Dilution	Source
BrdU	Mouse IgG	Monoclonal	1:2000	Chemicon, Temecula, CA, USA (MAB3424)
Ki67	Rabbit IgG	Polyclonal	1:400	Abcam Inc., Cambridge, MA, USA (ab66155)
Tbr2/EOMES	Chicken IgG	Polyclonal	1:200	Lifespan Biosciences, Inc., Seattle, WA, USA (LSC123463/31819)
Sox2	Rabbit IgG	Polyclonal	1:1000	Abcam Inc., Cambridge, MA, USA (ab97959)
DCX	Rabbit IgG	Polyclonal	1:1000	Abcam Inc., Cambridge, MA, USA (ab18723)
Prox1	Rabbit IgG	Polyclonal	1:1000	Abcam Inc., Cambridge, MA, USA (ab37128)
Parvalbumin	Rabbit IgG	Polyclonal	1:1000	Abcam Inc., Cambridge, MA, USA (ab11427)
NeuN	Rabbit IgG	Polyclonal	1:1000	EMD Millipore, Billerica, MA, USA (ABN78)
Iba1	Rabbit IgG	Polyclonal	1:500	Wako/Fisher, Cambridge, MA, USA (019-19741)
Nestin	Rabbit IgG	Polyclonal	1:400	Abcam Inc., Cambridge, MA, USA (ab27952)
Mash1	Rabbit IgG	Polyclonal	1:50	Novus Biologicals, Littleton, CO, USA (NBP1-51269)
Dlx2	Rabbit IgG	Polyclonal	1:1000	Abcam Inc., Cambridge, MA, USA (ab18188)
β-2 microglobulin	Rabbit IgG	Polyclonal	1:50	Santa Cruz Biotechnology Inc., Dallas, TX, USA (sc-15366)
Cleaved caspase-3	Rabbit IgG	Polyclonal	1:1200	Cell Signaling Technology, Danvers, MA, USA (#9661)

Molecular Probes Eugene, OR, USA). All sections were covered slips with anti-fade mounting reagent (ProLong, Molecular Probes).

Quantification

Bioquant Nova Advanced Image Analysis (R&M Biometric, Nashville, TN, USA) was used for image capture and analysis (Crews et al., 2004). Images were captured by using an Olympus BX50 Microscope and Sony DXC-390 video camera linked to a computer. In the hippocampal DG, positive cells (BrdU+, Ki67+, Tbr2+, SOX2+, Prox1+, PV+, β-2 microglobulin+ and activated caspase-3+IR) were counted using prolife counting in the granule cell layer of the dorsal DG of HIP (Bregma from −2.30 mm to −4.52 mm; Paxinos and Watson, 1997) and expressed as cells per square millimeter with both sides of 3–5 section per animals, and the average value per mm^2 was used (See Supplementary Figure S1D). For DCX+IR, the granule cell layer of the hippocampal DG was outlined and pixel density was measured for the outlined area (pixels/mm^2). In the SVZ, positive cells (Nestin+, Dlx2+, BrdU+ and activated caspase-3+IR) were counted using prolife counting similar to that described by Kuhn et al. (1996) and Crews et al. (2006) in the special regions interested and expressed as cells per square millimeter with both sides of 3–5 section per animals, and the average value per mm^2 was used. For SOX2+, Mash1+ and DCX+IR, pixel densities were measured for the outlined area (pixels/mm^2; see Supplementary Figure S1D). In the SVZ, Bregma from 1.20 mm to −0.30 mm (Paxinos and Watson, 1997), positive immunoreactivities were measured at series of three 50 μm boxes along the length of SVZ as described (Kuhn et al., 1996; Crews et al., 2006).

Confocal analyses were conducted using LesicaSP2 AOBS Upright Laser Scanning Confocal in Michael Hooker Microscopy Facility of University of North Carolina. In the granule cell layer of hippocampal DG, 50–100 BrdU positive cells per sample were analyzed for co-labeling with Tbr2 or Iba1 or NeuN. The percentages of co-label in BrdU+IR were calculated.

Statistical Analysis

All values (including body weight, positive cells and pixel values) were reported as mean ± SEM and analyzed using analysis of variance (ANOVA) (IBM SPSS Statistics 22). The change of body weight was analyzed using a mixed ANOVA (group × day) with day as the repeated measure. Significant effects of AIE exposure on the body weight at different days were used with Student's *t*-test. For the effect of AIE exposure on the expression of positive cells or pixel values, ANOVA was used to test statistical significance, and followed by comparison of each group mean with Independent-Samples *T*-Test, and the *p*-value was used for statistical significance. The significant difference were considered if $p < 0.05$ at least. In this study, the percentage of the maturational decline between P57 and P95 with marker+IR expression was (P57 − P95)/P57 * 100; and the maturational increase between P57 and P95 with marker+IR expression was (P95 − P57)/P57 * 100. The percentage of AIE-induced decrease was (Control − AIE)/Control * 100; and AIE-induced increase was (AIE − Control)/Control * 100. Pearson correlations were used to determine the correlation across all markers in either the hippocampal DG or SVZ region.

RESULTS

AIE Exposure Reduces Hippocampal Dentate Gyrus Neurogenesis

Neurogenesis in adults involves NPC proliferation followed by maturation to an integrated functional neuron over a period of several weeks. We used multiple markers of NPCs that distinguish stages of neurogenesis and neuronal maturation on P57 (3 days after last ethanol exposure) and P95 (41 days after abstinence). We first determined proliferation using two indices, Ki67 and BrdU. Ki67, an endogenous marker of cell proliferation, is a nuclear protein expressed in all phases of the cell cycle, except the resting phase. We administered BrdU 2 h before sacrifice, which marks dividing cells during S-phase of the mitotic process. We found that AIE exposure did not affect BrdU+ and Ki67+IR expression 3 days after last ethanol exposure (P57), however, 41 days later (P95) there was a difference. Control levels of both BrdU+IR (42.6 ± 5.4%) [$F_{(1,27)} = 69.31$, $p < 0.001$] and Ki67+ (42.3 ± 5.4%) [$F_{(1,27)} = 42.39$, $p < 0.001$] declined with age from P57 to P95 (**Figure 1**), consistent with the known maturational decline in hippocampal neurogenesis. Interestingly,

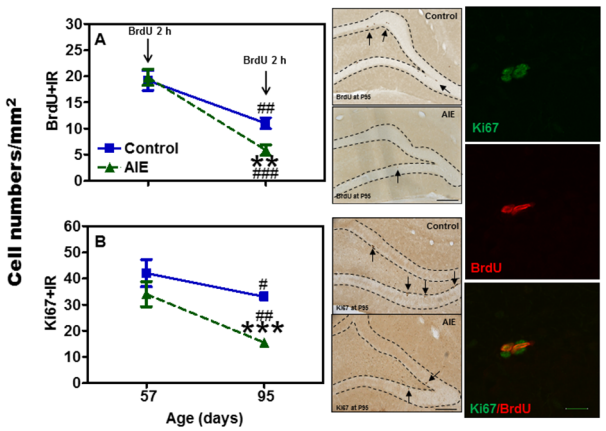


FIGURE 1 | Effects of adolescent intermittent ethanol (AIE, 5 g/kg, i.g., 2 days on/2 days off) exposure on BrdU+ (2 h, **A**) and Ki67+IR **(B)** expression in the hippocampal dentate gyrus (DG) of male rat brain at P57 and P95. Figure on left side: AIE exposure remarkably decreased BrdU+ (47%, **A**) and Ki67+IR (46%, **B**) expression at P95, but not at P57. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group. BrdU+ **(A)** and Ki67+IR **(B)** expression had the maturational decline in both control (BrdU+IR: 43%; Ki67+IR: 42%) and AIE group (BrdU+IR: 70%; Ki67+IR: 63%) from P57 to P95. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with control or AIE group at P57, respectively. The data were expressed as the numbers of BrdU+ **(A)** or Ki67+IR **(B)** positive cells, each point is mean ± SEM per mm^2 (n = 7∼8/group). Middle panels: BrdU+ and Ki67+IR expression in the granular cell layer of the hippocampal DG at P95 (Immunohistochemical staining, Bar scale = 100 μm). Right panels: photomicrographs of confocal images in the granular cell layer of hippocampal DG, Ki67+ (green) and BrdU+ (red), Bar scale = 15 μm.

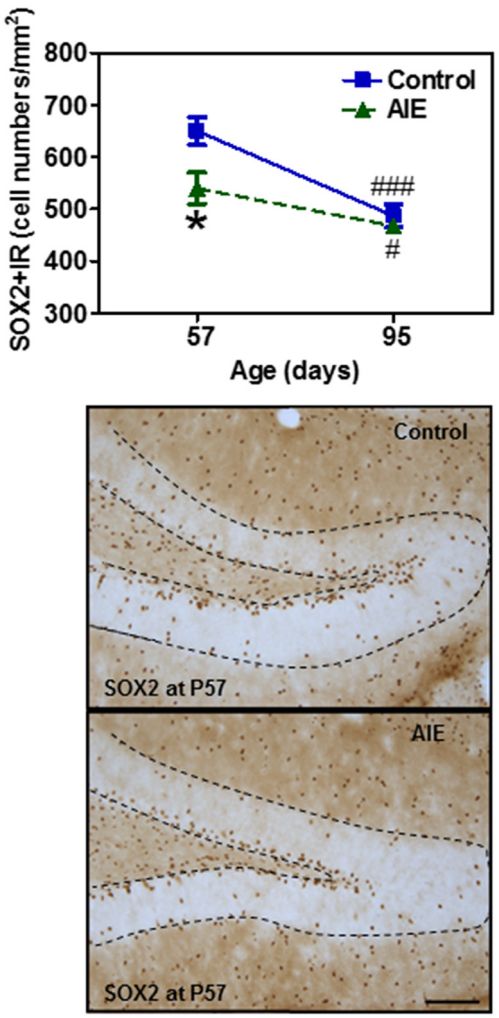


FIGURE 2 | Effects of AIE (5 g/kg, i.g., 2 days on/2 days off) exposure on SOX2+IR expression in the hippocampal DG of male rat brain at P57 and P95. Figure on the top: AIE exposure remarkably decreased SOX2+IR (17%) expression at P57, but not at P95. $^{*}p < 0.05$ compared with control group. SOX2+IR expression had the maturational decline in both control (28%) and AIE group (13%) from P57 to P95. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with control or AIE group at P57, respectively. The data were expressed as the numbers of SOX2+IR positive cells, each point is mean ± SEM per mm^2 (n = 7∼8/group). Below panels: SOX2+IR expression in the granular cell layer of the hippocampal DG at P57 (Immunohistochemical staining, Bar scale = 50 μm).

the maturational decline appeared to be accelerated in AIE (BrdU: 70.4 ± 5.0%, $p < 0.001$, Ki67: 63.3 ± 3.1%, $p < 0.001$) treated animals since at P95. AIE exposure decreased BrdU+ (46.8 ± 9.0%, $p < 0.01$, **Figure 1A**) and Ki67+IR (46.0 ± 4.6%, $p < 0.001$, **Figure 1B**) in the DG compared to control P95. For Ki67+IR study, two-way ANOVA showed that there was a main significant effect of group [$F_{(1,27)} = 10.59$, $p < 0.01$]. These findings suggest AIE exposure alters the maturation of proliferating NPCs in the DG.

To assess milestones of neurogenesis stages, we determined SOX2 and Tbr2 that mark NPCs of radial glial-like (Type-1) and intermediate progenitor cells (Type-2). Interestingly, both SOX2+ (**Figure 2**) and Tbr2+IR (**Figure 3**) show significant AIE-induced changes on P57 that are resolved by P95. AIE exposure significantly reduced SOX2+IR expression at P57 (16.9 ± 4.6%, $p < 0.05$, **Figure 2**), but not at P95. Both control and AIE group showed the maturational decline between P57 and P95 with SOX2+IR expression (control: 27.9 ± 3.4%, $p < 0.001$; AIE: 13.4 ± 1.9%, $p < 0.01$). Two-way ANOVA indicated that there was a main significant effect of group [$F_{(1,27)} = 5.14$, $p < 0.05$], age [$F_{(1,27)} = 35.99$, $p < 0.001$] and group × age interaction [$F_{(1,27)} = 4.87$, $p < 0.05$] on SOX2+IR. However, the maturational decline in AIE group was less than in control ($p < 0.01$, from P57 to P95). We determined both Tbr2+IR expression and cellular cluster size. AIE exposure significantly reduced Tbr2+IR expression (33.5 ± 4.9%, $p < 0.01$, **Figure 3A**) on P57, but not at P95. There was maturational decline of Tbr2+IR expression in control (62.5 ± 3.0%, $p < 0.001$) and AIE (58.5 ± 6.0%, $p < 0.001$). Two-way ANOVA indicated that there was a main significant effect of group [$F_{(1,27)} = 23.72$, $p < 0.01$], age [$F_{(1,27)} = 129.95$, $p < 0.0001$), and group × age interaction [$F_{(1,27)} = 7.04$, $p < 0.05$] on Tbr2+IR (**Figure 3**). Determination of mean cluster size of Tbr2+IR cells in the AIE animals (2.8 ± 0.2 cell numbers/cluster) were significantly smaller than controls (3.5 ± 0.2 cell numbers/cluster) at P57 ($p < 0.05$),

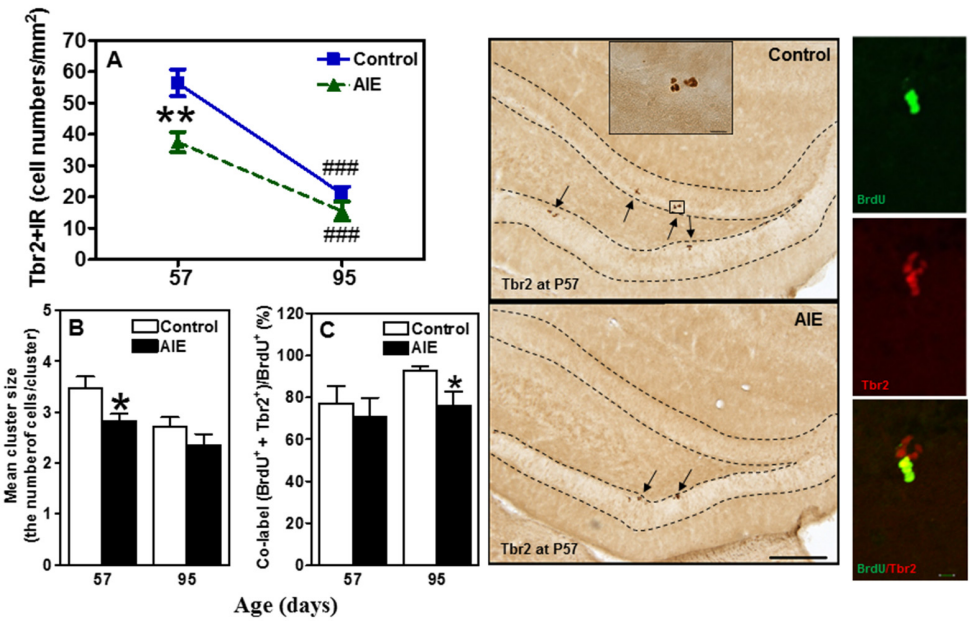


FIGURE 3 | Effects of AIE (5 g/kg, i.g., 2 days on/2 days off) exposure on Tbr2+IR expression in the hippocampal DG of male rat brain at P57 and P95. **(A)** AIE exposure remarkably decreased 34% Tbr2+IR expression at P57, but not at P95. $^{**}p < 0.01$ compared with control group. Tbr2+IR expression had the maturational decline in both control (63%) and AIE group (59%) from P57 to P95. $^{###}p < 0.001$ compared with control or AIE group at P57, respectively. The data were expressed as the numbers of Tbr2+IR positive cells, each point is mean ± SEM per mm^2 (n = 7∼8/group). **(B)** Tbr2+IR cell clusters and cell numbers per cluster were counted in the granular cell layer of the DG of control and AIE group at P57 and P95, and mean cell numbers per cluster were used. The mean cluster size of AIE group were significantly smaller than control group at P57 ($^{*}p < 0.05$). **(C)** AIE exposure resulted in a reduction of co-localization of Tbr2/BrdU+ cells in BrdU+ cells compared with control group ($^{*}p < 0.05$) at P95. Middle panels: Tbr2+IR expression in the granular cell layer of the hippocampal DG at P57 (Immunohistochemical staining, Bar scale = 100 μm, inset 10 μm). Right panels: photomicrographs of confocal images in the granular cell layer of the hippocampal DG, BrdU+ (green) and Tbr2+ (red), Bar scale = 10 μm.

however, they were not different on P95 (Control 2.7 ± 0.2 vs. AIE 2.4 ± 0.2 cell numbers/cluster, **Figure 3B**). Tbr2+/BrdU+ co-localization in controls was 77.3 ± 8.2% on P57 and 92.6 ± 2.2% on P95, with AIE animals having slightly fewer co-localized cells compared to control group at P95 (18.7 ± 5.8%, $p < 0.05$). Tbr2+/BrdU+ co-localization in AIE group was 70.9 ± 8.9% on P57 and 76.1 ± 6.0% on P95 (**Figure 3C**). These results suggest that AIE exposure reduces P57 radial glia-like and intermediate neuronal progenitor cells altering maturation of hippocampal DG.

To determine immature neuron formation, we used DCX and Prox1 markers as well as the mature GABAergic marker PV. All three markers decreased with age between P57 and P95 in the control group, DCX+ (48.6 ± 3.9%, $p < 0.001$, **Figure 4A**), Prox1+ (32.9 ± 5.0%, $p < 0.001$, **Figure 4B**) and PV+IR (28.7 ± 5.9%, $p < 0.05$, **Figure 4C**) consistent with the maturational decline in DG neurogenesis. AIE exposure resulted in a reduction with Prox1+ (15.3 ± 4.8%, $p < 0.05$) and PV+IR expression (26.8 ± 6.0%, $p < 0.05$), and had a reductive trend in DCX+IR ($t = 2.069$, $p = 0.059$) compared with P57 controls. At P95, AIE exposure reduced DCX+ (29.1 ± 5.1%, $p < 0.01$) and PV+IR (28.2 ± 5.1%, $p < 0.05$), but not Prox1+IR (**Figure 4**). These findings indicate a persistent loss of DCX+IR immature neurons and PV GABAergic neurons in adults following AIE exposure.

Survival and Differentiation of NPCs in Hippocampal Dentate Gyrus after AIE Exposure

To investigate the survival and differentiation of neurogenesis, BrdU was injected daily for 14 days to label dividing NPCs (P54-P68) and rats sacrificed weeks later allowing BrdU+ NPCs to mature. Surprisingly, we found that AIE exposure remarkably increased BrdU+IR expression 42.11 ± 6.92% [$F_{(1,16)} = 21.40$, $p < 0.001$, **Figure 5A**]. Co-localization studies indicated that BrdU+ co-labeled with mature neuronal marker NeuN+ was 80.1 ± 1.1% in controls that was lower in the AIE group (74.4 ± 1.3%, $p < 0.05$, **Figure 5C**), suggesting slightly fewer neurons after AIE exposure. Interestingly, a small percentage of BrdU+ cells positive for microglial Iba1+ was increased by AIE from a control level of 1.5 ± 0.6% to 3.7 ± 0.3%, ($p < 0.01$) in AIE treated rats (**Figure 5B**). These findings suggest an altered differentiation of NPCs in hippocampal DG following AIE exposure.

AIE Exposure Increases β-2 Microglobulin and Activated Caspase-3 Expression in the Hippocampal Dentate Gyrus

To investigate mechanisms, we assessed immune signaling with β-2 microglobulin (β2M) and cell death using activated caspase-3

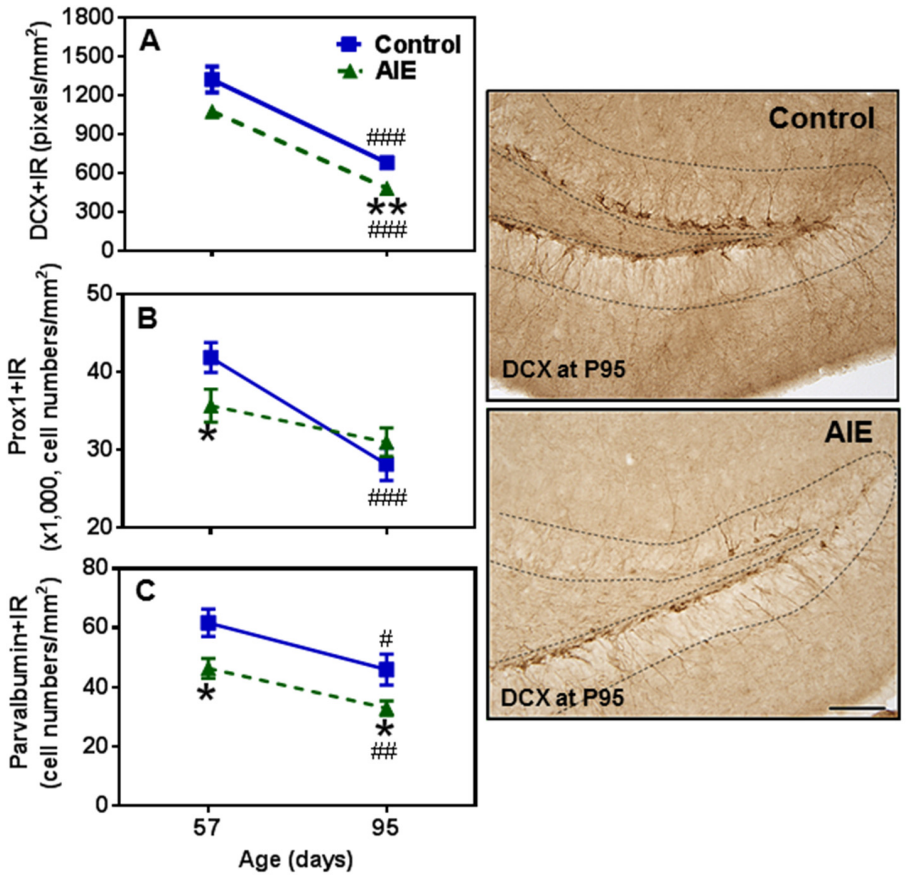


FIGURE 4 | Effects of AIE (5 g/kg, i.g., 2 days on/2 days off) exposure on DCX+ **(A)** Prox1+ **(B)** and parvalbumin (PV)+IR **(C)** expression in the hippocampal DG of male rat brain at P57 and P95. AIE exposure remarkably reduced Prox1+ (**B**, 15%) and PV+IR (**C**, 27%) expression at P57 compared with control group, and reduced DCX+ (**A**, 29%) and PV+IR (**C**, 28%) expression at P95. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group. There was a significantly maturational decline with DCX+ (**A**, control: 49%; AIE: 56%), Prox1+ (**B**, control: 33%) or PV+IR (**C**, control: 29%; AIE: 29%) expression in control or AIE group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with control or AIE group at P57, respectively. The data were expressed as pixels of DCX+ **(A)** or the numbers of Prox1+ **(B)** or PV+IR **(C)** positive cells, each point is mean ± SEM per mm^2 (n = 7∼8/group). Right panels: DCX+IR expression in the granular cell layer of the hippocampal DG (Immunohistochemical staining, Bar scale = 50 μm).

(as cleaved caspase-3). β2M is a major histocompatability complex 1 (MHC1) protein that is expressed on microglia and neurons, is involved in synaptic stripping and is known to inhibit hippocampal neurogenesis (Smith et al., 2015; Yousef et al., 2015). Caspase-3 is a protease which is activated during cell death, particularly apoptosis, and an antibody recognizes the active protease providing a marker of dying cells. Previous studies have found that injection of β2M into young adult mice results in loss of DG Tbr2+ and DCX+ NPCs (Smith et al., 2015), similar to our finding in young adults following AIE exposure. We found that AIE exposure did not alter DG β2M+IR expression at P57, whereas AIE-treated animals had about double the number of cells expressing β2M+IR at P95 (**Figure 6**). AIE-treated animals showed maturational increase expression from P57 to P95 (46.6 ± 11.6%, $p < 0.05$), not in control group. Two-way ANOVA of β2M+IR cells showed that there was a main effect of group [$F_{(1,27)} = 13.27$, $p < 0.001$] and age [$F_{(1,27)} = 6.82$, $p < 0.05$], but a trend effect of group × age interaction [$F_{(1,27)} = 3.59$, $p = 0.069$]. Interestingly, activated caspase-3+IR expression was slightly increased by AIE at P57 (26.7 ± 8.7%, $p < 0.05$). Maturation of controls to P95 slightly increased activated caspase-3+IR, but the increase of AIE group at P95 was about double the level of controls (**Figure 7**), e.g., increases between P57 and P95 for controls is 15.5 ± 4.4%, ($p < 0.05$) compared with AIE increases of 29.2 ± 7.0%, ($p < 0.05$). At P95, activated caspase-3+IR in DG after AIE exposure was increased 41.2 ± 8.5%, ($p < 0.01$) compared with controls (**Figure 7**). Two-way ANOVA of activated caspase-3+IR cells indicated that there was a main effect of group [$F_{(1,28)} = 16.88$, $p < 0.0001$] and age [$F_{(1,28)} = 8.54$, $p < 0.01$], but no effect of group × age interaction [$F_{(1,28)} = 1.29$, $p = 0.266$]. Interestingly, the AIE-induced increase in activated caspase-3+IR positively correlated with the AIE-induced reduction of Ki67+IR at P57 [$F_{(1,5)} = 11.5$, $r = 0.83$, $p = 0.020$], consistent with increased cell death reducing proliferating NPCs (**Table 2**). Similarly, in

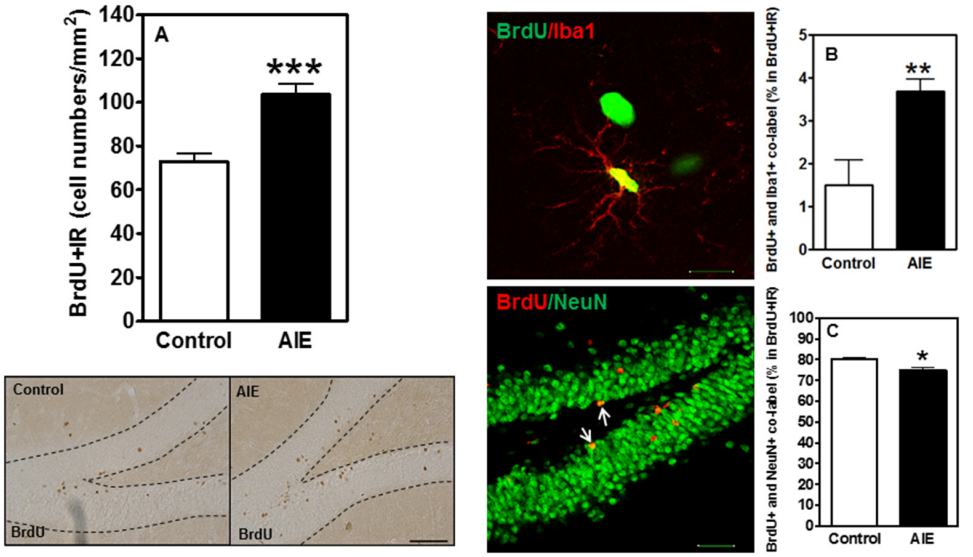


FIGURE 5 | Effects of AIE (5 g/kg, i.g., 2 days on/2 days off) exposure on survival BrdU+IR expression after abstinence in the hippocampal DG of male rat brain at P95. **(A)** BrdU+IR expression was measured after 6 weeks of abstinence, 4 weeks following labeling with BrdU (150 mg/kg, i.p. 14 days). AIE exposure significantly increased BrdU+IR expression in the DG **(A)** $^{***}p < 0.001$ compared with control group. The data were expressed as the numbers of BrdU+IR positive cells, each point is mean ± SEM per mm^2 (n = 8∼10/group). Left below panels: BrdU+IR expression in the granular cell layer of the hippocampal DG (Immunohistochemical staining, Bar scale = 50 μm). Middle panels show photomicrographs of confocal images in the granular cell layer of the hippocampal DG, top one: BrdU+ (green) and Iba1+ (red) (Bar scale = 15 μm), and bottom one: BrdU+ (red) and NeuN+ (green) (Bar scale = 50 μm). **(B)** A small percentage of BrdU+ cells were positive Iba1+ 1.5% in control vs. 3.7% in AIE ($^{**}p < 0.01$). **(C)** 74% BrdU+ co-labeled with NeuN+ in AIE was less than in control group (80%, $^{*}p < 0.05$).

adulthood at P95 the AIE-induced increase in activated caspase-3+IR positively correlated with the AIE-induced reduction DCX+IR [$F_{(1,6)}$ = 8.3, r = 0.76, p = 0.028] and Prox1+IR [$F_{(1,6)}$ = 7.5, r = 0.75, p = 0.034], consistent with AIE-induced increases in cell death contributing to loss of adult neurogenesis (**Table 2**). Thus, DG cell death and expression of β2M+IR is increased by AIE exposure and appears to contribute to the loss of both proliferating NPCs and maturing neuron loss in adulthood.

Subventricular Zone Neurogenesis and AIE Exposure

SVZ and hippocampal DG differ in adult neuroprogenitors and neurogenesis. SOX2+ and Mash1+IR mark SVZ early progenitors, and both showed a maturational decline between P57 and P95 (**Figure 8**). SOX2+IR did not differ between groups at P57, but the maturational decline in controls was 24.5 ± 4.5% ($p < 0.05$) compared to the AIE decline of 41.1 ± 3.2% ($p < 0.001$) that resulted in reduced expression of SOX2+IR at P95 (18.3 ± 4.1%, $p < 0.05$, **Figure 8A**). Two-way ANOVA revealed that there was a main effect of age [$F_{(1,26)}$ = 33.92, $p < 0.0001$], without effect of group [$F_{(1,26)}$ = 0.37, p = 0.549], group × age interaction [$F_{(1,26)}$ = 3.05, p = 0.092] on SOX2+IR. Similarly, Mash1+IR was not significantly decreased by AIE at P57, but by P95 AIE exposure decreased Mash1+IR to 59.2 ± 6.3% ($p < 0.05$, **Figure 8B**) of control. Two-way ANOVA showed that there was a significant effect of age [$F_{(1,27)}$ = 6.02, $p < 0.05$], group [$F_{(1,27)}$ = 8.81, $p < 0.01$], without effect of group × age interaction [$F_{(1,27)}$ = 0.79, p = 0.382] on Mash1+IR.

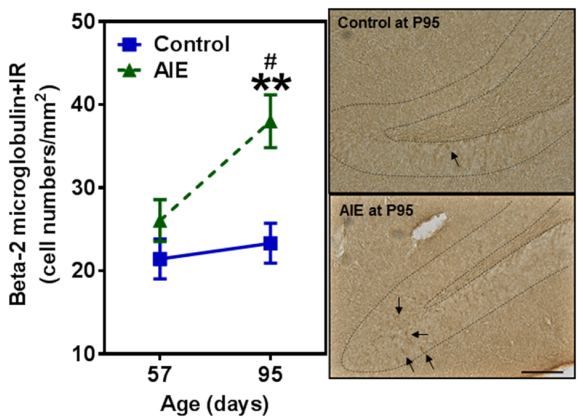


FIGURE 6 | Effects of AIE (5 g/kg, i.g., 2 days on/2 days off) exposure on β2M +IR expression in the hippocampal DG of male rat brain at P57 and P95. Figure on the left: AIE exposure remarkably increased β2M+IR expression (63%) compared with control group at P95. $^{**}p < 0.01$ compared with control group. β2M+IR expression had a significantly maturational increase in AIE group (47%). $^{\#}p < 0.05$ compared with AIE group at P57. The data were expressed as the numbers of β2M+IR positive cells, each point is mean ± SEM per mm^2 (n = 7∼8/group). Right panels: β2M+IR expression in the granular cell layer of the hippocampal DG (Immunohistochemical staining, Bar scale = 50 μm).

Interestingly, no correlation of SVZ markers was found at P57 in either controls or AIE exposure animals (**Table 2**). Determination of BrdU+IR 2 h. after injection (S-phase marker

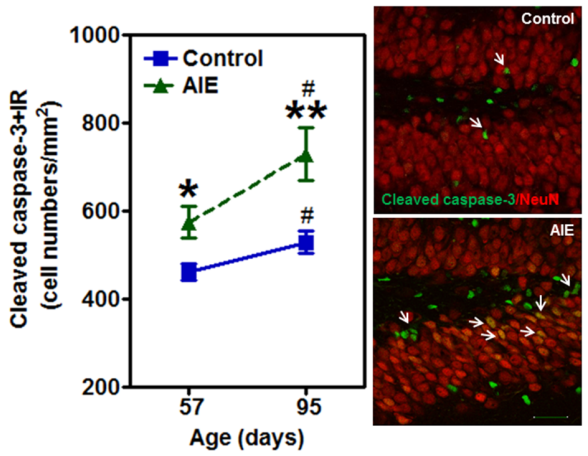


FIGURE 7 | Effects of AIE (5 g/kg, i.g., 2 days on/2 days off) exposure on activated caspase-3+IR expression in the hippocampal DG of male rat brain at P57 and P95. Figure on the left: AIE exposure remarkably increased the apoptosis marker activated caspase-3+IR from P57 (27%) to P95 (41%) compared with control group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group. Activated caspase-3+IR expression had a significantly maturational increase in control (16%) and AIE group (29%). $^{\#}p < 0.05$ compared with control or AIE group at P57, respectively. The data were expressed as the numbers of activated caspase-3+IR positive cells, each point is mean ± SEM per mm^2 (n = 8/group). Right panels: photomicrographs of confocal images in the granular cell layer of the hippocampal DG, activated caspased-3+ (green) and NeuN+ (red), Bar scale = 30 μm.

of cell proliferation) did not find a change in SVZ (**Table 3**). Also nestin+IR (a marker of neural stem cell, type B) expression in the SVZ at P57 and P95 were not altered by AIE exposure (**Table 3**). These finding suggest SVZ neurogenesis does not show immediate changes following AIE exposure, but following maturation to adulthood at P95 led to an AIE induced loss of early progenitor markers Mash1 and SOX2 in the SVZ.

To further investigate SVZ neurogenesis, we determined neuroprogenitor markers Dlx2+IR and DCX+IR as well as NPC survival using BrdU+IR. Similar to the other SVZ markers studied, AIE exposure did not markedly alter neurogenesis markers at P57, just after AIE exposure, but after maturation to P95 AIE exposure was found to significantly reduce Dlx2+IR (12.7 ± 4.9% $p < 0.05$) and DCX+IR (19.6 ± 5.8%, $p < 0.05$) expression compared with controls (**Figures 9A,B**). Dlx2+IR did not show a maturational decline, but DCX+IR had maturational decline between P57 and P95 in the control (25.5 ± 5.0%, $p < 0.05$) and AIE group (32.7 ± 4.9%, $p < 0.01$) in the SVZ (**Figure 9B**), respectively. Two-way ANOVA revealed that there was a main effect of age [$F_{(1,27)} = 1.73$, $p = 0.199$], without effect of group [$F_{(1,27)} = 6.73$, $p = 0.015$] and group × age interaction [$F_{(1,27)} = 0.04$, $p = 0.853$] on Dlx2+IR. And there was a significant effect of age [$F_{(1,24)} = 20.19$, $p < 0.0001$], group [$F_{(1,24)} = 4.19$, $p = 0.05$], without effect of group × age interaction [$F_{(1,24)} = 0.19$, $p = 0.671$] on DCX+IR. The AIE-induced decrease in Dlx2+ and DCX+IR at P95 correlated across individuals (**Table 2**). SVZ progenitor survival was determined following repeated BrdU (150 mg/kg i.p.) daily injected at P54 followed by weeks of maturation to P95 as described above. We found that AIE exposure markedly decreased SVZ BrdU+IR expression 41 days after abstinence in the SVZ (20.5 ± 2.8%, $p < 0.05$). One-way ANOVA revealed that there was significant effect of group [$F_{(1,16)} = 9.25$, $p < 0.01$] (see Supplementary Figure S2). Although these findings indicate AIE exposure caused a loss of adult SVZ neurogenesis, unlike hippocampus, we did not find a change in β2M+ and activated caspase-3+IR expression in the SVZ compared with controls at P57 or P95 (**Table 3**). These findings indicate that AIE exposure alters SVZ leading to a progressive persistent loss of SVZ neurogenesis that appears to increase with age.

DISCUSSION

In this study we investigated the impact of AIE exposure, a model of human adolescent binge drinking, on hippocampal DG and SVZ neurogenesis. Neurogenesis is a dynamic self-renewal

TABLE 2 | Pearson correlations across markers in the hippocampal dentate gyrus (DG) and subventricular zone (SVZ) of male rat brain.

Markers	Markers in the DG			Markers in the SVZ	
	Ki67	DCX	Prox1	SOX2	Dlx2
BrdU	-	$F_{(1,5)} = 11.2$, $r = 0.83$ $p = 0.020$ (AIE, P57)	-	-	$F_{(1,5)} = 9.9$, $r = 0.82$ $p = 0.025$ (Control, P95)
	-	$F_{(1,6)} = 10.0$, $r = 0.79$ $p = 0.020$ (AIE, P95)	-	-	-
SOX2	$F_{(1,5)} = 13.3$, $r = 0.85$ $p = 0.015$ (AIE, P57)	-	-	N/A	-
DCX	-	N/A	-	-	$F_{(1,6)} = 6.9$, $r = 0.73$ $p = 0.04$ (AIE, P95)
Activated caspase-3	$F_{(1,5)} = 11.5$, $r = 0.83$, $p = 0.020$ (AIE, P57)	-	-	$F_{(1,6)} = 14.9$, $r = 0.85$ $p = 0.008$ (AIE, P95)	-
	-	$F_{(1,6)} = 8.3$, $r = 0.76$ $p = 0.028$ (AIE, P95)	$F_{(1,6)} = 7.5$, $r = 0.75$ $p = 0.034$ (AIE, P95)	-	-

AIE (5 g/kg, i.g., 2 days on/2 days off) group at P57 and P95 with significant F, r and p values. Only correlation of p < 0.05, p < 0.01 are shown. (-), no any correlation.

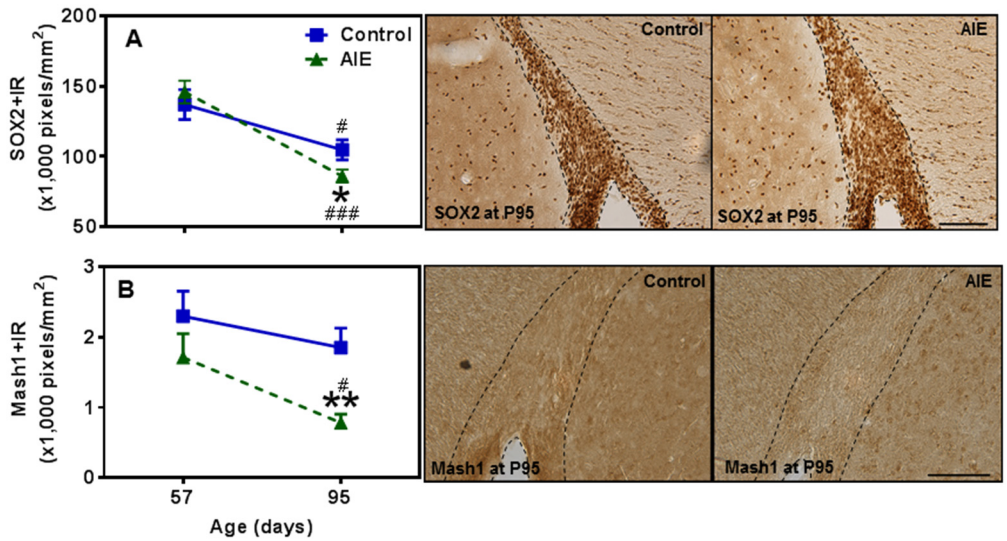


FIGURE 8 | Effects of AIE (5 g/kg, i.g., 2 days on/2 days off) exposure on SOX2+ and Mash1+IR expression in the subventricular zone (SVZ) of male rat brain at P57 and P95. AIE exposure remarkably decreased SOX2+ (18%, **A**) and Mash1+IR (59%, **B**) at P95 compared with control group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group. There was a significant maturational decline with SOX2+ (**A**, control: 25%; AIE: 41%), and Mash1+IR (**B**, AIE: 56%) expression in control or AIE group. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with control or AIE group at P57, respectively. The data were expressed as pixels of SOX2+ and Mash1+IR, each point is mean ± SEM per mm^2 (n = 7∼8/group). Right panels: SOX2+ and Mash1+IR expression in the SVZ at P95 (Immunohistochemical staining, Bar scale = 100 μm on SOX2+IR; 50 μm on Mash1+IR).

multistage process that involves the proliferation, migration, differentiation, and functional integration of new neurons into the preexisting neural circuitry that persists into adulthood in these two brain regions. We report here that AIE exposure decreases hippocampal progenitor makers SOX2+, Ki67+, Tbr2+ and BrdU+IR proliferation as well as markers of neuronal maturation, e.g., neurogenesis markers DCX+, Prox1+ and PV+IR (**Figure 10**). These finding are consistent with previous studies of hippocampal neurogenesis that AIE vapor treatment reduces adult Ki67+, DCX+ and BrdU+IR survival markers of neurogenesis in association with increased disinhibitory behavior and increased activated caspase-3+IR (Ehlers et al., 2013a). Similarly, AIE treatment with i.p injection of ethanol has been found to decrease adult hippocampal Ki67+ and DCX+IR while increasing HDAC activity and decreasing BDNF in association with increased anxiety (Sakharkar et al., 2016). Previous studies have found adolescents are more sensitive to ethanol inhibition of neurogenesis than adults (Crews et al., 2006). Studies by Nixon's group have investigated a continuous intoxication 4 day binge alcohol dependence model in adolescence and reported decreased hippocampal NPC survival 28 days after ethanol exposure, increased markers of cell death, and reduced DCX+IR, but no change in hippocampal Ki67+IR just after alcohol exposure ended (Morris et al., 2010; McClain et al., 2011) similar to our findings just after AIE exposure at P57. They have found that adolescent binge alcohol exposure inhibits neurogenesis through alterations in NPC cell cycle kinetics, especially decreasing the proportion of NPCs in S-phase. Further, the 4 day binge model found reduced SOX2+IR did not persist following adolescent alcohol exposure (McClain et al., 2011) similar to our finding of SOX2+IR returning to control levels at P95, in adulthood. Overall, all studies agree adolescent ethanol exposure inhibits hippocampal neurogenesis through multiple mechanisms including increases in cell death. Recent studies have found that the AIE-induced persistent loss of neurogenesis that is not found following identical treatment in adults (Broadwater et al., 2014). The AIE studies presented here add maturational changes and new

TABLE 3 | Adolescent intermittent ethanol (AIE, 5 g/kg, i.g., 2 days on/2 days off) exposure did not impact the expression of BrdU+ (2 h after single injection), nestin+, β-2 microglobulin+ and activated caspase-3+IR expression in the SVZ of male rat brain at P57 and P95.

Age (day)	Group	BrdU + IR (2 h) (cell number × 1000/mm^2)	Nestin + IR (cell number × 1000/mm^2)	β-2 microglobulin + IR (cell number × 1000/mm^2)	Activated caspase-3 + IR (cell number × 1000/mm^2)
57	Control	1.90 ± 0.11	23.87 ± 1.12	2.81 ± 0.50	6.68 ± 0.41
	AIE	2.04 ± 0.13	22.24 ± 0.57	2.63 ± 0.27	6.36 ± 0.28
95	Control	1.95 ± 0.13	23.58 ± 0.72	4.01 ± 0.37	6.61 ± 0.38
	AIE	1.80 ± 0.16	23.33 ± 0.76	4.47 ± 0.034$^{\#\#}$	6.63 ± 0.33

$^{\#\#}p < 0.01$, *compared to AIE group at P57.*

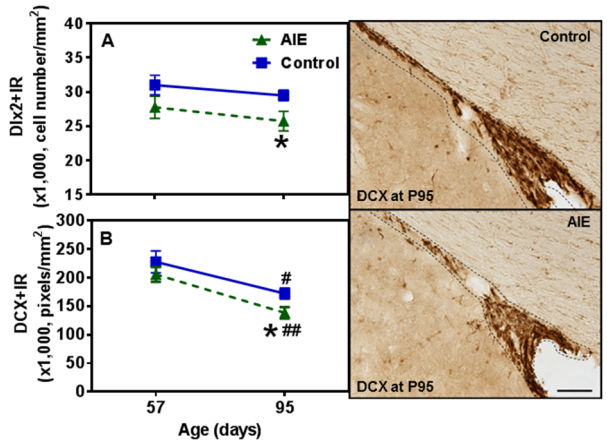


FIGURE 9 | Effects of AIE (5 g/kg, i.g., 2 days on/2 days off) exposure on Dlx2+ and DCX+IR expression in the SVZ of male rat brain at P57 and P95. Figure on the left: AIE remarkably decreased Dlx2+ (13%, **A**) and DCX+IR (20%, **B**) at P95 compared with control group. $^{*}p < 0.05$ compared with control group. There was a significant maturational decline of DCX+IR (control: 26%; AIE: 33%) in control and AIE group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with control or AIE group at P57, respectively. The data were expressed as the cell numbers of Dlx2+IR, and pixels of DCX+IR, each point is mean ± SEM per mm^2 ($n = 7$∼8/group). Right panels: DCX+IR expression in the SVZ at P95 (Immunohistochemical staining, Bar scale = 100 μm).

progenitor stage specific markers (Lindsey and Tropepe, 2006; Kempermann et al., 2015). Assessment of NPC proliferation markers in hippocampal DG, e.g., Ki67+ and BrdU+IR at 2 h, indicates AIE exposure accelerate the maturational declines in NPC proliferation between P57, late adolescence, and young adulthood (P95). In contrast, early neuroprogenitor transcription factor markers of radial-glial and intermediate neuronal progenitors, e.g., SOX-2+ and Tbr2+IR, respectively, are decreased after AIE exposure (P57), but decline less during abstinent maturation to P95, consistent with AIE exposure insulting early progenitors that are maturing. Conditional null mutant Sox2 mice have reduced neurogenesis, stem/precursor cells and a smaller hippocampus in adulthood consistent with Sox2 and neurogenesis being required for normal hippocampal maturation (Favaro et al., 2009). Similarly, previous studies indicate Tbr2 is a transcription factor uniquely expressed in DG NPCs that regulates progenitor cell fate in adult DG that is lost as neurons mature (Hodge et al., 2008, 2012). Prox1 is also a marker of the type-2 intermediate progenitors (Tbr2+ cells) in the DG (Urbán and Guillemot, 2014). AIE exposure resulted in the reduction of SOX2+, Tbr2+ and Prox1+IR expression in the DG at P57, suggesting that AIE-reduced radial glia and intermediate progenitor cells mainly happened after last ethanol exposure, but not on 41 days after abstinence in the hippocampal DG. Although AIE exposure causes an overall reduction in adult neurogenesis, the pools of SOX2+, Tbr2+ and Prox1+IR cells return to control levels in young adulthood (P95). Co-localized Tbr2+ and BrdU+ staining was reduced at P95 following AIE exposure consistent with fewer NPCs in S phase of cell cycle and a reduced rate of NPC proliferation and/or increased cell death. A reduced rate of progenitor proliferation is consistent with the reduced Ki67+ and BrdU+IR at 2 h, found after AIE exposure at P95. Further, we find AIE exposure increased activated caspase-3+IR cells at P57 that was further increased at P95. We also find increased activated caspase-3+IR at P57 correlates with decreased Ki67+IR in hippocampus DG consistent with increased cell death in early progenitors just after AIE exposure. In AIE exposed young adults at P95, activated caspase-3+IR cells only correlate with DCX+ cells suggesting increased cell death is an important mechanism contributing to the persistent loss of neurogenesis following AIE.

The mechanisms of persistent loss of neurogenesis following AIE exposure may be related to increased expression of proinflammatory cytokines and other innate immune signaling molecules. Previous studies have found that AIE exposure induces multiple proinflammatory cytokines, Toll-like receptors (TLRs) and RAGE, as well as HMGB1 an agonist at these immune receptors (Vetreno and Crews, 2012, 2014; Vetreno et al., 2013), that persists after AIE exposure and increases during maturation to young adulthood (Vetreno and Crews, 2012). Similarly, another study found that AIE exposure reduced adult hippocampal neurogenesis is associated with increases in adult hippocampal expression of the proinflammatory cytokines TNFα and MCP1 as well as the protease MMP9 and innate immune transcription factor NFκB (Vetreno and Crews, 2014). Further, this study found that treatment with lipopolysaccharide, a TLR4 agonist known to induce brain cytokines (Qin et al., 2007), reduces adult hippocampal neurogenesis (Vetreno and Crews, 2014). Another related finding is that AIE exposure reduces hippocampal BDNF expression (Sakharkar et al., 2016) suggesting loss of trophic support with increased neuroimmune gene expression after AIE exposure. β2M is a MHC1 protein that is expressed on monocytes and other cells as well as on brain microglia and neurons, where it is involved in synaptic stripping and is known to inhibit hippocampal neurogenesis (Smith et al., 2015; Yousef et al., 2015). In the current study, we found a marked increase in β2M+IR cells at P95, but not just after AIE exposure at P57, suggesting a progressive induction in expression of neuroimmune signaling β2M+IR cells. Proinflammatory cytokines (Fan and Pang, 2017) and TLRs (Rolls et al., 2007) can inhibit neurogenesis through increased progenitor death and/or increased differentiation to glia. Interestingly, β2M+IR expression similar to the receptor RAGE+IR (Vetreno and Crews, 2012; Vetreno et al., 2013) is not induced just after AIE treatment, but is triggered by AIE exposure and induced during maturation to young adulthood. Our study focused on adolescent maturation to adulthood and found increased β2M+IR expression and the greatest reductions in neurogenesis in young adulthood. Previous studies have reported that age-related increases in the DG β2M cause the age-related loss of neurogenesis during senescence (Smith et al., 2015; Yousef et al., 2015). Our findings are consistent with AIE-induced β2M+IR reflecting a progressive increase in neuroimmune signaling that reduces neurogenesis through increased cell death of hippocampal neuroprogenitors.

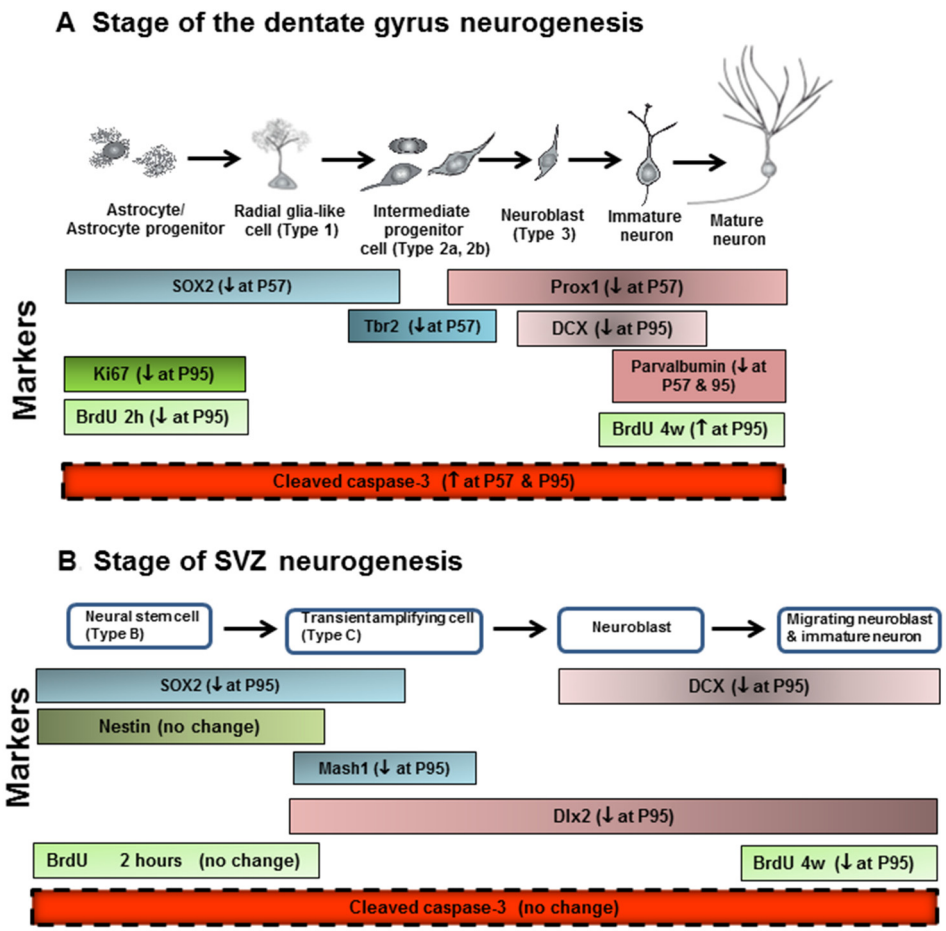


FIGURE 10 | Neurogenesis stages in the DG of the adult hippocampus and SVZ of lateral ventricle. **(A)** Schematic diagram representing the progression of neuronal development (Bonaguidi et al., 2012), and specific markers in the different developmental stages of the adult DG, such SOX2, Tbr2, Prox1, DCX and PV. Ki67 protein is present during all active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent from resting cells (G_0). Bromodeoxyuridine (BrdU), used in the detection of proliferating cells in living tissues as exogenous marker, is incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle). For the proliferation study, animals were sacrificed 2 h after injecting BrdU with 300 mg/kg (i.p.). For the survival study, animals were sacrificed 4 weeks after injecting BrdU with 150 mg/kg (i.p.) for 14 days. **(B)** Schematic diagram representing the progression of neuronal development, and specific markers in the different developmental stages of the adult SVZ, such as SOX2, nestin, Mash1, DCX and Dlx2. ↓ at P57, decreased at postnatal day 57; ↓ at P95, decreased at postnatal day 95; ↑ at P57, increased at postnatal days 57; ↑ at P95, increased at postnatal days 95.

In the present study, we add new data on the impact of AIE on SVZ neurogenesis (**Figure 10**). SVZ NPCs are different from hippocampal NPCs (Kriegstein and Alvarez-Buylla, 2009; Ming and Song, 2011). In the adult mammalian brain, new neurons are generated in the SVZ and migrate through the white matter into the neocortex (Gould et al., 1999). Neurogenesis in adult SVZ is found along the walls of the lateral ventricles and has stages of adult SVZ neurogenesis that differ from DG (Ming and Song, 2005). Proliferating radial glia-like SOX2+ cells (type B cells), give rise to transient amplifying neuroblasts and immature Mash1+ neuroprogenitors (type C cells) that migrate through the rostral migratory stream (RMS). Hansson et al. (2010) in an adult rat 7 weeks chronic relapsing alcoholic model found that DCX+IR and SOX2+IR in the SVZ was reduced just after treatment and the reduced SVZ neurogenesis persisted for 21 days of abstinence. Other studies in adult mice have reported weeks of ethanol self-administration reduce SVZ BrdU+ cells (Campbell et al., 2014). We previously found that acute ethanol dose-dependently decreased cell proliferation in the SVZ of adolescent rats (Crews et al., 2006). A previous AIE exposure study in Sprague-Dawley rats that were sacrificed on P74, 26 days after the last ethanol exposure, found decreased hippocampal and SVZ neurogenesis with age, similar to that reported here, as well as the persistent loss in adulthood, although in SVZ the AIE reduction was not statistically significant at P74 (Broadwater et al., 2014), consistent with our finding of the AIE reduced SVZ neurogenesis increasing with age and being significant at P95. We studied AIE-treated rats 41 days after the last AIE exposure and found a significant, persistent loss of adult

neurogenesis as indicated by decreased SOX2+, Mash1+, DCX+ and Dlx2+ cells in the SVZ. This is consistent with an emerging loss of SVZ neurogenesis during age-related maturation that requires time, e.g., at P95 we report here a significant loss of SVZ neurogenesis, but our studies reported here at P57 and Broadwater et al. (2014) at P74 found no change or a trend, respectively. Taken together, these studies suggest AIE exposure causes a progressive persistent change in the neurogenic niche of SVZ that weeks after AIE exposure ends reduces SOX2+ neural stem cells, Mash1+ progenitors, Dlx2+ neuroblasts and DCX+ immature neurons. These decreases in SVZ occur without an increase in activated caspase-3+ cells suggesting increased cell death is not a major contributor to the persistent loss of SVZ neurogenesis like it is in hippocampus DG. The AIE exposure induced loss of neurogenesis appears to be related to a progressive loss of SVZ neuroprogenitors. Mash1 is also known as achaete-scute homolog 1 (ASCL1) and is a transcription factor associated with stem cells neuronal differentiation commitment and Dlx2 is a neuroprogenitor-immature neuron transcription factor that are both decreased in young adults after AIE exposure consistent with a loss of early stem cells as well as differentiating progenitors. DCX marks immature neurons and BrdU (4 weeks) assesses neurogenesis. AIE exposure reduced both DCX+ and BrdU+IR (4 weeks) assessments indicating loss of neurogenesis. Thus, together these findings suggest AIE exposure leads to a persistent and growing loss of SVZ neuroprogenitors and neurogenesis.

Although our study did not investigate AIE-induced changes in behavior, loss of neurogenesis may contribute to AIE exposure induced behavior changes in adults. AIE exposure has been found to reduce adult cognitive flexibility and increase negative affect-anxiety (Crews et al., 2016). Loss of hippocampal neurogenesis has been proposed to reduce cognitive flexibility (Anacker and Hen, 2017) and to be associated with stress-induced depression-like behavior and the ability of anti-depressants to reverse depression. In addition, AIE-induced loss of neurogenesis has been found to correlate with loss of hippocampal volume (Ehlers et al., 2013b), a finding that mimics the reduced hippocampal volume found in human alcoholics and depressed individuals. Thus, the AIE induced loss of neurogenesis may contribute to cognitive and mood changes in adulthood that increase risks of adult psychopathology.

AUTHOR CONTRIBUTIONS

FTC and WL designed the study. WL performed the experiments and collected data. WL and FTC analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

FUNDING

The present study has been supported by the National Institute of Alcohol Abuse and Alcoholism grants P60AA011605, U54AA019767; NADIA project, UO1AA020023, U24AA020024.

ACKNOWLEDGMENTS

We thank to the UNC-Bowles Center for Alcohol Studies for support. Authors would like to thank Ryan P. Vetreno for reviewing this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnbeh.2017.00151/full#supplementary-material

REFERENCES

Abrous, D. N., Koehl, M., and Le Moal, M. (2005). Adult neurogenesis: from precursors to network and physiology. *Physiol. Rev.* 85, 523–569. doi: 10.1152/physrev.00055.2003

Anacker, C., and Hen, R. (2017). Adult hippocampal neurogenesis and cognitive flexibility—linking memory and mood. *Nat. Rev. Neurosci.* 18, 335–346. doi: 10.1038/nrn.2017.45

Bonaguidi, M. A., Song, J., Ming, G. L., and Song, H. (2012). A unifying hypothesis on mammalian neural stem cell properties in the adult hippocampus. *Curr. Opin. Neurobiol.* 22, 754–761. doi: 10.1016/j.conb.2012.03.013

Briones, T. L., and Woods, J. (2013). Chronic binge-like alcohol consumption in adolescence causes depression-like symptoms possibly mediated by the effects of BDNF on neurogenesis. *Neuroscience* 254, 324–334. doi: 10.1016/j.neuroscience.2013.09.031

Broadwater, M. A., Liu, W., Crews, F. T., and Spear, L. P. (2014). Persistent loss of hippocampal neurogenesis and increased cell death following adolescent, but not adult, chronic ethanol exposure. *Dev. Neurosci.* 36, 297–305. doi: 10.1159/000362874

Brown, J. P., Couillard-Despres, S., Cooper-Kuhn, C. M., Winkler, J., Aigner, L., and Kuhn, H. G. (2003). Transient expression of doublecortin during adult neurogenesis. *J. Comp. Neurol.* 467, 1–10. doi: 10.1002/cne.10874

Campbell, J. C., Stipcevic, T., Flores, R. E., Perry, C., and Kippin, T. E. (2014). Alcohol exposure inhibits adult neural stem cell proliferation. *Exp Brain Res.* 232, 2775–2784. doi: 10.1007/s00221-014-3958-1

Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., et al. (2005). Doublecortin expression levels in adult brain reflect neurogenesis. *Eur. J. Neurosci.* 21, 1–14. doi: 10.1111/j.1460-9568.2004.03813.x

Crews, F. T., Mdzinarishvili, A., Kim, D., He, J., and Nixon, K. (2006). Neurogenesis in adolescent brain is potently inhibited by ethanol. *Neuroscience* 137, 437–445. doi: 10.1016/j.neuroscience.2005.08.090

Crews, F. T., Nixon, K., and Wilkie, M. E. (2004). Exercise reverses ethanol inhibition of neural stem cell proliferation. *Alcohol* 33, 63–71. doi: 10.1016/j.alcohol.2004.04.005

Crews, F. T., Vetreno, R. P., Broadwater, M. A., and Robinson, D. L. (2016). Adolescent alcohol exposure persistently impacts adult neurobiology and behavior. *Pharmacol. Rev.* 68, 1074–1109. doi: 10.1124/pr.115.012138

Dahl, R. E. (2004). Adolescent development and the regulation of behavior and emotion: introduction to part VIII. *Ann. N Y Acad. Sci.* 1021, 294–295. doi: 10.1196/annals.1308.034

Ehlers, C. L., Liu, W., Wills, D. N., and Crews, F. T. (2013a). Periadolescent ethanol vapor exposure persistently reduces measures of hippocampal neurogenesis that are associated with behavioral outcomes in adulthood. *Neuroscience* 244, 1–15. doi: 10.1016/j.neuroscience.2013.03.058

Ehlers, C. L., Oguz, I., Budin, F., Wills, D. N., and Crews, F. T. (2013b). Peri-adolescent ethanol vapor exposure produces reductions in hippocampal volume that are correlated with deficits in prepulse inhibition of the startle. *Alcohol. Clin. Exp. Res.* 37, 1466–1475. doi: 10.1111/acer.12125

Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., et al. (1998). Neurogenesis in the adult human hippocampus. *Nat. Med.* 4, 1313–1317. doi: 10.1038/3305

Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., et al. (2014). Neurogenesis in the striatum of the adult human brain. *Cell* 156, 1072–1083. doi: 10.1016/j.cell.2014.01.044

Fan, L. W., and Pang, Y. (2017). Dysregulation of neurogenesis by neuroinflammation: key differences in neurodevelopmental and neurological disorders. *Neural Regen. Res.* 12, 366–371. doi: 10.4103/1673-5374.202926

Favaro, R., Valotta, M., Ferri, A. L., Latorre, E., Mariani, J., Giachino, C., et al. (2009). Hippocampal development and neural stem cell maintenance require Sox2-dependent regulation of Shh. *Nat. Neurosci.* 12, 1248–1256. doi: 10.1038/nn.2397

Gould, E., Reeves, A. J., Graziano, M. S., and Gross, C. G. (1999). Neurogenesis in the neocortex of adult primates. *Science* 286, 548–552. doi: 10.1126/science.286.5439.548

Hansson, A. C., Nixon, K., Rimondini, R., Damadzic, R., Sommer, W. H., Eskay, R., et al. (2010). Long-term suppression of forebrain neurogenesis and loss of neuronal progenitor cells following prolonged alcohol dependence in rats. *Int. J. Neuropsychopharmacol.* 13, 583–593. doi: 10.1017/s1461145710000246

He, J., and Crews, F. T. (2007). Neurogenesis decreases during brain maturation from adolescence to adulthood. *Pharmacol. Biochem. Behav.* 86, 327–333. doi: 10.1016/j.pbb.2006.11.003

He, J., Nixon, K., Shetty, A. K., and Crews, F. T. (2005). Chronic alcohol exposure reduces hippocampal neurogenesis and dendritic growth of newborn neurons. *Eur. J. Neurosci.* 21, 2711–2720. doi: 10.1111/j.1460-9568.2005.04120.x

Herrera, D. G., Yague, A. G., Johnsen-Soriano, S., Bosch-Morell, F., Collado-Morente, L., Muriach, M., et al. (2003). Selective impairment of hippocampal neurogenesis by chronic alcoholism: protective effects of an antioxidant. *Proc. Natl. Acad. Sci. U S A* 100, 7919–7924. doi: 10.1073/pnas.1230907100

Hodge, R. D., and Hevner, R. F. (2011). Expression and actions of transcription factors in adult hippocampal neurogenesis. *Dev. Neurobiol.* 71, 680–689. doi: 10.1002/dneu.20882

Hodge, R. D., Kahoud, R. J., and Hevner, R. F. (2012). Transcriptional control of glutamatergic differentiation during adult neurogenesis. *Cell. Mol. Life Sci.* 69, 2125–2134. doi: 10.1007/s00018-011-0916-y

Hodge, R. D., Kowalczyk, T. D., Wolf, S. A., Encinas, J. M., Rippey, C., Enikolopov, G., et al. (2008). Intermediate progenitors in adult hippocampal neurogenesis: Tbr2 expression and coordinate regulation of neuronal output. *J. Neurosci.* 28, 3707–3717. doi: 10.1523/JNEUROSCI.4280-07.2008

Kempermann, G., Song, H., and Gage, F. H. (2015). Neurogenesis in the Adult Hippocampus. *Cold Spring Harb. Perspect. Biol.* 7:a018812. doi: 10.1101/cshperspect.a018812

Kempermann, G., Wiskott, L., and Gage, F. H. (2004). Functional significance of adult neurogenesis. *Curr. Opin. Neurobiol.* 14, 186–191. doi: 10.1016/j.conb.2004.03.001

Kriegstein, A., and Alvarez-Buylla, A. (2009). The glial nature of embryonic and adult neural stem cells. *Annu. Rev. Neurosci.* 32, 149–184. doi: 10.1146/annurev.neuro.051508.135600

Kuhn, H. G., Dickinson-Anson, H., and Gage, F. H. (1996). Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J. Neurosci.* 16, 2027–2033.

Lavado, A., Lagutin, O. V., Chow, L. M., Baker, S. J., and Oliver, G. (2010). Prox1 is required for granule cell maturation and intermediate progenitor maintenance during brain neurogenesis. *PLoS Biol.* 8:e1000460. doi: 10.1371/journal.pbio.1000460

Lavado, A., and Oliver, G. (2007). Prox1 expression patterns in the developing and adult murine brain. *Dev. Dyn.* 236, 518–524. doi: 10.1002/dvdy.21024

Lindsey, B. W., and Tropepe, V. (2006). A comparative framework for understanding the biological principles of adult neurogenesis. *Prog. Neurobiol.* 80, 281–307. doi: 10.1016/j.pneurobio.2006.11.007

Liu, W., and Crews, F. T. (2015). Adolescent intermittent ethanol exposure enhances ethanol activation of the nucleus accumbens while blunting the prefrontal cortex responses in adult rat. *Neuroscience* 293, 92–108. doi: 10.1016/j.neuroscience.2015.02.014

Lledo, P. M., Alonso, M., and Grubb, M. S. (2006). Adult neurogenesis and functional plasticity in neuronal circuits. *Nat. Rev. Neurosci.* 7, 179–193. doi: 10.1038/nrn1867

Mandyam, C. D., and Koob, G. F. (2012). The addicted brain craves new neurons: putative role for adult-born progenitors in promoting recovery. *Trends Neurosci.* 35, 250–260. doi: 10.1016/j.tins.2011.12.005

McClain, J. A., Hayes, D. M., Morris, S. A., and Nixon, K. (2011). Adolescent binge alcohol exposure alters hippocampal progenitor cell proliferation in rats: effects on cell cycle kinetics. *J. Comp. Neurol.* 519, 2697–2710. doi: 10.1002/cne.22647

Ming, G. L., and Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* 70, 687–702. doi: 10.1016/j.neuron.2011.05.001

Ming, G. L., and Song, H. (2005). Adult neurogenesis in the mammalian central nervous system. *Annu. Rev. Neurosci.* 28, 223–250. doi: 10.1146/annurev.neuro.28.051804.101459

Morris, S. A., Eaves, D. W., Smith, A. R., and Nixon, K. (2010). Alcohol inhibition of neurogenesis: a mechanism of hippocampal neurodegeneration in an adolescent alcohol abuse model. *Hippocampus* 20, 596–607. doi: 10.1002/hipo.20665

Nixon, K., and Crews, F. T. (2002). Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. *J. Neurochem.* 83, 1087–1093. doi: 10.1046/j.1471-4159.2002.01214.x

Nixon, K., and Crews, F. T. (2004). Temporally specific burst in cell proliferation increases hippocampal neurogenesis in protracted abstinence from alcohol. *J. Neurosci.* 24, 9714–9722. doi: 10.1523/JNEUROSCI.3063-04.2004

Nixon, K., Kim, D. H., Potts, E. N., He, J., and Crews, F. T. (2008). Distinct cell proliferation events during abstinence after alcohol dependence: microglia proliferation precedes neurogenesis. *Neurobiol. Dis.* 31, 218–229. doi: 10.1016/j.nbd.2008.04.009

O'Malley, P. M., Johnston, L. D., and Bachman, J. G. (1998). Alcohol use among adolescents. *Alcohol. Health Res. World* 22, 85–93.

Paxinos, G., and Watson, C. (1997). *The Rat Brain in Stereotaxic Coordinates.* San Diego, CA: Academic Press.

Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J. S., et al. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 55, 453–462. doi: 10.1002/glia.20467

Rao, M. S., and Shetty, A. K. (2004). Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. *Eur. J. Neurosci.* 19, 234–246. doi: 10.1111/j.0953-816x.2003.03123.x

Rolls, A., Shechter, R., London, A., Ziv, Y., Ronen, A., Levy, R., et al. (2007). Toll-like receptors modulate adult hippocampal neurogenesis. *Nat. Cell Biol.* 9, 1081–1088. doi: 10.1038/ncb1629

Sakharkar, A. J., Vetreno, R. P., Zhang, H., Kokare, D. M., Crews, F. T., and Pandey, S. C. (2016). A role for histone acetylation mechanisms in adolescent alcohol exposure-induced deficits in hippocampal brain-derived neurotrophic factor expression and neurogenesis markers in adulthood. *Brain Struct. Funct.* 221, 4691–4703. doi: 10.1007/s00429-016-1196-y

Smith, L. K., He, Y., Park, J. S., Bieri, G., Snethlage, C. E., Lin, K., et al. (2015). β2-microglobulin is a systemic pro-aging factor that impairs cognitive function and neurogenesis. *Nat. Med.* 21, 932–937. doi: 10.1038/nm.3898

Song, J., Olsen, R. H., Sun, J., Ming, G. L., and Song, H. (2016). Neuronal circuitry mechanisms regulating adult mammalian neurogenesis. *Cold Spring Harb. Perspect. Biol.* 8:a018937. doi: 10.1101/cshperspect.a018937

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., et al. (2013). Dynamics of hippocampal neurogenesis in adult humans. *Cell* 153, 1219–1227. doi: 10.1016/j.cell.2013.05.002

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* 24, 417–463. doi: 10.1016/s0149-7634(00)00014-2

Staples, M. C., and Mandyam, C. D. (2016). Thinking after drinking: impaired hippocampal-dependent cognition in human alcoholics and animal models of alcohol dependence. *Front. Psychiatry* 7:162. doi: 10.3389/fpsyt.2016.00162

Stevenson, J. R., Schroeder, J. P., Nixon, K., Besheer, J., Crews, F. T., and Hodge, C. W. (2009). Abstinence following alcohol drinking produces depression-like behavior and reduced hippocampal neurogenesis in mice. *Neuropsychopharmacology* 34, 1209–1222. doi: 10.1038/npp.2008.90

Urbán, N., and Guillemot, F. (2014). Neurogenesis in the embryonic and adult brain: same regulators, different roles. *Front. Cell. Neurosci.* 8:396. doi: 10.3389/fncel.2014.00396

Vetreno, R. P., and Crews, F. T. (2012). Adolescent binge drinking increases expression of the danger signal receptor agonist HMGB1 and Toll-like receptors in the adult prefrontal cortex. *Neuroscience* 226, 475–488. doi: 10.1016/j.neuroscience.2012.08.046

Vetreno, R. P., and Crews, F. T. (2014). Current hypotheses on the mechanisms of alcoholism. *Handb. Clin. Neurol.* 125, 477–497. doi: 10.1016/b978-0-444-62619-6.00027-6

Vetreno, R. P., and Crews, F. T. (2015). Binge ethanol exposure during adolescence leads to a persistent loss of neurogenesis in the dorsal and ventral hippocampus that is associated with impaired adult cognitive functioning. *Front. Neurosci.* 9:35. doi: 10.3389/fnins.2015.00035

Vetreno, R. P., Qin, L., and Crews, F. T. (2013). Increased receptor for advanced glycation end product expression in the human alcoholic prefrontal cortex is linked to adolescent drinking. *Neurobiol. Dis.* 59, 52–62. doi: 10.1016/j.nbd.2013.07.002

Wechsler, H., Dowdall, G. W., Davenport, A., and Castillo, S. (1995). Correlates of college student binge drinking. *Am. J. Public Health* 85, 921–926. doi: 10.2105/ajph.85.7.921

Yousef, H., Conboy, M. J., Morgenthaler, A., Schlesinger, C., Bugaj, L., Paliwal, P., et al. (2015). Systemic attenuation of the TGF-β pathway by a single drug simultaneously rejuvenates hippocampal neurogenesis and myogenesis in the same old mammal. *Oncotarget* 6, 11959–11978. doi: 10.18632/oncotarget.3851

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: 04 May 2017
doi: 10.3389/fnbeh.2017.00078

Adolescent Ethanol Exposure Leads to Stimulus-Specific Changes in Cytokine Reactivity and Hypothalamic-Pituitary-Adrenal Axis Sensitivity in Adulthood

*Andrew S. Vore[1], Tamara Doremus-Fitzwater[2], Anny Gano[1] and Terrence Deak[1]**

[1]*Behavioral Neuroscience Program, Department of Psychology, Binghamton University, Binghamton, NY, USA,* [2]*Department of Psychology, Ithaca College, Ithaca, NY, USA*

Adolescent alcohol use comprises a significant public health concern and is often characterized by binge-like consumption patterns. While ethanol exposure in adulthood has been shown to alter the stress response, including the Hypothalamic–Pituitary–Adrenal (HPA) axis, few studies have examined whether binge-like ethanol exposure during adolescence results in enduring changes in HPA axis sensitivity in adulthood. In the present studies, adolescent Sprague-Dawley rats were given intragastric (i.g.) intubations of ethanol (4 g/kg) or vehicle once per day for three consecutive days, beginning on postnatal day (P) 30 (±1). This exposure was followed by a 2-day period of rest/withdrawal. Rats received a total of either two (Experiments 1, 2 and 3) or four (Experiment 4) cycles of ethanol exposure and were subsequently allowed to age normally until adulthood. In Experiment 1, adult, (P71–75), ethanol- or vehicle-exposed rats received a 60 min restraint stress challenge. In Experiment 2, rats received a 50 μg/kg injection of lipopolysaccharide (LPS). In Experiment 3, rats received a challenge of 2.5 g/kg ethanol (intraperitoneally; i.p.). In Experiment 4, male and female ethanol- or vehicle- exposed rats received a 50 μg/kg injection of LPS. In all experiments, blood samples were collected for later assessment of corticosterone (CORT), blood ethanol concentrations (BECs), and the cellular fraction of blood was analyzed for cytokine gene expression. As expected, all three challenges led to a time-dependent surge in CORT. Gene expression analyses of cytokines (Interleukin [IL]-6, IL-1β, and Tumor necrosis factor alpha [TNFα]) from the cellular fraction of blood revealed unique, time-dependent patterns of cytokine expression depending upon the nature of the adult challenge incurred (restraint, LPS, or EtOH). Importantly, adolescent ethanol exposure led to attenuated restraint and LPS-induced cytokine

OPEN ACCESS

Edited by:
Mary M. Torregrossa, University of Pittsburgh, USA

Reviewed by:
Jessica R. Barson, Drexel University College of Medicine, USA
Heather Trantham-Davidson, Medical University of South Carolina, USA

****Correspondence:***
Terrence Deak
tdeak@binghamton.edu

Received: *07 February 2017*
Accepted: *12 April 2017*
Published: *04 May 2017*

Citation:
Vore AS, Doremus-Fitzwater T, Gano A and Deak T (2017) Adolescent Ethanol Exposure Leads to Stimulus-Specific Changes in Cytokine Reactivity and Hypothalamic-Pituitary-Adrenal Axis Sensitivity in Adulthood. Front. Behav. Neurosci. 11:78. doi: 10.3389/fnbeh.2017.00078

Abbreviations: ACTH, Adrenocorticotropic hormone; AIE, Adolescent intermittent ethanol; ANOVA, Analysis of variance; AUD, Alcohol use disorder; AVP, Vasopressin; BALs, Blood alcohol levels; BECs, Blood ethanol concentrations; C, Celcius; CORT, Corticosterone; CRH, Corticotropin-releasing hormone; CTA, Conditioned taste aversion; HPA, Hypothalamic-Pituitary-Adrenal; HSF1, Heat shock factor protein 1; Hsp70, 70 kilodalton heat shock protein; IACUC, Institutional animal care and use committee; i.g., Intragastric; IκBα, Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IL, Interleukin; i.p., Intraperitoneal; LPS, Lipopolysaccharide; MYD88, Myeloid differentiation primary response gene; NFκB, Nuclear factor kappa-light-chain-enhancer of activated B cells; P, Postnatal day; PAE, Prenatal alcohol exposure; PVN, Paraventricular nucleus of the hypothalamus; RT-PCR, Real time reverse transcription polymerase chain reaction; TLR4, Toll-like receptor 4.

expression in males, whereas female rats displayed an absence of cytokine alterations, and a tendency toward heightened HPA axis reactivity. These findings suggest that adolescent ethanol exposure may cause lasting alterations in cytokine regulation and HPA axis sensitivity that (a) persist into adulthood; (b) may vary depending on the nature of the challenge incurred during adulthood; and that (c) are sex-specific.

Keywords: adolescent intermittent ethanol, rat, sex differences, HPA axis, cytokine

INTRODUCTION

Alcohol use and abuse disorders comprise a substantial public health and financial burden, resulting in an estimated 3.3 million deaths per year globally and $223.5 billion in financial burden to the United States alone (Centers for Disease Control and Prevention, 2014; World Health Organization, 2014). It has been estimated that almost 75% of this impact can be attributed to binge drinking, defined as ethanol consumption that results in Blood Ethanol Concentrations (BECs) of 0.08 mg/dL or higher (Centers for Disease Control and Prevention, 2014). Alcohol consumption is prevalent in adolescence and a large percentage of adolescent individuals that consume alcohol exhibit binge-like consumption patterns. These patterns of intake typically begin in early to late adolescence, peaking in early adulthood (21–25 years of age), with consumption tapering across adulthood in individuals that exhibit normative alcohol use. Binge-like alcohol consumption can be particularly harmful, and early, high frequency binge drinking has been shown to correlate with later alcohol use disorder (AUD) development (Grant and Dawson, 1997; Crews et al., 2016).

Adolescence is a time of unique sensitivity to the consequences of ethanol exposure. Mirroring what is seen in humans, adolescent rodents consume notably higher quantities of ethanol than adult counterparts (Doremus et al., 2005; Spear, 2016). Adolescent rats show altered reactivity to ethanol exposure with increased sensitivity to the positive effects of ethanol exposure and decreased sensitivity to the negative effects (Doremus et al., 2003; Varlinskaya and Spear, 2004a). Adolescent animals have also exhibited increased locomotor sensitization to ethanol and higher sensitivity to the motivational effects of ethanol (Pautassi et al., 2008) compared to adult counterparts that received similar quantities of ethanol. This differential reaction to a spectrum of consequences of ethanol exposure likely contributes to adolescent animals consuming higher quantities of ethanol, since insensitivity to negative consequences of high dose ethanol use (sedation, motor impairment, aversion) and hypersensitivity to various positive elements would seem to promote future ethanol consumption (Varlinskaya and Spear, 2004b).

A more concerning element of adolescent ethanol exposure is its ability to cause potentially lifelong changes in development. Adolescence is a critical time of neural refinement during which the brain undergoes a series of structural and functional changes. Increases in myelination, decreases in gray matter volumes, and a reduced total number of synapses indicate that adolescence is a time during which novel connections are being made and prior connections are being refined (Giedd, 2008). Underlying neurodevelopmental changes during adolescence may confer added potential for insult from substances such as ethanol (Guerri and Pascual, 2010). A common functional consequence of adolescent ethanol exposure is the retention of an adolescent-like phenotype into adulthood. This "locking in" of the adolescent phenotype into adulthood has been shown to occur in a variety of phenomena and is not seen in adult rats following exposure to similar quantities of ethanol. Behavioral phenomenon such as resilience to withdrawal-induced anxiety (Doremus et al., 2003), reduced sensitivity to the sedative effects of ethanol (Matthews et al., 2008), as well as reduced sensitivity to conditioned taste aversion (CTA) induction (Diaz-Granados and Graham, 2007) have all demonstrated this locking in-like effect following adolescent ethanol exposure when challenged during adulthood. This phenomenon has also been demonstrated to extend beyond just ethanol challenge effects. Observed baseline differences in impulsivity, novelty seeking, and many other phenomena have also shown to linger into adulthood following adolescent ethanol exposure (see Spear and Swartzwelder, 2014 for a recent review).

Ethanol alone is a potent stimulator of the hypothalamic-pituitary-adrenal (HPA) axis as demonstrated by dose-dependent increases in plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT; Rivier et al., 1984; Doremus-Fitzwater et al., 2014). Importantly, cross sectional studies comparing adolescent and adult rats have indicated that stressors such as hypoxia and restraint stress result in prolonged ACTH and CORT responses when compared to adult rodents (Romeo et al., 2016), though differences in peak CORT responses were not observed. A similar difference occurs following ethanol challenge as well, where adolescents exhibit delayed recovery (or shutoff) of the axis relative to adults. While this effect was more pronounced in female rats, both male and female adolescent rats have shown increased CORT at several points after ethanol when compared to adult animals challenged with the same dose of ethanol (Willey et al., 2012). In contrast, prior exposure to ethanol in adolescence has been shown to blunt the HPA-axis response to subsequent ethanol exposure up to 24 days later (Lee and Rivier, 2003). Thus, intrinsic differences in the HPA axis response to stress between adolescents and adults are now well-precedented in the literature, though the mechanisms underlying these differences remain obscure.

Beyond effects on the HPA axis, ethanol also has substantial effects on cytokine release and the immune system. Cytokines

are a class of proteins that play a key role in propagation of the inflammatory response evoked by immunological threats, tissue damage, and other challenges such as stress (see Deak et al., 2015 for a recent review). In particular, the pro-inflammatory cytokines IL-1, TNF-α and IL-6 are rapidly induced by immune challenges or psychological stress, and often act synergistically with one another to influence host defense and tissue repair. These cytokines also potently induce HPA axis activation, with glucocorticoids in turn influencing subsequent cytokine expression and activity. In this way, HPA-immune interactions serve as an exemplar of bi-directional communication between the brain and the immune system.

From a longitudinal perspective, recent studies have begun to explore whether the adolescent period also represents a critical period during which ethanol exposure can produce life-long changes in HPA axis sensitivity and immune function. For instance, adolescent ethanol exposure (6 exposures to 3 g/kg intraperitoneal (i.p.)) in male rats resulted in reduced baseline CORT and sensitized CORT release following acute or repeated ethanol challenge in adulthood (Przybycien-Szymanska et al., 2010, 2011). These changes in CORT patterns correlated with decreased vasopressin (AVP) and increased corticotropin-releasing hormone (CRH) within the paraventricular nucleus of the hypothalamus (PVN) of adolescent ethanol exposed rats following acute and binge ethanol challenge, suggesting alterations in central regulation of HPA axis regulation. In contrast, a more recent study showed that adolescent ethanol exposure (daily exposure to ethanol vapor for 6 h maintaining blood alcohol levels, BALs of 200 mg/dL) blunted the HPA axis response to adult ethanol challenge (3.2–4.5 g/kg intragastric (i.g.); Allen et al., 2016). Though dose, route and frequency of ethanol exposure during adolescence are likely explanations for the differences between these studies, a more fundamental question regarding the potential for stressor-specific outcomes in adults with a history of adolescent ethanol exposure still remains.

With this in mind, our primary goal was to establish how binge-like ethanol exposure during adolescence would alter cytokine and HPA axis responses to stress challenges incurred during adulthood. In doing so, we adopted an adolescent alcohol exposure procedure that has previously been shown to produce changes lasting to adulthood in HPA axis regulation, including alterations in CORT release as well as changes in AVP and CRH following binge and acute ethanol exposure (Przybycien-Szymanska et al., 2010, 2011). Following intermittent binge ethanol exposure in adolescence, three distinct studies were executed in which rats were given different challenges in adulthood to test whether axis sensitivity may depend upon the nature/modality of the stress challenge experienced as adults. Thus, adult rats with a history of adolescent ethanol exposure were challenged by restraint, lipopolysaccharide (LPS) administration, or adult ethanol exposure (Experiments 1–3 respectively). Though our primary interest was HPA axis sensitivity indicated by plasma CORT responses, we also had a secondary interest in how adolescent intermittent ethanol (AIE) would influence the expression of cytokines within the cellular fraction of whole blood as a secondary index of stress sensitivity, and due to the potential mechanistic role that plasma cytokines might play in HPA axis responses (Rivest et al., 2000; Szelényi, 2001). In human research, analysis of peripheral cytokine levels has been shown to be useful in monitoring general immune activation and to correlate well with expected levels of neuroimmune activation (Sullivan et al., 2006). In addition, elevated levels of peripheral pro-inflammatory cytokines have been shown to correlate with psychiatric illness in a specific subset of individuals (Fillman et al., 2016). Finally, due to the literature demonstrating that male animals display profound HPA axis blunting following adolescent ethanol exposure, we examined potential sex differences in HPA axis reactivity following adolescent alcohol exposure.

MATERIALS AND METHODS

General Methods

Subjects

In the first three studies, male Sprague-Dawley rats ($N = 50$) were purchased from Harlan Laboratories and shipped at postnatal day (P) 22 ± 1. In the fourth study, male and female Sprague-Dawley rats ($N = 30$) were bred in-house using breeders originally derived from Harlan (Envigo). With day of birth being deemed postnatal day (P) 0, rats were allowed to develop normally until P21 at which point animals were weaned and pair-housed with a same-sex partner from a different litter in standard Plexiglas bins. In all cases, rats were given 1 week to acclimate to colony conditions and were then handled (P29) for 2–3 min to acclimate them to human contact prior to experimentation. Colony conditions were maintained at 22 ± 1°C on a 12:12 light:dark cycle. Animals were pair-housed in standard Plexiglas bins with *ad libitum* access to food and water. Rats housed in pairs were always assigned to the same experimental group. In all experiments, rats were treated in accordance with Public Health Service (PHS) policy and all experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Binghamton University.

Adolescent Alcohol Exposure Procedure

Starting at early adolescence (P30–32) rats received 4.0 g/kg intragastric (i.g.) intubations of ethanol or an equivolume dose of vehicle (tap water) once per day at approximately 1000 h. When administered i.g., ethanol solution was prepared daily using 95% ethanol stock diluted to a final working concentration of 20% in tap water. Weights were taken daily at least 90 min prior to intubations. Animals received three consecutive days of intubations followed by a 2-day period of rest/withdrawal during which animals remained undisturbed in their home cage. This cycle of intubations (3 days on, 2 days off) was then repeated a second time (Experiments 1–3), reaching its conclusion on P40–42. In Experiment 4, this cycle was administered four times, spanning ages of approximately P30–P50. Rats were then reared to adulthood without further manipulation until weights were again collected on P73–75.

At this time, rats were randomly divided into cohorts for independent experiments.

Drug Preparations

When injected i.p., ethanol was prepared in a 20% solution mixed fresh daily using 95% ethanol stock with sterile physiological saline used as the vehicle. LPS (from serotype E0111:B4; Sigma Chemical Co.) solution was initially diluted 1.0 mg/mL using sterile (pyrogen-free) physiological saline and stored in frozen aliquots at −20°C until needed. On the day of experimentation, an aliquot of LPS was thawed and mixed fresh daily to the working concentration of 50 μg/mL LPS and delivered on a 1 mL/kg basis, also in pyrogen-free physiological saline.

Blood Sampling and Processing Procedure

In each experiment, rats were removed from their home cage and briefly placed into restraint tubes for the acquisition of blood samples. Blood samples were collected using the tail clip method in which the last 0.5–1.0 mm of the tail was transected. The tail was then stroked until whole blood was obtained as previously described (Hueston and Deak, 2014b). Rats were immediately returned to their home cage following each time point except in conditions where rats were assigned to the restraint stress condition. Animals were returned to their home cage within 2 min after first intrusion. Following collection, plasma was immediately separated through refrigerated centrifugation and stored at −20°C until analysis. RNA was extracted from the remaining fractionated layers of leukocytes and erythrocytes. Prior studies have shown that RNA extraction of whole blood is possible (Schwochow et al., 2012). Due to elimination of the plasma fraction and the lack of nuclei or cell organelles such as mitochondria in erythrocytes, it was reasoned that extraction on the combined buffy layer and erythrocyte fractions would largely reflect gene expression changes in leukocytes. The combined layers were stored at −80°C until analysis of expression of blood cytokine levels using Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR).

Corticosterone Measures

Quantitative determinations of plasma CORT levels were assessed using a CORT EIA kit (Cat No: ADI-901-097; Enzo Life Sciences, Farmingdale, NY, USA) as described in Hueston and Deak (2014a) with an inter-assay variability of 7.616% and an assay sensitivity of 27.0 pg/mL. Manufacturer's instructions were followed for all steps except that samples were heat inactivated by immersion in 75°C water for a period of 60 min to denature endogenous CBG.

Reverse-Transcription Polymerase Chain Reaction

All RT-PCR was conducted using procedures described in previous work (Hueston and Deak, 2014a). Blood pellets were stored at −80°C until the time of RNA extraction. Total RNA in the sample was homogenized in Trizol RNA reagent (Invitrogen) using a TissueLyser and 5 mm stainless steel beads (Qiagen). Total RNA was then extracted through the use of RNeasy mini columns (Qiagen) following manufacturer's instructions. RNA yield and purity were evaluated using a Nanodrop system (ThermoScientific) and cDNA was synthesized using a QuantiTect reverse transcription kit (Qiagen). All RT-PCR was run using a CFX384 real-time PCR detection system (Bio-Rad). All results were normalized to β-Actin as a housekeeper gene. Primer sequences for all targets run can be found in **Table 1**.

Blood Ethanol Concentrations

BECs were determined from 5 μL samples of plasma using an Analox AM-1 alcohol analyzer (Analox Instruments, Lunenburg, MA, USA; as described in Doremus-Fitzwater et al., 2015). The machine was calibrated using a 200 mg/dL standard and tested every 15 samples using an alcohol quality control standard of known concentration purchased from Analox Instruments. Samples below 10–15 mg/dL were within a range considered to be noise and as such were interpreted as 0 values.

Statistical Analyses

All analyses of CORT levels, BECs and cytokine expression were analyzed using a mixed factorial ANOVA ($p < 0.05$). Fisher's Least Significant Difference ($p < 0.05$) was used as a *post hoc* examination in instances where two way interactions were noted to identify loci of significant difference. Outliers were defined as data points that were more extreme than ±2 standard deviations from a given experimental group's mean. If an outlier was identified within a given PCR reaction, it was excluded from that specific analysis but not across the remainder of the gene targets. Before analyzing individual cytokine data, β-Actin was examined to determine differences that may exist between groups in expression of this target gene of reference. All gene expression was reported quantified relative to expression of this reference gene.

TABLE 1 | Primer sequences and accession numbers.

Primer	Accession number	Oligo	Sequence
β-Actin	NM_031144.3	Forward	5′-GTCGTACCACTGGCATTGTG-3′
		Reverse	5′-GCCATCTCTTGCTCGAAGTC-3′
IL-6	NM_012589	Forward	5′-TAGTCCTTCCTACCCCAACTTCC-3′
		Reverse	5′-TTGGTCCTTAGCCACTCCTTC-3′
IL-1β	NM_031512	Forward	5′-AGGACCCAAGCACCTTCTTT-3′
		Reverse	5′-AGACAGCACGAGGCATTTTT-3′
TNF-α	NM_012675	Forward	5′-GGGGCCACCACGCTCTTCTG-3′
		Reverse	5′-CGACGTGGGCTACGGGTTG-3′
IκBα	NM_080899	Forward	5′-CTGTTGAAGTGTGGGGCTGA-3′
		Reverse	5′-AGGGCAACTCATCTTCCGTG-3′

Experiment 1: Axis Reactivity to Restraint Stress Challenge Following Adolescent Ethanol Exposure

In the first experiment, restraint stress was chosen as a challenge due to its ability to reliably produce activation of the HPA axis and for reported differences between adolescent and adult CORT responses to restraint stress (Romeo et al., 2016). Male rats (n = 6–8 per group; N = 14) were given two cycles of intermittent ethanol exposure or vehicle intubations as described above. At adulthood (P73–75), rats from both groups were placed into clear Plexiglas restraint tubes and a baseline blood sample was collected immediately. Rats remained in restraint and subsequent blood samples were collected at 15, 30 and 60 min time points. Immediately after the 60 min time point, rats were returned to their home cage and a final recovery sample was collected 60 min later (i.e., 120 min following stress onset; see **Figure 1A**).

Experiment 2: Axis Reactivity to Lipopolysaccharide Challenge Following Adolescent Ethanol Exposure

In the second experiment, LPS challenge was chosen as a representative, immune-based challenge that is known to activate the HPA axis through cytokine-mediated mechanisms. Male rats (n = 8 per group; N = 16) were given two cycles of intermittent ethanol exposure or vehicle intubations as described above. During adulthood (P73–75) rats were injected with 50 μg/kg (i.p.) LPS or equivolume vehicle. Blood was collected at baseline, 45, 90 and 180 min post injection.

Experiment 3: Axis Reactivity to Ethanol Challenge Following Adolescent Ethanol Exposure

In the third experiment, rats were given an acute ethanol challenge during adulthood to test for durable changes in HPA axis sensitivity to the same challenge(s) utilized during adolescence, and to provide a referent to other published studies (Przybycien-Szymanska et al., 2010, 2011; Allen et al., 2016). Male rats (n = 8–10 per group; N = 18) were given two cycles of intermittent ethanol exposure or vehicle intubations as described above. During adulthood (P73–75), the test day began with collection of a baseline blood sample, after which rats were given a 2.5 g/kg i.p. ethanol or equivolume vehicle. Subsequent blood samples were collected 30, 60, 120 and 240 min post injection (see **Figure 1A**).

Experiment 4: Sex-Differences in the Consequences of Adult LPS Challenge Following Adolescent Ethanol Exposure

The goals of the fourth experiment were to: (a) replicate the initial effect of reduced CORT and cytokine responses observed in adults with a history of adolescent alcohol exposure; (b) extend the period of alcohol exposure more broadly across the adolescent period; and (c) determine whether the impact of adolescent alcohol exposure on adult stress sensitivity would be sex-specific. Because early life shipping can produce variations in stress history and thereby influence stress reactivity (Laroche et al., 2009a,b; Vargas et al., 2016), rats for Experiment 4 were bred in house. Thus, in Experiment 4, rats (n = 6–8 per group; N = 30) were given four cycles of vehicle or ethanol intubations utilizing the procedure described above. After reaching adulthood, all rats received a 50 μg/kg i.p. LPS injection. Blood was collected at baseline, 45, 90, 180 and 360 min post injection.

RESULTS

Experiments 1–3

Plasma Corticosterone Response to Challenges

Data were analyzed using a mixed between-subjects (adolescent Ethanol or Vehicle exposure) and repeated-measures (time course) analysis of variance (ANOVA). Restraint challenge showed significant CORT elevation peaking around 30 min and CORT levels returning to baseline by 120 min ($F_{(4,48)}$ = 62.07, $p < 0.001$; **Figure 1B**). LPS challenge produced comparable increases in CORT across the 45 and 90 min time points, which then decreased by the 180 min time point ($F_{(3,42)}$ = 22.64, $p < 0.001$; **Figure 1C**). Adult ethanol challenge increased CORT within 30 min and sustained high CORT levels through the 120 min time point ($F_{(4,64)}$ = 70.65, $p < 0.001$). CORT still showed notable elevation at 240 min (**Figure 1D**), though the response was clearly resolving at this time point. Although no statistically significant effects of adolescent ethanol history on CORT response were observed on in response to the adult challenges, visual inspection of the data suggested a moderate attenuation of CORT in response to LPS challenge.

Blood Ethanol Concentrations Following Ethanol Challenge

As expected, rats injected with ethanol exhibited a marked and time-dependent elevation in BECs that peaked at 30–60 min and tapered over the remainder of the time course ($F_{(4,64)}$ = 200.15, $p < 0.001$; **Figure 1E**). Rats with a history of adolescent alcohol exposure showed trended higher mean BECs at both 30 and 60 min after ethanol, though this effect was not statistically significant. It should be noted that mean peak BECs were approximately 25 mg/dL higher at both time points relative to rats that received vehicle during adolescence. Potential differences in alcohol pharmacokinetics in adolescent-exposed rats could therefore be an area worth examining in more targeted studies.

Blood Pellet mRNA Expression Levels Following Restraint Challenge

While restraint challenge produced no significant alterations in IL-6 expression (**Figure 2A**), significantly increased IL-1β expression was noted at 30 min ($F_{(4,48)}$ = 10.16, $p < 0.001$) and IL-1β remained elevated throughout the remainder of the time course (**Figure 2D**). No alterations in TNF-α expression

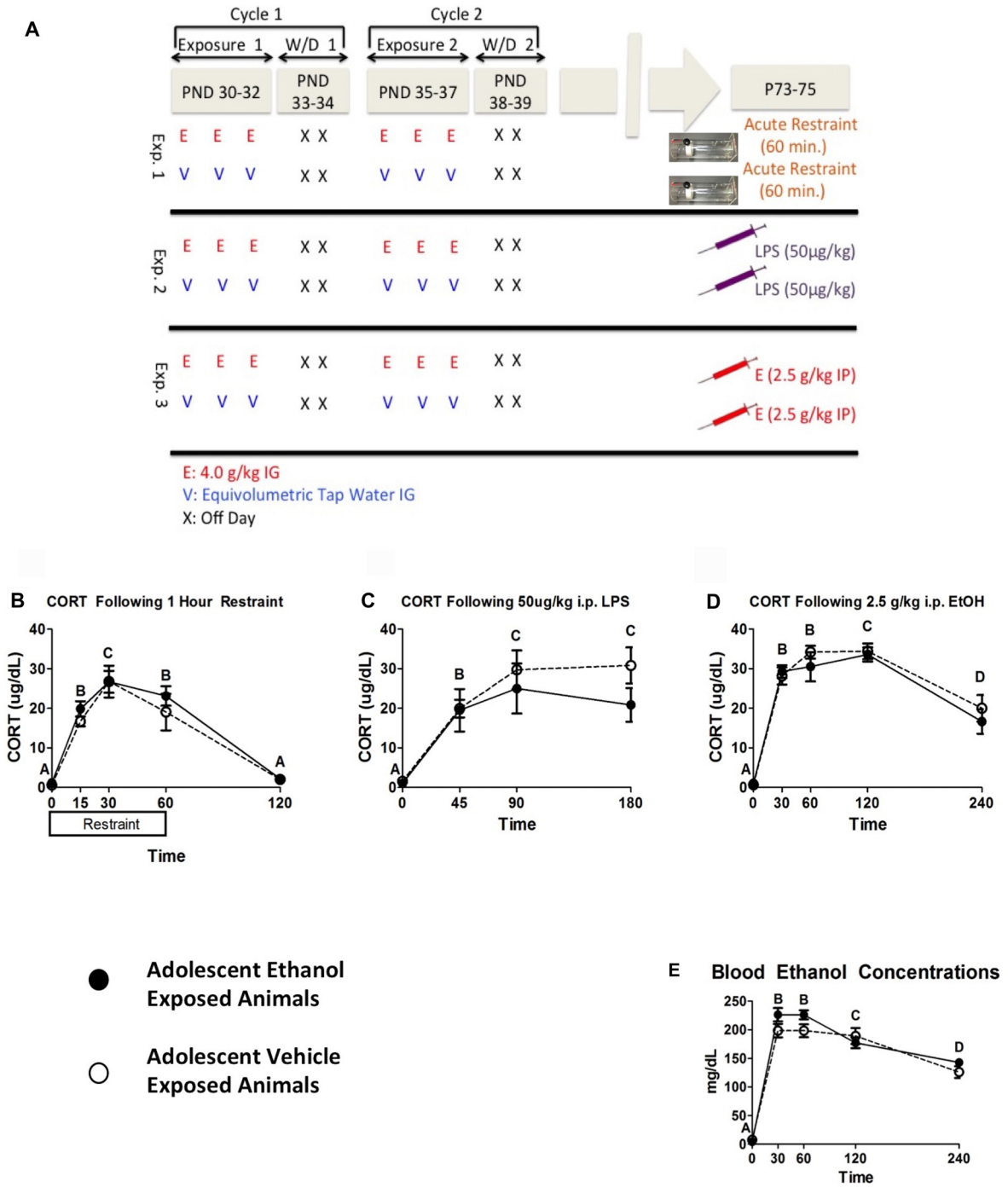


FIGURE 1 | Plasma Data from Experiments 1–3. This figure shows a schematic diagramming Experiments 1–3 **(A)**. It also shows plasma corticosterone (CORT) levels following 1 h of restraint **(B)**, 50 µg/kg i.p. lipopolysaccharide (LPS) injection **(C)**, and 2.5 g/kg i.p. EtOH injection **(D)** and corresponding blood ethanol concentrations (BECs) in the ethanol challenge group **(E)**. Plasma was sampled 0, 15, 30, 60 and 120 min following restraint, 0, 45, 90 and 180 min following LPS challenge, and 0, 30, 60, 120 and 240 min following ethanol challenge. A significant main effect of time point is denoted by a lettering system in which time points that differ in letter are significantly different.

were noted (**Figure 2G**) however; significant increases in Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) expression at 30 and 60 min ($F_{(4,48)}$ = 13.53, $p < 0.001$) were seen that had returned to baseline levels by the 120 min time point (**Figure 2J**). A pattern showing that adolescent exposure to ethanol

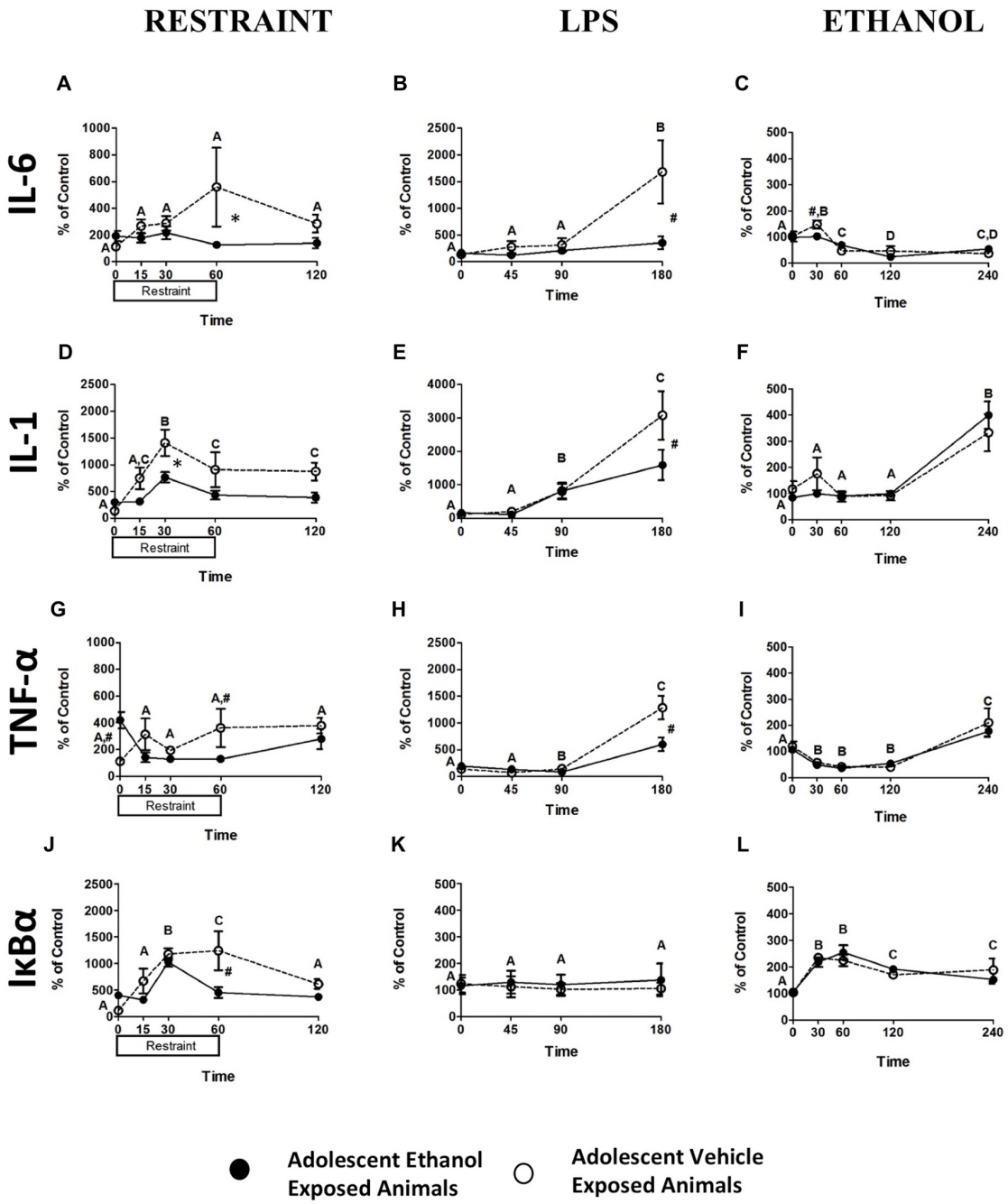


FIGURE 2 | Cytokine Expression from Experiments 1–3. This figure shows mRNA expression changes following restraint, LPS and ethanol challenge in IL-6 (**A–C** respectively), IL-1 (**D–F** respectively), TNF-α (**G–I** respectively), IκBα (**J–L** respectively). Samples were collected at 0, 15, 30, 60 and 120 min following restraint, 0, 45, 90 and 180 min following LPS challenge, and 0, 30, 60, 120 and 240 min following ethanol challenge. Data is expressed relative to ultimate control (vehicle animals at the baseline time point) normalized to β-Actin. A significant main effect of time point is denoted by a lettering system in which time points that differ in letter are significantly different. A pound sign indicates a significant interaction between adolescent exposure and challenge stimulus at a given time point. An asterisk indicates a trend interaction between adolescent exposure and challenge stimulus at a given time point. All *post hoc* comparisons were made using Fisher's Least Significant Difference *post hoc* test ($p < 0.05$).

attenuated response to adult restraint challenge was seen in IL-6, IL-1β, TNF- α, and IκBα expression. A trend showing attenuation of IL-6 expression in the adolescent ethanol exposed rats was seen at the 60-min time point ($F_{(4,48)} = 2.55$, $p = 0.051$; **Figure 2A**). A similar trend showed that IL-1β expression was attenuated in the adolescent ethanol exposed

rats at the 30-min time point ($F_{(4,48)}$ = 2.44, p = 0.06; **Figure 2D**). Finally, a similar reduction was seen at the 60-min time point in both TNF-α ($F_{(4,48)}$ = 5.49, $p < 0.005$; **Figure 2G**) and IκBα ($F_{(4,48)}$ = 4.77, $p < 0.005$; **Figure 2J**) expression.

Blood Pellet mRNA Expression Levels Following LPS Challenge

Blood analysis revealed significant changes in expression of IL-6, IL-1β and TNF-α following LPS challenge. An increase in IL-6 expression at 180 min ($F_{(3,42)}$ = 9.33, $p < 0.001$) as well as an overall decrease in IL-6 expression in rats exposed to ethanol in adolescence ($F_{(1,14)}$ = 4.96, $p < 0.05$) was noted (**Figure 2B**). A distinct increase in IL-1β expression ($F_{(3,42)}$ = 23.94, $p < 0.001$) occurred at 90 min and appeared to still be escalating at 180 min (**Figure 2E**). A similar pattern of results was found for TNF-α expression ($F_{(3,42)}$ = 56.27, $p < 0.001$) with a profound increase occurring at 180 min and a decrease in overall expression in the adolescent exposed animals ($F_{(1,14)}$ = 6.09, $p < 0.05$; **Figure 2H**). It was also found that adolescent ethanol exposure resulted in significant attenuation of IL-6 ($F_{(3,42)}$ = 5.55, $p < 0.05$; **Figure 2B**), IL-1β ($F_{(3,42)}$ = 3.07, $p < 0.05$; **Figure 2E**), and TNF-α ($F_{(3,42)}$ = 10.60, $p < 0.001$; **Figure 2H**) expression at the 180 min time point in contrast to vehicle-exposed counterparts. Neither alterations in IκBα expression resultant from LPS challenge nor any significant effects of AIE were noted (**Figure 2K**).

Blood Pellet mRNA Expression Levels Following Ethanol Challenge

Data were analyzed using a mixed between subjects (adolescent Ethanol or Vehicle exposure) and repeated measures (0, 30, 60, 120 and 240 min post-injection) ANOVA. Analysis revealed significant changes in overall IL-6, IL-1β, TNF-α and IκBα expression across time. A significant decrease in overall IL-6 expression ($F_{(4,64)}$ = 21.89, $p < 0.001$) was noted from 60 min post-challenge to 240 min (**Figure 2C**). Significant increases in IL-1β expression did not begin to occur until after 120 min showing continued escalation at the 240 min time point ($F_{(4,64)}$ = 27.40, $p < 0.001$; **Figure 2F**). TNF-α also showed a significant reduction in expression ($F_{(4,64)}$ = 39.58, $p < 0.001$) during the 30, 60 and 120 min time points and began to show rebound elevation at 240 min (**Figure 2I**). IκBα showed an increase in expression through the 60 min time point that had begun to taper by the 240 min time point ($F_{(4,64)}$ = 17.27, $p < 0.001$; **Figure 2L**). Rats that received ethanol exposure during adolescence demonstrated significantly reduced IL-6 expression in contrast to vehicle-exposed rats ($F_{(4,64)}$ = 3.03, $p < 0.05$; **Figure 2C**). No effects of adolescent ethanol exposure on IL-1β, TNF-α, or IκBα were observed.

Experiment 4 Results

Corticosterone Response to Challenge

Data were analyzed using a mixed between-subjects (adolescent Ethanol or Vehicle exposure; male or female) and repeated-measures (time course) ANOVA. Sex-specific analyses were then run using a mixed between subjects (adolescent Ethanol or Vehicle exposure) and repeated measures (time course) ANOVA. When ANOVAs for each sex were run separately, LPS challenge produced significant increases in both male and female CORT release with female animals showing overall higher levels of CORT than males. A significant main effect of time point was found in both females ($F_{(4,48)}$ = 40.86, $p < 0.001$; **Figure 3C**) as well as in males ($F_{(4,56)}$ = 22.58, $p < 0.001$; **Figure 3B**). CORT increased at the 45-min time point and remained at peak through the 360-min time point in both males ($F_{(4,56)}$ = 22.58, $p < 0.001$; **Figure 3B**) and females ($F_{(4,48)}$ = 40.86, $p < 0.001$; **Figure 3C**). Although there was a trend indicating that adolescent ethanol exposure increased CORT release following LPS challenge in female rats ($F_{(4,48)}$ = 1.68, p = 0.17), no such effect was seen in male rats (**Figures 3B,C**). A planned comparison examining CORT in female adolescent ethanol and vehicle exposed animals at the 90 min timepoint revealed a significant sensitization of LPS induced CORT in adolescent ethanol exposed animals when compared to vehicle exposed animals at the same timepoint ($t_{(12)}$ = 2.34, $p < 0.05$).

Blood Pellet mRNA Expression Levels Following LPS Challenge

When the data were analyzed using an omnibus ANOVA including sex as a variable, a significant sex by adolescent interaction was noted ($F_{(1,26)}$ = 7.20, $p < 0.05$; **Figures 4A,B**). When ANOVAs for each sex were run separately, IL-6 showed a trend in the male adolescent ethanol exposed rats showing attenuation of expression in contrast to vehicle exposed animals ($F_{(4,56)}$ = 2.04, p = 0.10; **Figure 4A**). No such effects were noted in female animals (**Figure 4B**). While adolescent ethanol exposure visibily reduced IL-6 expression in male rats it had little recognizable effect on IL-6 expression in the females.

When data were analyzed using an omnibus ANOVA including sex as a variable, rats that received adolescent ethanol exposure showed significantly reduced levels of IL-1β expression when compared to vehicle exposed counterparts ($F_{(4,104)}$ = 2.95, $p < 0.05$) however, this appears to be largely a result of the differences noted in the male animals (**Figures 4C,D**). When ANOVAs for each sex were run separately, a significant attenuation of IL-1β expression was replicated in the male, adolescent ethanol exposed rats at the 180 min time point ($F_{(4,56)}$ = 3.44, $p < 0.05$; **Figure 4C**). Interestingly, no such interaction was noted for the female animals although a significant increase in overall expression was noted at the 90 and 180 min time points ($F_{(4,48)}$ = 14.63, $p < 0.001$; **Figure 4D**).

When data were analyzed using an omnibus ANOVA including sex as a variable, males showed much greater TNF-α increase in expression over the time course in contrast to female counterparts, regardless of adolescent exposure ($F_{(4,104)}$ = 2.71, $p < 0.05$; **Figures 4E,F**). Regardless of sex, TNF-α expression increased with peak levels being reached by the 360 min time point ($F_{(4,104)}$ = 11.06, $p < 0.001$; **Figures 4E,F**). When ANOVAs for each sex were run separately, TNF-α expression in males

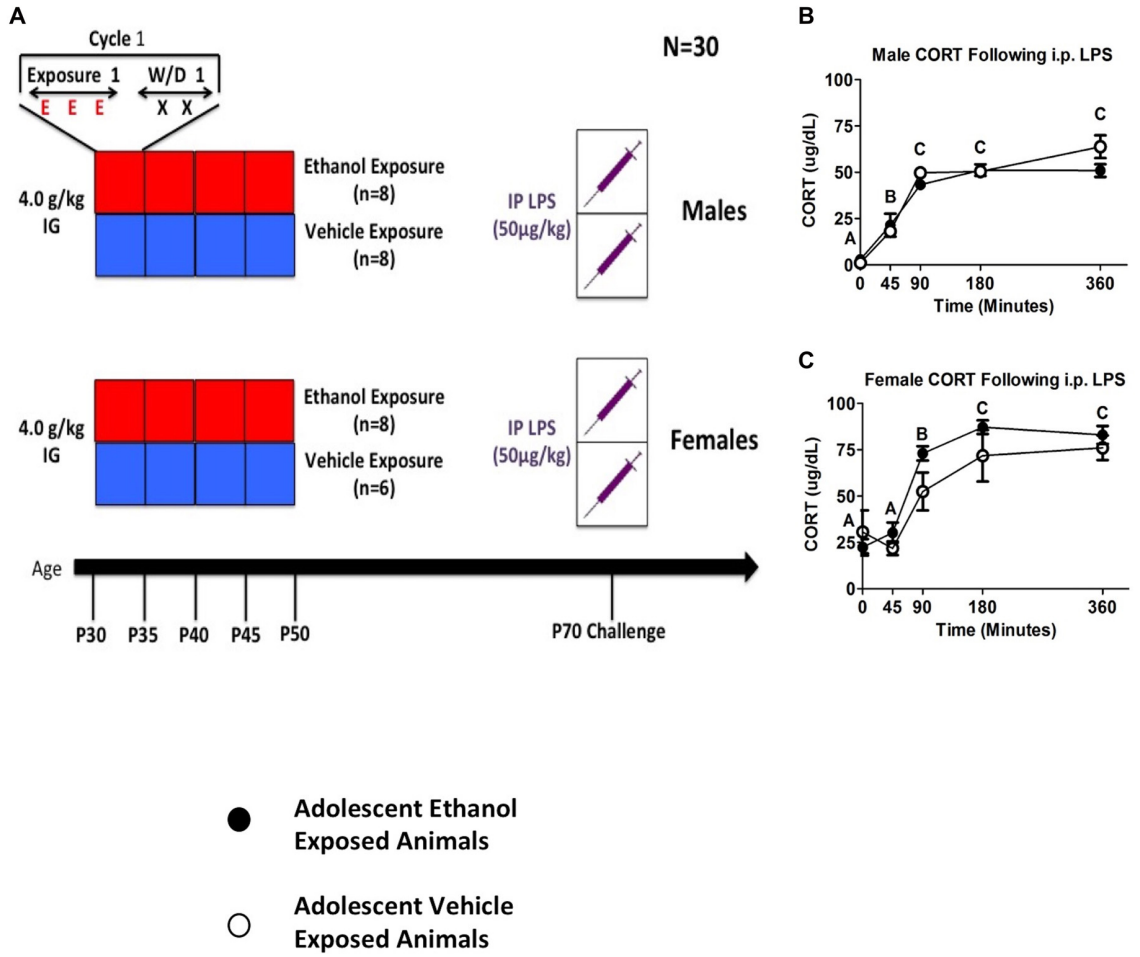


FIGURE 3 | Plasma CORT from Experiment 4. The figure shows a schematic diagramming Experiment 4 **(A)**. It also shows plasma CORT levels in male and female rodents following 50 μg/kg i.p. LPS injection at 0, 45, 90, 180 and 360 min time points **(B, C)**. A significant main effect of time point is denoted by a lettering system in which time points that differ in letter are significantly different.

showed a similar pattern of expression as in the previous experiment with a noticeable but not significant attenuation of expression developing in the adolescent ethanol exposed animals. Males showed much higher overall levels of expression than females ($F_{(1,26)} = 5.07$, $p < 0.05$; **Figures 4E,F**). Females also showed a significant increase in overall TNF-α expression at the 360 min time point ($F_{(4,48)} = 4.90765$, $p < 0.05$; **Figure 4F**).

IκBα closely mirrored the pattern of expression observed in Experiment 1. When data were analyzed using an omnibus ANOVA including sex as a variable, a significant three way interaction between adolescent exposure, sex and treatment ($F_{(4,104)} = 3.60$, $p < 0.01$) revealed that again adolescent ethanol exposure resulted in significant attenuation of IκBα exposure but exclusively in the male animals (**Figures 4G,H**). Female animals showed little to no consequence of adolescent ethanol exposure. Interestingly while in both female and male rats IκBα expression peaked at approximately the 1500% mark in the vehicle exposed male rats expression continued to escalate until the 360 min time point reaching almost double the magnitude of expression seen in the remaining three groups (**Figures 4G,H**).

DISCUSSION

Our primary goal in these studies was to examine how AIE would alter the HPA axis response to different types of challenge in adulthood. This set of studies shows that not only does adolescent ethanol exposure produce lasting changes in HPA axis sensitivity into adulthood, but that these changes are both sexually dimorphic and stimulus specific. Whereas adolescent ethanol exposed male rats showed blunting of peripheral cytokine expression in several key pro-inflammatory cytokines in response to restraint and LPS administered during adulthood, minimal differences were observed in response to ethanol challenge. These findings suggest that the HPA axis is not debilitated by adolescent ethanol challenge and

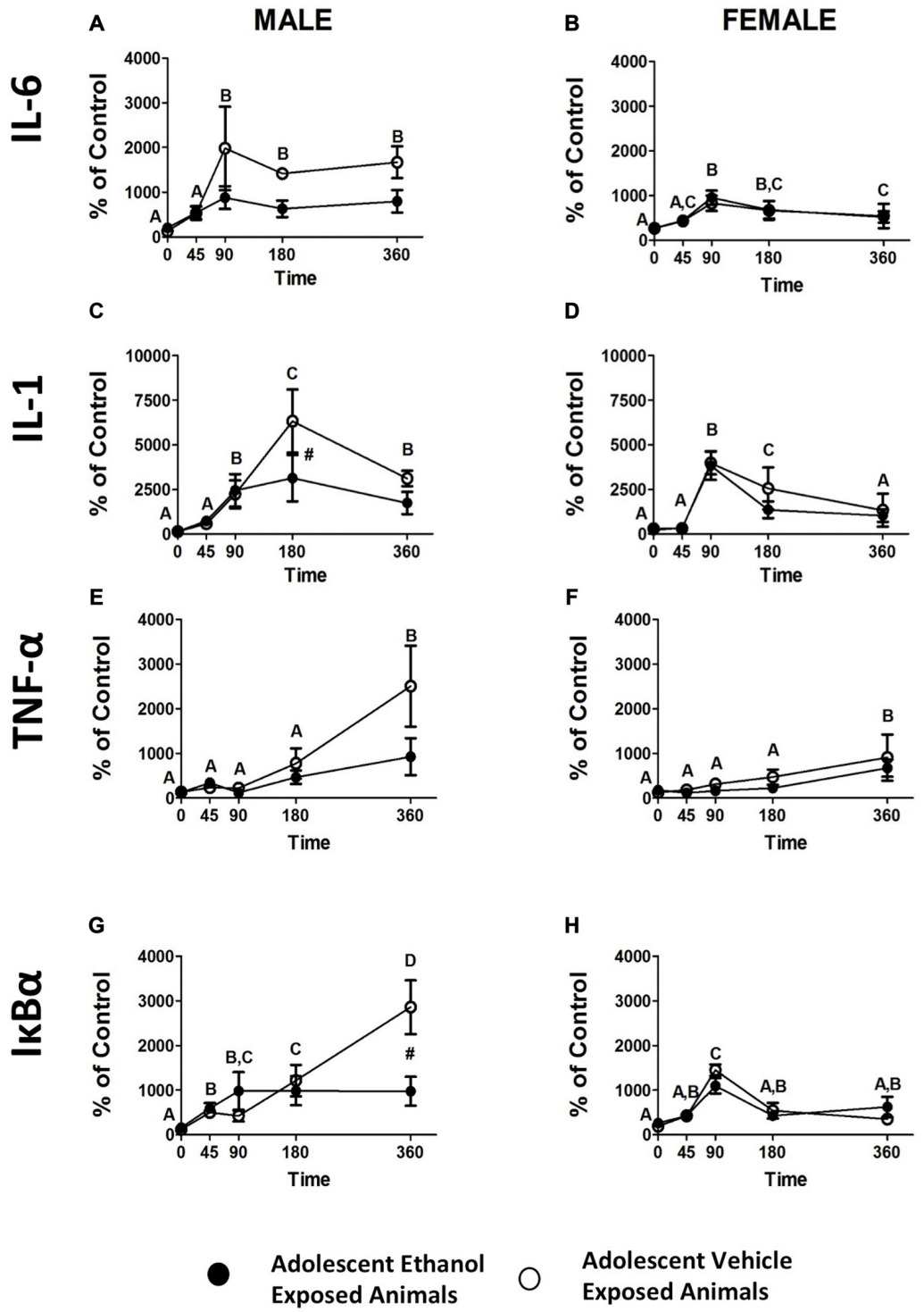


FIGURE 4 | Cytokine expression in male and female rats from experiment 4. This figure shows mRNA expression changes in male and female rats following i.p. LPS injection in IL-6 **(A,B)**, IL-1 **(C,D)**, TNF-α **(E,F)** and IκBα **(G,H)**. Samples were collected at 0, 45, 90, 180 and 360 min following LPS injection. Data is expressed relative to ultimate control (Vehicle animals at the baseline time point) normalized to β-Actin. A significant main effect of time point is denoted by a lettering system in which time points that differ in letter are significantly different. A pound sign indicates a significant interaction between adolescent exposure and challenge stimulus at a given time point. All *post hoc* comparisons were made using Fisher's Least Significant Difference *post hoc* test ($p < 0.05$).

might suggest adaptations that occur extrinsic to the axis. The three challenges used in the study each represent unique modalities for stimulating the HPA axis, restraint being psychological, LPS being immune, and ethanol being the same stimulus used to invoke the changes. Each stimulus produced a unique profile of cytokine expression in blood and the effects of AIE were different between challenge modality.

Alcohol is known to produce immune dysfunction. However, unlike other illnesses, the immune dysfunction that occurs is rarely at a level that would be considered clinical, rather it becomes notable following exposure to a subsequent challenge (Szabo and Saha, 2015). One example of this is the increased susceptibility to bacterial infections such as pneumonia and tuberculosis that occurs in heavy drinkers and alcohol dependent individuals (Cook, 1998; Ogunsakin et al., 2016). In addition, evidence from both human and animal studies has shown that prenatal ethanol exposure can result in increased vulnerability to infection and disease that may persist across the lifespan (Gauthier, 2015). In this study, a pronounced immunosuppressive effect of AIE in response to subsequent LPS challenge was seen in male rats while no difference was noted in females. In contrast, no effect of AIE on the CORT response in male rats was noted, yet a sensitization was observed in females. Although the functional significance for males and females with a history of adolescent ethanol exposure remains unclear, these divergent outcomes suggest that males may be more prone to infection, whereas females may be more prone to stress-related disorders (depression, anxiety) that are often co-morbid with HPA axis dysregulation (Weinberg et al., 2008). In this way, adolescent ethanol exposure may contribute to sex differences in specific disease vulnerabilities that emerge later in life.

Prenatal Alcohol Exposure (PAE) has also been shown to alter HPA axis reactivity in adulthood, suggesting there may be multiple developmental windows during which ethanol can impact later stress sensitivity. For instance, PAE-exposed animals show hyperactivity of the CORT response to restraint stress imposed later in life (Weinberg, 1992; Wieczorek et al., 2015). This hyperactivity has also been shown in response to LPS challenge, IL-1β challenge, local inflammatory challenge probed via turpentine injection, and other types of stressors (Lee and Rivier, 1996; Raineki et al., 2014). The consequences of PAE on adult challenge also appear to be sexually dimorphic as well as stimulus specific. PAE has led to an enhanced HPA axis response in adults to stressors such as acute restraint and forced swim in female rats but not in males (Weinberg et al., 2008). In contrast, solely PAE-exposed male rodents have shown a hyper-reactive HPA response to prolonged restraint and exposure to cold (Kim et al., 1999; Weinberg et al., 2008). Both sexes have shown hyper-reactivity of the axis to immune challenges such as LPS and IL-1β challenge (Weinberg et al., 2008). The results of the present studies clearly demonstrate that adolescent ethanol exposure is capable of causing changes in HPA axis reactivity lasting into adulthood. Based on the results of this study, the effects of adolescent ethanol exposure on later adult challenge appear to be stimulus specific and show sex specific effects, much like has been shown in studies involving PAE.

Prior work from our lab demonstrated that male adolescent rats displayed a blunted cytokine response in the CNS following either LPS challenge or ethanol challenge relative to adult comparators (Doremus-Fitzwater et al., 2015). One potential interpretation of the blunted cytokine responses observed in adolescent-exposed males in the present studies is that this may represent a "locking-in-like" effect. It is noteworthy, however, that, circulating concentrations of endotoxin following LPS challenge were substantially reduced in adolescents compared to adults, suggesting that adolescents may not process LPS in the same way as their adult counterparts. Whether this effect is through differential uptake and transit of LPS into the bloodstream, enhanced clearance of LPS from blood, or other immunological differences between adolescents and adults remains unclear. Nevertheless, plasma endotoxin concentrations were predictive of the HPA axis response (Doremus-Fitzwater et al., 2015). Unfortunately, it was not possible to measure plasma endotoxin concentrations in the present studies due to the limited available sample. Future studies will be necessary to test whether adolescent alcohol exposure impacts subsequent immune processing of LPS in a manner consistent with a "locking in-like" effect. The lack of AIE effects on cytokine expression in female rodents could suggest that female adolescent and adults would not demonstrate the previously noted differences in response to LPS challenge or that females displayed some form of resiliency to the changes that resulted in the "locking-in-like" effect.

Future studies should further probe the mechanisms by which adolescent alcohol exposure influences adult cytokine reactivity and stress sensitivity. LPS invokes an inflammatory response at least in part through its effects on Toll-like receptor 4 (TLR4), activating the NFKB pathway, and ultimately stimulating inflammatory cytokine expression and release (Doremus-Fitzwater et al., 2015). Female rodents have shown an enhanced ethanol-induced inflammatory response during adolescence, as exhibited by increased inflammatory cytokine levels in serum and in brain, however, this effect was not observed in male rats (Pascual et al., 2016). This difference may be mediated by up-regulated TLR4 expression during intoxication that are more pronounced in female rodents than in males, as supported by the fact that TLR4 KO mice do not exhibit these cytokine differences (Pascual et al., 2016). Binge ethanol expression has also been shown to interfere with LPS induced NFKB activity. Ethanol induces Heat shock factor protein 1 (HSF1) and 70 kilodalton heat shock protein (hsp70), two enzymes that ultimately inhibit TLR4/Myeloid differentiation primary response gene (MYD88) signaling via inhibition of the NFKB pathway (Muralidharan et al., 2014). This results in something akin to endotoxin tolerance in monocytes and macrophages pretreated with ethanol (Muralidharan et al., 2014). Lingering inhibition of TLR4/MYD88 signaling resulting from binge ethanol exposure could lead to a reduced cytokine response in male rats that could be normalized in females through sensitized TLR4 expression in female rats following AIE. While this is just one potential explanation for the effects seen in

this study, future studies should address the mechanism of these changes and TLR4 signaling pathways might be a good place to start.

Although the results of this study showed few consequences of adolescent ethanol exposure on the response to adult ethanol challenge, the existing literature examining similar manipulations is conflicting. The work of Pak using a similar model of ethanol administration has shown sensitization of the axis response in males following adult ethanol challenge (Przybycien-Szymanska et al., 2010, 2011). In contrast, a different study in which male adolescent rats received AIE and were challenged with ethanol in adulthood showed blunting of the axis response to later ethanol challenge (Allen et al., 2016). There are a number of factors that could account for this disparity. In the first set of studies, the number of ethanol cycles as well as the challenge dose was different, and the experiments were conducted using Wistar rats. In the second set of studies the method of adolescent administration was via daily vapor inhalation, whereas our studies utilized an intermittent, intragastric administration procedure. This could suggest that the dose, route, and schedule of ethanol exposure during adolescence may be critical determinants of long-term changes in HPA axis reactivity and cytokine gene induction.

One important question to address in future studies will be the mechanisms underlying the apparent HPA axis sensitization observed in females as a result of adolescent ethanol exposure. In particular, it will be important to identify whether these changes reflect altered function of neural circuits that govern HPA axis regulation, or intrinsic differences in sensitivity of endocrine glands comprising the HPA axis. This can be readily tested through a systematic series of hormonal challenges (CRH or ACTH injection) as we have done under other circumstances (Hueston and Deak, 2014b; also see Spencer and Deak, 2016 for an overview). Such studies are planned for the near future. It will also be important to extend these physiological findings to behavioral circumstances as well. For instance, the suppressed cytokine response evoked by LPS would predict that males with a history of adolescent ethanol exposure should display reduced sickness behavior under conditions of acute illness as adults. Given the HPA dysregulation observed in females with an adolescent history of ethanol, one might predict heightened anxiety and/or depression in adulthood. Given the established association between age of first alcohol use and subsequent vulnerability to addiction (Ystrom et al., 2014), it will also be important to assess whether adolescent ethanol exposure might increase vulnerability to addiction, particularly in females since stress dysregulation has been posited as one pathway towards addiction (Miller and Spear, 2006; Koob, 2008). These and many other issues will need to be explored in future studies.

As with any pre-clinical model of ethanol exposure, the mode of ethanol delivery and subsequent BECs achieved require careful consideration. We elected to utilize intragastric intubation for these studies because it mimics the normal route of administration for ethanol, and allows for binge-like doses to be achieved consistently and reliably. Such BECs would be difficult to achieve through voluntary consumption in rat, though that may be a path for future studies. BECs during the adolescent intubation regimen were not assessed due to concerns that the blood sampling procedure might have an enduring influence on the study outcomes. However, prior studies from our lab reported peak BECs of approximately 180 mg/dl after delivery of 4 g/kg (i.g.) of ethanol, which is consistent with a binge-like range of BECs (Buck et al., 2011; Doremus-Fitzwater et al., 2015). Because BECs were not assessed in the present studies, we cannot rule out the possibility that the sex-specific outcomes observed here might be due to intrinsic differences in ethanol metabolism (or other pharmacokinetic differences) between males and females (Truxell et al., 2007). Finally, our studies did not include a group of non-intubated controls to assess the influence of the intubation procedure itself. We occasionally use non-intubated controls and have not seen any evidence emerge to demonstrate long-term effects of the intubation procedure itself thus far. For practical purposes, however, we cannot do so in every study design. Nevertheless, these issues raise a more general question regarding whether any stress challenge (not just ethanol) imposed during adolescence might lead to similar outcomes. This is an interesting question that will have to be resolved in future studies. Overall, any ethanol delivery approach has both strengths and limitations and the study outcomes should be considered within an appropriate framework.

While this study yielded several interesting results, there are several procedural differences that must be considered in the interpretation of study outcomes. For instance, Experiments 1–3 utilize rats shipped from a vendor post weaning, whereas Experiment 4 utilized rats bred in house. The use of shipped animals for developmental studies is not ideal (see Laroche et al., 2009a,b; Spencer and Deak, 2016; Vargas et al., 2016), though it remains common practice for many investigators who do not have access to a breeding facility. Despite this limitation, Experiments 2 and 4 both yielded similar, durable influences of adolescent ethanol on LPS-induced cytokines as adults, even though the overall magnitude of cytokine responses across studies varied widely. A second disparity in study design was the number of ethanol cycles that the animals received during adolescence. Two cycles of intermittent ethanol exposure were utilized in Experiments 1–3, whereas four cycles were utilized in Experiment 4. Ultimately, the increase in number of cycles did not alter the directionality of the effects seen although it did appear to increase their magnitude. The model of ethanol administration is an important consideration because other studies have suggested that it is not only the amount of ethanol that is the dominant factor in creating durable effects resulting from AIE, but also the number of periods of abstinence between ethanol exposures (Lopez and Becker, 2005; Diaz-Granados and Graham, 2007). Indeed, periods of acute withdrawal that occur throughout intermittent ethanol exposure may play a critical role in causing some of the lasting changes of ethanol (Spear, 2016). Finally, to truly attribute the results of this study to an adolescent critical period, follow up studies that incorporate an adult control (that receive the same quantity of ethanol over the same time frame) and that control for the time difference between AIE and challenge between the two studies would be needed. Despite these minor limitations, the present study

yielded important and highly consistent results that warrant further investigation.

A final element of the study that affects interpretation of the results is the use of the cellular fraction of blood to study peripheral cytokine expression (Wang et al., 2016; Opstad et al., 2017). Although it is more common in contemporary studies to utilize plasma protein measures to detect cytokines, cytokines in plasma have very short half-lives and the assays to measure them require comparatively large samples. Thus, a viable alternative for procuring cytokine signals is to utilize gene expression in the cellular fraction, which can be done in very small samples, thereby allowing for the use of a within-subjects experimental design. Although there is always a question as to how such gene expression changes might align with functional protein measures, the detailed time course information gathered in the present study can be used to inform selection of more discrete time points for protein measures. Such studies are already planned in the near future. Furthermore, while the functional relationship between peripheral cytokines and central cytokines is unclear, prior studies have suggested that peripheral cytokine release may play a role in activating subsequent central action. LPS induced TNF-α in serum has been shown to play a role in triggering a proinflammatory response in the brain (Qin et al., 2007, 2008). Further research probing the relationship between these elements of the immune response would be worthwhile.

In conclusion, the effects of ethanol during adolescence appear to be mitigated by many factors. The results of this study as well as other work suggest that how the ethanol is administered, the nature/modality of the subsequent challenge stimulus, and the sex of the animals all may interact with AIE. While the rates of binge drinking in both adolescents and adults is very high, men are almost twice as likely to engage in binge drinking as women (Centers for Disease Control and Prevention, 2012). Differences in how men and women may be affected by ethanol, particularly during periods of developmental vulnerability, may contribute to the difference in AUD prevalence that exists between men and women (Nolen-Hoeksema, 2004). Despite all of this, it has been reported that women suffer more negative consequences of heavy ethanol use, including increased susceptibility to physical illness at lower levels of exposure than has been seen in men (Nolen-Hoeksema, 2004). Several drugs of abuse have been linked to weakened natural immune system function during use and at times lingering afterwards. Ethanol abuse specifically has been shown to produce a pronounced immunosuppressive effect and can increase the chances of individuals with ethanol being vulnerable to infection (Szabo, 1997; Szabo and Saha, 2015). Alterations in HPA axis reactivity and cytokine function could help to explain some of ethanol's effects on the immune system and the potential for adolescent ethanol use to cause lasting alterations in immune function. This could lead to a lifetime of heightened immune vulnerability to an array of types of challenge stimuli. Better understanding of how adolescent ethanol exposure affects the immune system and the longevity of such changes could help to better understand the risk of individuals exposed to future insults and help identify targets to reverse negative alterations.

AUTHOR CONTRIBUTIONS

ASV, TD-F, AG and TD made substantial contributions to the conception or design of the work as well as participated in the acquisition, analysis, or interpretation of data for the work. In addition, they played a significant role in drafting the work or revising it critically for important intellectual content and submitted for final approval of the version to be published. All authors 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

Research reported in this publication was supported by the National Institute on Alcohol Abuse and Alcoholism of the National Institute of Health under Award Number P50AA017823 to TD and the Center for Development and Behavioral Neuroscience at Binghamton University. Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the above stated funding agencies.

ACKNOWLEDGMENTS

Special thanks to Dennis Lovelock, Jackie Paniccia, Amy Perkins and Maryam Sammakia for playing a critical role in the execution of these studies.

REFERENCES

Allen, C. D., Grigoleit, J. S., Hong, J., Bae, S., Vaughan, J., and Lee, S. (2016). Exposure to alcohol during adolescence exerts long-term effects on stress response and the adult brain stress circuits. *Neuroscience* 339, 64–71. doi: 10.1016/j.neuroscience.2016.09.031

Buck, H. M., Hueston, C. M., Bishop, C., and Deak, T. (2011). Enhancement of the hypothalamic-pituitary-adrenal axis but not cytokine responses to stress challenges imposed during withdrawal from acute alcohol exposure in Sprague-Dawley rats. *Psychopharmacology (Berl)* 218, 203–215. doi: 10.1007/s00213-011-2388-z

Centers for Disease Control and Prevention. (2012). Vital signs: binge drinking prevalence, frequency, and intensity among adults—U.S., 2010. *MMWR Morb. Mortal. Wkly. Rep.* 61, 14–19.

Centers for Disease Control and Prevention. (2014). Alcohol use and your health. Available online at: http://www.cdc.gov/alcohol/fact-sheets/alcohol-use.htm

Cook, R. T. (1998). Alcohol abuse, alcoholism, and damage to the immune system— a review. *Alcohol. Clin. Exp. Res.* 22, 1927–1942. doi: 10.1111/j.1530-0277.1998.tb05900.x

Crews, F. T., Vetreno, R. P., Broadwater, M. A., and Robinson, D. L. (2016). Adolescent alcohol exposure persistently impacts adult neurobiology and behavior. *Pharmacol. Rev.* 68, 1074–1109. doi: 10.1124/pr.115.012138

Deak, T., Quinn, M., Cidlowski, J. A., Victoria, N. C., Murphy, A. Z., and Sheridan, J. F. (2015). Neuroimmune mechanisms of stress: sex differences, developmental plasticity, and implications for pharmacotherapy of stress-related disease. *Stress* 18, 367–380. doi: 10.3109/10253890.2015.1053451

Diaz-Granados, J. L., and Graham, D. L. (2007). The effects of continuous and intermittent ethanol exposure in adolesence on the aversive properties of ethanol during adulthood. *Alcohol. Clin. Exp. Res.* 31, 2020–2027. doi: 10.1111/j.1530-0277.2007.00534.x

Doremus, T. L., Brunell, S. C., Rajendran, P., and Spear, L. P. (2005). Factors influencing elevated ethanol consumption in adolescent relative to adult rats. *Alcohol. Clin. Exp. Res.* 29, 1796–1808. doi: 10.1097/01.alc.0000183007.65998.aa

Doremus, T. L., Brunell, S. C., Varlinskaya, E. I., and Spear, L. P. (2003). Anxiogenic effects during withdrawal from acute ethanol in adolescent and adult rats. *Pharmacol. Biochem. Behav.* 75, 411–418. doi: 10.1016/s0091-3057(03)00134-5

Doremus-Fitzwater, T. L., Buck, H. M., Bordner, K., Richey, L., Jones, M. E., and Deak, T. (2014). Intoxication- and withdrawal-dependent expression of central and peripheral cytokines following initial ethanol exposure. *Alcohol. Clin. Exp. Res.* 38, 2186–2198. doi: 10.1111/acer.12481

Doremus-Fitzwater, T. L., Gano, A., Paniccia, J. E., and Deak, T. (2015). Male adolescent rats display blunted cytokine responses in the CNS after acute ethanol or lipopolysaccharide exposure. *Physiol. Behav.* 148, 131–144. doi: 10.1016/j.physbeh.2015.02.032

Fillman, S. G., Weickert, T. W., Lenroot, R. K., Catts, S. V., Bruggemann, J. M., Catts, V. S., et al. (2016). Elevated peripheral cytokines characterize a subgroup of people with schizophrenia displaying poor verbal fluency and reduced Broca's area volume. *Mol. Psychiatry* 21, 1090–1098. doi: 10.1038/mp.2015.90

Gauthier, T. W. (2015). Prenatal alcohol exposure and the developing immune system. *Alcohol. Res.* 37, 279–285.

Giedd, J. N. (2008). The teen brain: insights from neuroimaging. *J. Adolesc. Health* 42, 335–343. doi: 10.1016/j.jadohealth.2008.01.007

Grant, B. F., and Dawson, D. A. (1997). Age at onset of alcohol use and its association with DSM-IV alcohol abuse and dependence: results from the national longitudinal alcohol epidemiologic survey. *J. Subst. Abuse* 9, 103–110. doi: 10.1016/s0899-3289(97)90009-2

Guerri, C., and Pascual, M. (2010). Mechanisms involved in the neurotoxic, cognitive and neurobehavioral effects of alcohol consumption during adolescence. *Alcohol* 44, 15–26. doi: 10.1016/j.alcohol.2009.10.003

Hueston, C. M., and Deak, T. (2014a). The inflamed axis: the interaction between stress, hormones, and the expression of inflammatory-related genes within key structures comprising the hypothalamic-pituitary-adrenal axis. *Physiol. Behav.* 124, 77–91. doi: 10.1016/j.physbeh.2013.10.035

Hueston, C. M., and Deak, T. (2014b). On the time course, generality and regulation of plasma progesterone release in male rats by stress exposure. *Endocrinology* 155, 3527–3537. doi: 10.1210/en.2014-1060

Kim, C. K., Giberson, P. K., Yu, W., Zoeller, R. T., and Weinberg, J. (1999). Effects of prenatal ethanol exposure on hypothalamic-pituitary-adrenal responses to chronic cold stress in rats. *Alcohol. Clin. Exp. Res.* 23, 301–310. doi: 10.1111/j.1530-0277.1999.tb04114.x

Koob, G. F. (2008). A role for brain stress systems in addiction. *Neuron* 59, 11–34. doi: 10.1016/j.neuron.2008.06.012

Laroche, J., Gasbarro, L., Herman, J. P., and Blaustein, J. D. (2009a). Enduring influences of peripubertal/adolescent stressors on behavioral response to estradiol and progesterone in adult female mice. *Endocrinology* 150, 3717–3725. doi: 10.1210/en.2009-0099

Laroche, J., Gasbarro, L., Herman, J. P., and Blaustein, J. D. (2009b). Reduced behavioral response to gonadal hormones in mice shipped during the peripubertal/adolescent period. *Endocrinology* 150, 2351–2358. doi: 10.1210/en.2008-1595

Lee, S., and Rivier, C. (1996). Gender differences in the effect of prenatal alcohol exposure on the hypothalamic-pituitary-adrenal axis response to immune signals. *Psychoneuroendocrinology* 21, 145–155. doi: 10.1016/0306-4530(95)00038-0

Lee, S., and Rivier, C. (2003). Long-term influence of an initial exposure to alcohol on the rat hypothalamic-pituitary axis. *Alcohol. Clin. Exp. Res.* 27, 1463–1470. doi: 10.1097/01.alc.0000086065.06203.dd

Lopez, M. F., and Becker, H. C. (2005). Effect of pattern and number of chronic ethanol exposures on subsequent voluntary ethanol intake in C57BL/6J mice. *Psychopharmacology* 181, 688–696. doi: 10.1007/s00213-005-0026-3

Matthews, D. B., Tinsley, K. L., Diaz-Granados, J. L., Tokunaga, S., and Silvers, J. M. (2008). Chronic intermittent exposure to ethanol during adolescence produces tolerance to the hypnotic effects of ethanol in male rats: a dose-dependent analysis. *Alcohol* 42, 617–621. doi: 10.1016/j.alcohol.2008.09.001

Miller, M. W., and Spear, L. P. (2006). The alcoholism generator. *Alcohol. Clin. Exp. Res.* 30, 1466–1469. doi: 10.1111/j.1530-0277.2006.00177.x

Muralidharan, S., Ambade, A., Fulham, M. A., Deshpande, J., Catalano, D., and Mandrekar, P. (2014). Moderate alcohol induces stress proteins HSF1 and hsp70 and inhibits proinflammatory cytokines resulting in endotoxin tolerance. *J. Immunol.* 193, 1975–1987. doi: 10.4049/jimmunol.1303468

Nolen-Hoeksema, S. (2004). Gender differences in risk factors and consequences for alcohol use and problems. *Clin. Psychol. Rev.* 24, 981–1010. doi: 10.1016/j.cpr.2004.08.003

Ogunsakin, O., Hottor, T., Mehta, A., Lichtveld, M., and McCaskill, M. (2016). Chronic ethanol exposure effects on vitamin D levels among subjects with alcohol use disorder. *Environ. Health Insights* 10, 191–199. doi: 10.4137/ehi.s40335

Opstad, T. B., Brusletto, B. S., Arnesen, H., Pettersen, A. Å., and Seljeflot, I. (2017). Cigarette smoking represses expression of cytokine IL-12 and its regulator miR-21-An observational study in patients with coronary artery disease. *Immunobiology* 222, 169–175. doi: 10.1016/j.imbio.2016.10.007

Pascual, M., Montesinos, J., Marcos, M., Torres, J. L., Costa-Alba, P., Garcia-García, F., et al. (2016). Gender differences in the inflammatory cytokine and chemokine profiles induced by binge ethanol drinking in adolescence. *Addict. Biol.* doi: 10.1111/adb.12461 [Epub ahead of print].

Pautassi, R. M., Myers, M., Spear, L. P., Molina, J. C., and Spear, N. E. (2008). Adolescent but not adult rats exhibit ethanol-mediated appetitive second-order conditioning. *Alcohol. Clin. Exp. Res.* 32, 2016–2027. doi: 10.1111/j.1530-0277.2008.00789.x

Przybycien-Szymanska, M. M., Mott, N. N., Paul, C. R., Gillespie, R. A., and Pak, T. R. (2011). Binge-pattern alcohol exposure during puberty induces long-term changes in HPA axis reactivity. *PLoS One* 6:e18350. doi: 10.1371/journal.pone.0018350

Przybycien-Szymanska, M. M., Rao, Y. S., and Pak, T. R. (2010). Binge-pattern alcohol exposure during puberty induces sexually dimorphic changes in genes regulating the HPA axis. *Am. J. Physiol. Endocrinol. Metab.* 298, E320–E328. doi: 10.1152/ajpendo.00615.2009

Qin, L., He, J., Hanes, R. N., Pluzarev, O., Hong, J. S., and Crews, F. T. (2008). Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. *J. Neuroinflammation* 5:10. doi: 10.1186/1742-2094-5-10

Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J. S., et al. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 55, 453–462. doi: 10.1002/glia.20467

Raineki, C., Hellemans, K. G., Bodnar, T., Lavigne, K. M., Ellis, L., Woodward, T. S., et al. (2014). Neurocircuitry underlying stress and emotional regulation in animals prenatally exposed to alcohol and subjected to chronic mild stress in adulthood. *Front. Endocrinol. (Lausanne)* 5:5. doi: 10.3389/fendo.2014.00005

Rivest, S., Lacroix, S., Vallières, L., Nadeau, S., Zhang, J., and Laflamme, N. (2000). How the blood talks to the brain parenchyma and the paraventricular nucleus of the hypothalamus during systemic inflammatory and infectious stimuli. *Proc. Soc. Exp. Biol. Med.* 223, 22–38. doi: 10.1111/j.1525-1373.2000.22304.x

Rivier, C., Bruhn, T., and Vale, W. (1984). Effect of ethanol on the hypothalamic-pituitary-adrenal axis in the rat: role of corticotropin-releasing factor (CRF). *J. Pharmacol. Exp. Ther.* 229, 127–131.

Romeo, R. D., Patel, R., Pham, L., and So, V. M. (2016). Adolescence and the ontogeny of the hormonal stress response in male and female rats and mice. *Neurosci. Biobehav. Rev.* 70, 206–216. doi: 10.1016/j.neubiorev.2016.05.020

Schwochow, D., Serieys, L. E., Wayne, R. K., and Thalmann, O. (2012). Efficient recovery of whole blood RNA— a comparison of commercial RNA extraction protocols for high-throughput applications in wildlife species. *BMC Biotechnol.* 12:33. doi: 10.1186/1472-6750-12-33

Spear, L. P. (2016). Consequences of adolescent use of alcohol and other drugs: studies using rodent models. *Neurosci. Biobehav. Rev.* 70, 228–243. doi: 10.1016/j.neubiorev.2016.07.026

Spear, L. P., and Swartzwelder, H. S. (2014). Adolescent alcohol exposure and persistence of adolescent-typical phenotypes into adulthood: a mini-review. *Neurosci. Biobehav. Rev.* 45, 1–8. doi: 10.1016/j.neubiorev.2014.04.012

Spencer, R. L., and Deak, T. (2016). A users guide to HPA axis research. *Physiol. Behav.* doi: 10.1016/j.physbeh.2016.11.014 [Epub ahead of print].

Sullivan, P. F., Fan, C., and Perou, C. M. (2006). Evaluating the comparability of gene expression in blood and brain. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 141b, 261–268. doi: 10.1002/ajmg.b.30272

Szabo, G. (1997). Alcohol's contribution to compromised immunity. *Alcohol Health Res. World* 21, 30–41.

Szabo, G., and Saha, B. (2015). Alcohol's effect on host defense. *Alcohol Res.* 37, 159–170.

Szelényi, J. (2001). Cytokines and the central nervous system. *Brain Res. Bull.* 54, 329–338. doi: 10.1016/S0361-9230(01)00428-2

Truxell, E. M., Molina, J. C., and Spear, N. E. (2007). Ethanol intake in the juvenile, adolescent and adult rat: effects of age and prior exposure to ethanol. *Alcohol. Clin. Exp. Res.* 31, 755–765. doi: 10.1111/j.1530-0277.2007.00358.x

Vargas, J., Junco, M., Gomez, C., and Lajud, N. (2016). Early life stress increases metabolic risk, HPA axis reactivity and depressive-like behavior when combined with postweaning social isolation in rats. *PLoS One* 11:e0162665. doi: 10.1371/journal.pone.0162665

Varlinskaya, E. I., and Spear, L. P. (2004a). Acute ethanol withdrawal (hangover) and social behavior in adolescent and adult male and female Sprague-Dawley rats. *Alcohol. Clin. Exp. Res.* 28, 40–50. doi: 10.1097/01.alc.0000108655.51087.df

Varlinskaya, E. I., and Spear, L. P. (2004b). Changes in sensitivity to ethanol-induced social facilitation and social inhibition from early to late adolescence. *Ann. N Y Acad. Sci.* 1021, 459–461. doi: 10.1196/annals.1308.064

Wang, W., Liu, Z., Su, J., Chen, W. S., Wang, X. W., Bai, S. X., et al. (2016). Macrophage micro-RNA-155 promotes lipopolysaccharide-induced acute lung injury in mice and rats. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 311, L494–L506. doi: 10.1152/ajplung.00001.2016

Weinberg, J. (1992). Prenatal ethanol effects: sex difference in offspring stress responsiveness. *Alcohol* 9, 219–223. doi: 10.1016/0741-8329(92)90057-h

Weinberg, J., Sliwowska, J. H., Lan, N., and Hellemans, K. G. (2008). Prenatal alcohol exposure: foetal programming, the hypothalamic-pituitary-adrenal axis and sex differences in outcome. *J. Neuroendocrinol.* 20, 470–488. doi: 10.1111/j.1365-2826.2008.01669.x

Wieczorek, L., Fish, E. W., O'Leary-Moore, S. K., Parnell, S. E., and Sulik, K. K. (2015). Hypothalamic-pituitary-adrenal axis and behavioral dysfunction following early binge-like prenatal alcohol exposure in mice. *Alcohol* 49, 207–217. doi: 10.1016/j.alcohol.2015.01.005

Willey, A. R., Anderson, R. I., Morales, M., Ramirez, R. L., and Spear, L. P. (2012). Effects of ethanol administration on corticosterone levels in adolescent and adult rats. *Alcohol* 46, 29–36. doi: 10.1016/j.alcohol.2011.08.005

World Health Organization (2014). Global status report on alcohol and health. Available online at: http://apps.who.int/iris/bitstream/10665/112736/1/9789240692763_eng.pdf

Ystrom, E., Kendler, K. S., and Reicborn-Kjennnerud, T. (2014). Early age of alcohol initiation is not the cause of alcohol use disorders in adulthood, but is a major indicator of genetic risk. A population-based twin study. *Addiction* 109, 1824–1832. doi: 10.1111/add.12620

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: 07 February 2017
doi: 10.3389/fnbeh.2017.00015

Effects of Intermittent Alcohol Exposure on Emotion and Cognition: A Potential Role for the Endogenous Cannabinoid System and Neuroinflammation

*Laura Sanchez-Marin †, Francisco J. Pavon †, Juan Decara, Juan Suarez, Ana Gavito, Estela Castilla-Ortega, Fernando Rodriguez de Fonseca * and Antonia Serrano **

Unidad de Gestion Clinica de Salud Mental, Instituto de Investigacion Biomedica de Malaga, Hospital Regional Universitario de Malaga, Universidad de Malaga, Malaga, Spain

OPEN ACCESS

Edited by:
Mary M. Torregrossa, University of Pittsburgh, USA

Reviewed by:
Tommy Pattij, VU University Medical Center, Netherlands
Consuelo Guerri, Centro de Investigación Príncipe Felipe, Spain

***Correspondence:**
Fernando Rodriguez de Fonseca
fernando.rodriguez@ibima.eu
Antonia Serrano
antonia.serrano@ibima.eu

† These authors have contributed equally to this work.

Received: *23 October 2016*
Accepted: *16 January 2017*
Published: *07 February 2017*

Citation:
Sanchez-Marin L, Pavon FJ, Decara J, Suarez J, Gavito A, Castilla-Ortega E, Rodriguez de Fonseca F and Serrano A (2017) Effects of Intermittent Alcohol Exposure on Emotion and Cognition: A Potential Role for the Endogenous Cannabinoid System and Neuroinflammation. Front. Behav. Neurosci. 11:15. doi: 10.3389/fnbeh.2017.00015

Intermittent alcohol exposure is a common pattern of adolescent alcohol use that can lead to binge drinking episodes. Alcohol use is known to modulate the endocannabinoid system (ECS), which is involved in neuronal communication, neuroplasticity, neuroinflammation and behavior. Adolescent male Wistar rats were exposed to 4-week intermittent alcohol intoxication (3 g/kg injections for 4 days/week) or saline ($N = 12$ per group). After alcohol deprivation, adult rats were assessed for emotionality and cognition and the gene expression of the ECS and other factors related to behavior and neuroinflammation was examined in the brain. Alcohol-exposed rats exhibited anxiogenic-like responses and impaired recognition memory but no motor alterations. There were brain region-dependent changes in the mRNA levels of the ECS and molecular signals compared with control rats. Thus, overall, alcohol-exposed rats expressed higher mRNA levels of endocannabinoid synthetic enzymes (N-acyl-phosphatidylethanolamine phospholipase D and diacylglycerol lipases) in the medial-prefrontal cortex (mPFC) but lower mRNA levels in the amygdala. Furthermore, we observed lower mRNA levels of receptors CB_1 CB_2 and peroxisome proliferator-activated receptor-α in the striatum. Regarding neuropeptide signaling, alcohol-exposed rats displayed lower mRNA levels of the neuropeptide Y signaling, particularly NPY receptor-2, in the amygdala and hippocampus and higher mRNA levels of corticotropin-releasing factor in the hippocampus. Additionally, we observed changes of several neuroinflammation-related factors. Whereas, the mRNA levels of toll-like receptor-4, tumor necrosis factor-α, cyclooxygenase-2 and glial fibrillary acidic protein were significantly increased in the mPFC, the mRNA levels of cyclooxygenase-2 and glial fibrillary acidic protein were decreased in the striatum and hippocampus. However, nuclear factor-κβ mRNA levels were lower in the mPFC and striatum and allograft inflammatory factor-1 levels were differentially expressed in the amygdala and hippocampus. In conclusion, rats exposed to adolescent intermittent alcohol displayed anxiety-like behavior and cognitive deficits in adulthood and these alterations were

accompanied by brain region-dependent changes in the gene expression of the ECS and other signals associated with neuroinflammation and behavior. An intermittent adolescent alcohol exposure has behavioral and molecular consequences in the adult brain, which might be linked to higher vulnerability to addictive behaviors and psychopathologies.

Keywords: rats, intermittent alcohol, adolescence, endocannabinoid system, neuroinflammation, anxiety, brain, recognition memory

INTRODUCTION

Alcohol is the most widely used recreational drug, and its consumption is increasing in young people and adolescents. A common pattern of alcohol intake among adolescents is the intermittent alcohol exposure, which can lead eventually to heavy episodic drinking. Thus, adolescent binge drinking is a major public health concern and it is associated with long-term health consequences, including mental problems (e.g., anxiety, mood and personality disorders) and substance use disorders, primarily alcoholism (Oesterle et al., 2004; Dawson et al., 2008; Read et al., 2008). Further, the potential role of alcohol use as a risk factor for adult psychiatric disorders cannot be discarded.

The molecular actions of alcohol on the brain are complex, and involve several mechanisms and signaling systems and some of these actions occur in both the adult and the adolescent brain. Brain maturation mainly happens during adolescence, when numerous plastic and dynamic processes are happening, and the vulnerability of the developing brain to the toxic effects of alcohol is higher. Therefore, early alcohol exposure can produce alterations in the brain structure and function, resulting in behavioral and cognitive deficits (Nagel et al., 2005; Zeigler et al., 2005; Guerri et al., 2009). These long-term harmful effects of alcohol exposure on behavior have also been confirmed in animal models (e.g., learning dysfunctions in adolescent rats exposed to repeated alcohol continue into adulthood) (Crews et al., 2000; Pascual et al., 2007).

Alcohol and other drugs of abuse induce changes in the Central Nervous System (CNS) that can lead to pathological behaviors related to addiction. In fact, the transition to alcoholism involves changes in the reinforcing and rewarding effects of alcohol use, a dysregulation of synaptic plasticity and the development of maladaptive stress responses (Kalivas and O'Brien, 2008; Koob, 2008). A variety of signals and neurotransmitters (e.g., dopamine, serotonin, glutamate, GABA, opioids, endocannabinoids...) in the CNS are implicated in the pathophysiology and development of alcoholism which play a prominent role in mediating behavioral and pharmacological effects of alcohol (Koob et al., 1998).

Over the past years, numerous studies have demonstrated the involvement of the endogenous cannabinoid system (ECS) in the behavioral and pharmacological effects of alcohol (Serrano and Parsons, 2011). The ECS includes endogenous ligands, cannabinoid receptors and the enzymatic machinery for the synthesis and inactivation of endocannabinoids. The main endocannabinoids are arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG), which bind to cannabinoid receptors to exert their effects. The cannabinoid receptors are G protein-coupled receptors and two types have been characterized and cloned, CB_1 and CB_2 (Howlett et al., 1990; Munro et al., 1993). Regarding the metabolic pathways for endocannabinoids, there are very complex enzymatic cascades for synthesis and inactivation that are crucial in regulating their levels. The primary pathway for AEA synthesis is mediated by a specific phospholipase D (NAPE-PLD) (Okamoto et al., 2004), while the synthetic pathway for 2-AG is mainly mediated by two sn-1-selective diacylglycerol lipases (DAGL-α and DAGL-β) (Bisogno et al., 2003). Finally, the inactivation of endocannabinoids is mediated by cellular reuptake and subsequent intracellular hydrolysis and both fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) have been identified as enzymes primarily responsible for the degradation of AEA and 2-AG, respectively (Cravatt et al., 1996; Dinh et al., 2002).

The ECS has been widely studied in recent years due to its anti-inflammatory and homeostatic properties, which is of interest because alcohol abuse is associated with the induction of neuroinflammatory and neurodegenerative processes. In this regard, binge-like alcohol exposure increases the nuclear factor-*kappa* β (NF-κβ)-DNA binding activity, up-regulates the expression of cyclooxygenase-2 (COX-2), causes microglia activation (Knapp and Crews, 1999; Obernier et al., 2002a; Crews et al., 2006) and induces brain injury in the cortex and hippocampus associated with cognitive deficits (Crews et al., 2000; Obernier et al., 2002b; Tajuddin et al., 2014; Antón et al., 2016). However, it is important to consider that all the mentioned effects of alcohol use depend on several factors, including age of the subject, amount of alcohol consumed, duration and pattern of alcohol consumption (Kovacs and Messingham, 2002; Goral et al., 2008).

Long-term effects of alcohol exposure in animal models have revealed clear deleterious impact on emotional and cognitive processing, as well as on underlying inflammatory responses triggered by the unique ability of alcohol to direct activate natural immunity through toll-like receptor-4 (TLR4) (Fernandez-Lizarbe et al., 2009; Pascual et al., 2011). In fact, recent studies have reported the role of TLR4 in behavioral and cognitive dysfunctions using models of intermittent alcohol exposure in adolescence (Montesinos et al., 2015, 2016).

In this context, the present study aimed to characterize the impact of an adolescent intermittent alcohol exposure in emotional behaviors (open field and elevated-plus maze), cognitive responses (novel object recognition memory) and gene expression of primary signaling systems related to neuroinflammation, anxiety and stress in adult rats. The medial prefrontal cortex, amygdala, striatum and hippocampus were selected for gene expression analysis since they

play a predominant role in alcohol-related behaviors and neuroadaptations.

MATERIALS AND METHODS

Animals and Ethical Statement

Twenty-four male Wistar rats (Charles River Laboratories, Barcelona, Spain) weighing 100–125 g on postnatal day (pnd) 24 were housed individually in a humidity and temperature-controlled vivarium on a 12 h light/dark cycle (lights off at 19:00 h). Rats were allowed to acclimatize to the new environment for 7 days before any experimental procedure was performed (pnd 31). Water and rat chow pellets were available *ad libitum*.

This study was carried out in accordance with the European Directive 2010/63/EU for the protection of animals used for scientific purposes and Spanish regulations (Real Decreto 53/2013 and 178/2004, Ley 32/2007 and 9/2003 and Decreto 320/2010) for the care and use of laboratory animals. The protocol was approved by the Ethic and research Committee at the Universidad de Malaga. All efforts were made to minimize animal suffering and social isolation, as well as to reduce the number of animals used.

Experimental Design

As shown in **Figure 1**, adolescent rats (pnd 31) were randomly assigned to the experimental alcohol group following 4 weeks of intermittent alcohol exposure ($n = 12$) or control group ($n = 12$).

Intermittent Alcohol Procedure

An intraperitoneal (i.p.) administration of ethanol solution was used as intermittent intoxication during 4 weeks. Rats were injected weekly with 3 g/kg of ethanol (20% in saline, w/v) for 4 consecutive days followed by 3 days of alcohol deprivation. After the adolescent alcohol exposure, rats were left undisturbed in their home-cages before performing behavioral tests to evaluate locomotor activity, cognitive responses and anxiety-like behaviors. Following a schedule similar to the alcohol group, control animals received an injection of saline and they were also individually maintained in their home-cages for controlling the effects of social isolation on behavioral experiments and biochemical determinations previously reported in adolescence (Skelly et al., 2015).

Determination of Blood Ethanol Concentration

Rats were tail-bled 1 h after the last alcohol exposure. Blood was collected into a microtube containing anticoagulant (4 µl heparin; 1000 USP units/ml) and centrifuged at 2000 × g for 10 min. Serum was extracted and assayed for ethanol concentration (BEC) using the alcohol oxidase method with an AM1 Alcohol Analyzer (Analox Instrument, London, UK).

Behavioral Studies

Behavioral tests were conducted by trained observers who were unaware of the experimental conditions.

Open Field Test

Motor and anxiety-like behaviors were studied in an opaque open field (100 × 100 × 40 cm) divided into 16 squares with two zones; the center (4 squares) and periphery (12 squares) of the field. The open field was illuminated using a ceiling halogen lamp that was regulated to yield 350 lux at the center of the field. On the experimental day, the animals were placed in the center and the locomotor activity and time spent in the center and periphery were scored for 15 min. The number of crossings (expressed as number lines/squares crossed) and the percent of time spent in the center (time spent in the center/total time × 100) were calculated.

This task was also used as habituation session for the novel object recognition test.

Novel Object Recognition Test

The novel object recognition procedure consists of habituation, familiarization, and test phases. Familiarization phase was conducted 24 h after the habituation phase. During the familiarization phase, animals were placed in the test field and allowed to explore for 3 min two identical objects. Then, animals were returned to their home-cage for 1 h. Test phase was conducted by placing rats for 3 min in the test field with 2 different objects, one was familiar and the other one was novel. Objects were made in plastic but different in shape, size and color. Between each test, the relative position of both objects were counterbalanced and permuted.

The time spent in exploring these objects were recorded in both phases. The exploration time was defined as the time that animals spent in licking, sniffing or touching each object, but it was not considered the time spent in standing or sitting on or leaning against each object (expressed in seconds). The novel object preference was determined using the discrimination ratio (expressed as the difference in time exploring the novel and the familiar objects/total exploration time).

Elevated Plus-Maze

The elevated plus-maze was made of opaque plastic and was composed of 2 oppositely positioned open arms (45 × 10 cm), 2 oppositely positioned closed arms of the same size and 50-cm-high walls. The arms were connected by a central and neutral area (10 × 10 cm). The entire apparatus was elevated 75 cm above a white floor and exposed to dim illumination (70 lux). At the beginning, rats were placed in the center of the maze, facing an open arm, and were allowed to freely explore the maze for 5 min. The number of entries (an arm entry was defined as all four paws in the arm zone) and the time spent in each arm were scored using a video monitor. The number of entries into the closed arms (expressed as number of entries into the closed arms) and the percent of time spent in the exposed arm (time spent in the open arms/total time × 100) were calculated.

Sample Collection

Two weeks after the last alcohol exposure (pnd 70), rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and brain samples were collected. The brains were quickly removed, immediately frozen on dry ice and stored at −80°C,

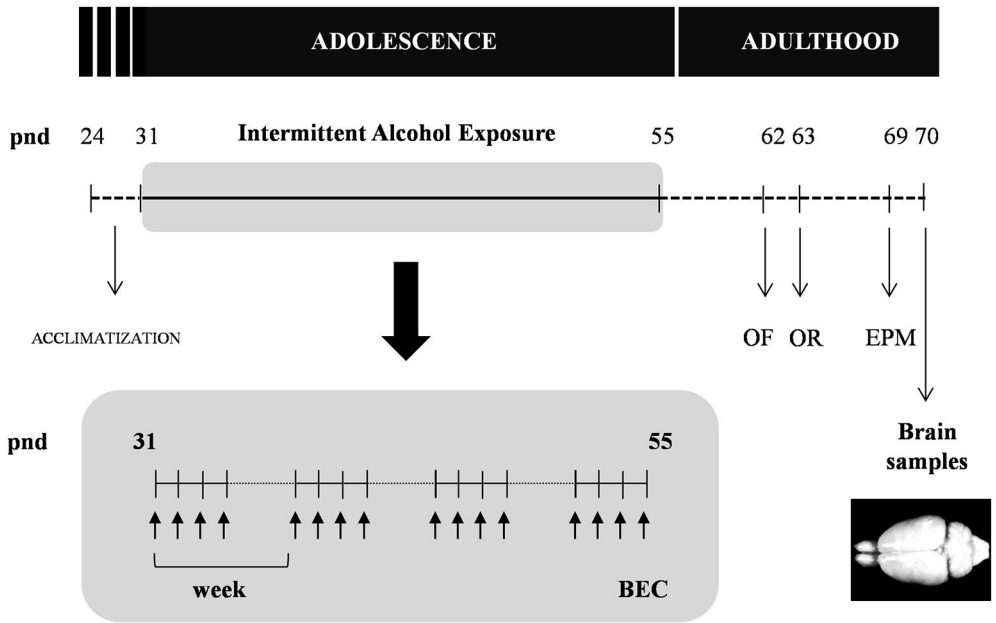


FIGURE 1 | Experimental design of the study. Rats were exposed to an intermittent alcohol exposure for 4 weeks (from pnd 31 to 55). During this period, rats were randomly assigned to alcohol (repeated i.p. administration) or control (saline) group. Blood ethanol concentrations (BEC) were determined 60 min after the last alcohol exposure. The analyzes of behavior on the open field (OF), novel object recognition (OR) and elevated plus-maze (EPM) were carried out in the different groups at 1 and 2 weeks after the alcohol exposure. All animals were sacrificed 2 weeks after the last alcohol exposure and brain samples were collected.

until determination of the gene expression of proteins using quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR).

Dissection of the Brain

The frozen brains were placed in acrylic rat brain matrices, and 2-mm thick slices were obtained using brain matrix razor blades. The medial prefrontal cortex (mPFC), striatum (accumbens, caudate, and putamen nucleus), entire amygdala and dorsal and ventral hippocampus were dissected out bilaterally and collected using a scalpel (mPFC and hippocampus) and a sample corer (striatum and amygdala). The localization of the brain regions was performed using a rat brain atlas and considering the bregma point as zero coordinate in the rostral-caudal coordinates: mPFC, +3.0 mm to +5.0 mm from bregma; striatum, +1.0 mm to −1.0 mm from bregma; amygdala, −1.0 mm to −3.0 mm from bregma; and hippocampus, −3.0 mm to −5.0 mm from bregma (Paxinos and Watson, 1998).

RNA Isolation and RT-qPCR Analysis

Total RNA was extracted from brain samples using Trizol Reagent (Gibco BRL Life Technologies, Baltimore, MD, USA) and the concentrations were quantified using a spectrophotometer to ensure ratios of absorbance at 260 to 280 nm of 1.8–2.0. The reverse transcription was performed using the Transcriptor Reverse Transcriptase kit and random hexamer primers (Transcriptor RT; Roche Diagnostic, Mannheim, Germany). The RT-qPCR was performed using an ABI PRISM® 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the FAM dye label format for the TaqMan Gene Expression Assays (Applied Biosystems). The absolute values from each sample were normalized relative to the housekeeping β-actin gene (*Actb*). The relative quantification was calculated using the ΔΔCt method and normalized to the control group. Primers for the RT-qPCR were obtained based on the Applied Biosystems genome database of rat mRNA references (http://bioinfo.appliedbiosystems.com/genome-database/gene-expression.html) (**Table 1**).

Statistical Analysis

All the data in the graphs are expressed as the means ± SEM. The statistical analysis was performed using GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA, USA) and the normal distribution of data was evaluated by D'Agostino-Pearson omnibus test. Student's *t*-test and Welch's *t*-test for unequal variances were conducted to compare continuous variables between the two experimental groups (Control and Alcohol). Two-way analysis of variance (ANOVA) was conducted in the Novel Object Recognition Test using alcohol exposure (control and alcohol) and type of object (familiar and novel) as factors and using Bonferroni as *post hoc* test. Benjamini–Hochberg false discovery rate (FDR) approach was used for multiple comparisons (Benjamini et al., 2001) of the mRNA expression analysis. The statistics (*t*-statistic and *F*-statistic) and degrees of freedom were indicated in the description of results. The significance level was established at 0.05 and a *p*-value of less than 0.05 was considered statistically significant. Additionally,

TABLE 1 | Primers references for TaqMan® Gene Expression Assays.

Gene symbol	Assay ID	GenBank accession number	Amplicon length
Actb	Rn00667869_m1	NM_031144.3	91
Cnr1	Rn02758689_s1	NM_012784.4	92
Cnr2	Rn01637601_m1	NM_020543.4	68
Dagla	Rn01454304_m1	NM_001005886.1	67
Daglb	Rn01453771_m1	NM_001107120.1	98
Faah	Rn00577086_m1	NM_024132.3	63
Mgll	Rn00593297_m1	NM_138502.2	78
Napepld	Rn01786262_m1	NM_199381.1	71
Ppara	Rn00566193_m1	NM_013196.1	98
Crh	Rn01462137_m1	NM_031019.1	112
Crhr1	Rn00578611_m1	XM_006247542.2	58
Crhr2	Rn00575617_m1	NM_022714.1	82
Npy	Rn00561681_m1	NM_012614.2	63
Npy1r	Rn02769337_s1	NM_001113357.1	98
Npy2r	Rn00576733_s1	NM_023968.1	65
Npy5r	Rn02089867_s1	NM_012869.1	107
Aif1	Rn00574125_g1	NM_017196.3	126
Gfap	Rn01253033_m1	NM_017009.2	75
Ptgs2	Rn01483830_g1	NM_017232.3	69
Rela	Rn01502266_m1	NM_199267.2	67
Tlr4	Rn00569848_m1	NM_019178.1	127
Tnf	Rn99999017_m1	NM_012675.3	108

FDR adjusted significance levels were indicated for the multiple comparisons in each case.

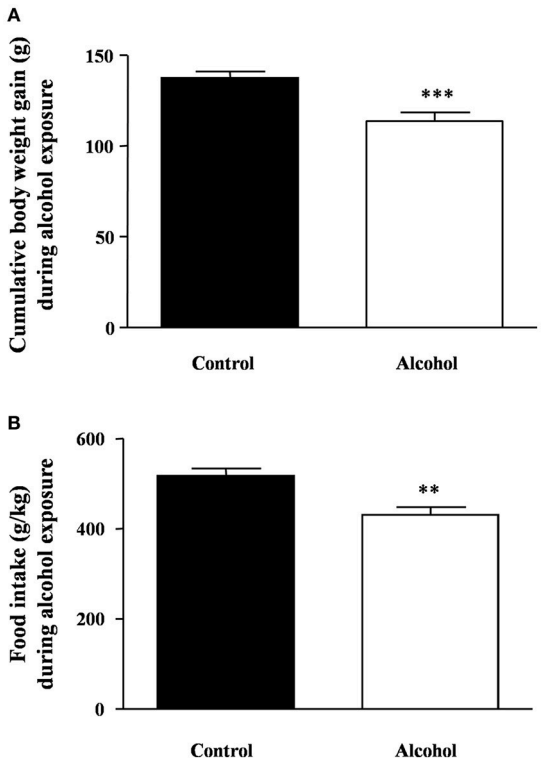


FIGURE 2 | Body weight gain and food intake during intermittent alcohol exposure in adolescent rats. Cumulative body weight gain **(A)** and food intake **(B)** after 4 weeks of alcohol exposure. Bars represent the mean ± SEM (8–12 animals per group). Data were analyzed by Student's *t*-test (Welch's correction). $^{**}p < 0.01$ and $^{***}p < 0.001$ denote significant differences compared with the control group.

RESULTS

In the present study, we examined adult rats exposed to intermittent alcohol exposures during adolescence. We assessed anxiety-like and cognitive behaviors as well as the mRNA expression of numerous enzymes and receptors involved in the ECS and other mediators linked to behavior, neuroinflammation and plasticity.

Body Weight Gain and Food Intake during Adolescent Intermittent Alcohol Exposure

As shown in **Figure 2A**, during the period of alcohol exposure, there was a significant decrease in body weight gain in the alcohol group compared with the control group [$t_{(22)} = 4.00$, $p < 0.001$]. Similarly, the averages of food intake during these 4 weeks were also significantly different [$t_{(22)} = 3.68$, $p = 0.001$] (**Figure 2B**).

After the last alcohol exposure, the BEC in the alcohol group reached an average of 201 ± 6 mg/dl.

Effects of Intermittent Alcohol Exposure on Locomotion and Anxiety-Like Behaviors

We next explored whether alcohol exposure during adolescence induced locomotor or emotional alterations.

One week after the last adolescent alcohol exposure, we measured spontaneous locomotor and exploratory activity using the open field test in rats. We observed no significant differences in the number of crossings between both groups (**Figure 3A**). Additionally, we measured anxiety-like behavior by time spent in the center area of the field (**Figure 3B**) and we detected a significant decrease in the time spent in the center area in the alcohol group compared with the control group [$t_{(14)} = 4.19$, $p < 0.001$].

The rats were also tested on the elevated plus-maze under novelty conditions 2 weeks after alcohol exposure. Regarding the locomotor activity in the elevated plus-maze, we found no differences in the number of entries in the closed arms between groups (**Figure 3C**). In this maze, we measured anxiety-like behavior by the time spent in the open arms and significant differences were found. As shown in **Figure 3D**, there was a significant decrease in the time of open arm exploration in the alcohol group compared with the control group [$t_{(14)} = 4.65$, $p < 0.001$].

Effects of Intermittent Alcohol Exposure on Novel Object Recognition

One week after adolescent alcohol exposure, the recognition memory was measured using the novel object recognition task. During the familiarization phase, none of the groups displayed any preference for the objects but the statistical analysis revealed

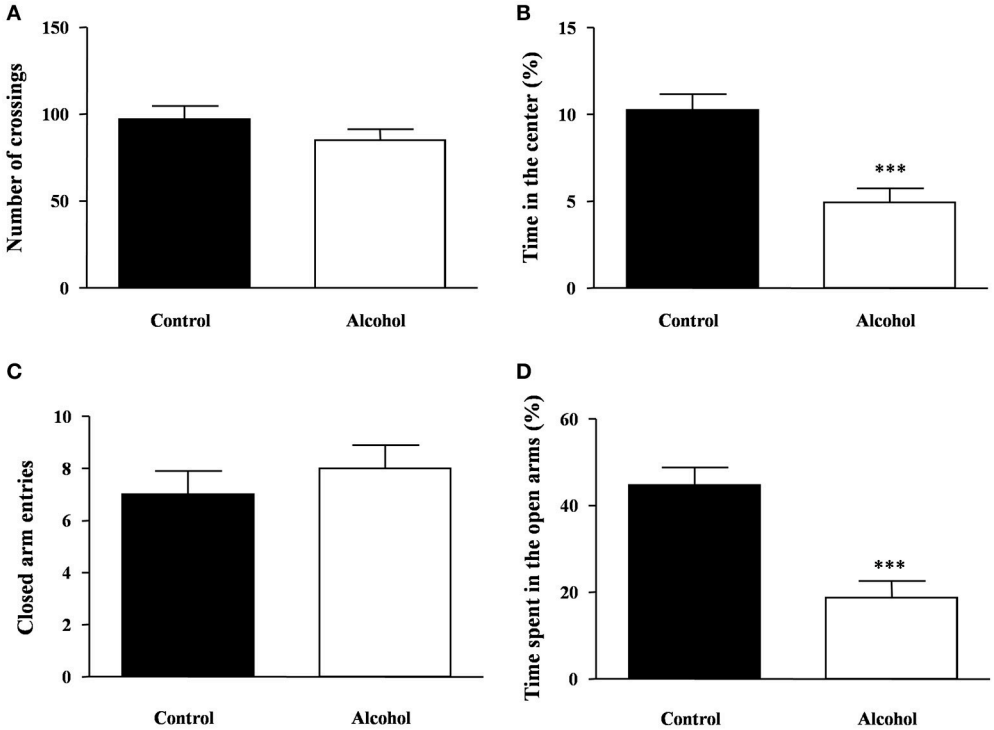


FIGURE 3 | Locomotion and anxiety-like behavior in adult rats exposed to intermittent adolescent alcohol. Number of crossings **(A)** and time spent in the center of the field **(B)** were evaluated in the open field test 1 week after the last alcohol exposure (pnd 62). Closed arm entries **(C)** and time spent exploring the open arms **(D)** were evaluated in the elevated plus-maze 2 weeks after the last alcohol exposure (pnd 69). Bars represent the mean ± SEM (8–12 animals per group). Data were analyzed by Student's *t*-test (Welch's correction). ***$p < 0.001$ denotes significant differences compared with the control group.

significant differences on the total exploration time (**Figure 4A**). Thus, the alcohol group spent less time to explore both objects than the control group [$t_{(21)} = 4.73$, $p < 0.001$].

One hour later, the test session was repeated using a novel object. A two-way ANOVA revealed a main effect of alcohol exposure on the exploration time [$F_{(1, 42)} = 12.76$, $p < 0.001$] but also an interaction between alcohol exposure and type of object [$F_{(1, 42)} = 14.40$, $p < 0.001$]. As shown in **Figure 4B**, the *post hoc* comparison indicated that the control group had a higher preference for the novel object relative to the familiar object ($p < 0.05$), whereas the alcohol group displayed a lower preference for the novel object ($p < 0.05$). Additionally, there was a significant decrease in the preference of the novel object in the alcohol group compared with the control group ($p < 0.001$).

Consequently, the alcohol group exhibited a negative discrimination ratio that indicated an impaired recognition memory [$t_{(21)} = 5.83$, $p < 0.001$] (**Figure 4C**).

Effects of Intermittent Alcohol Exposure on the Gene Expression of Endocannabinoid System

We examined the effect of adolescent alcohol exposure on the gene expression encoding relevant enzymes and receptors involved in endocannabinoid signaling [receptors: CB_1 (*Cnr1*), CB_2 (*Cnr2*) and peroxisome proliferator-activated receptor-α (PPAR-α) (*Ppara*); synthesis enzymes: NAPE-PLD (*Napepld*), DAGL-α (*Dagla*) and DAGL-β (*Daglb*); degradation enzymes: FAAH (*Faah*) and MAGL (*Mgll*)] following 2 weeks after the last exposure.

Medial Prefrontal Cortex

In the mPFC (**Figure 5A**), the observed effects were mostly activatory. The mRNA expression of *Ppara* [$t_{(7)} = 4.96$, $p = 0.002$]; the synthesis enzymes *Napepld* [$t_{(14)} = 6.12$, $p < 0.001$], *Dagla* [$t_{(7)} = 12.47$, $p < 0.001$] and *Daglb* [$t_{(7)} = 9.47$, $p < 0.001$]; and *Faah* [$t_{(14)} = 2.53$, $p = 0.024$] were significantly increased in the alcohol group relative to the control group before adjustment for multiple comparisons. The FDR adjusted significance level ($q = 0.031$) indicated that all differences in the mRNA levels were still significant.

Striatum

Conversely, the adolescent exposure during adolescence produced only inhibitory effects, as shown in **Figure 5B**. Thus, rats exposed to alcohol displayed a significant decrease in the striatal mRNA expression of the receptors: *Cnr1* [$t_{(7)} = 6.66$, $p < 0.001$], *Cnr2* [$t_{(7)} = 2.94$, $p = 0.022$] and *Ppara* [$t_{(8)} = 14.55$, $p < 0.001$] compared with the control group. However, the FDR adjusted significance level ($q = 0.013$) showed a significant decrease in the expression of *Cnr1* and *Ppara*.

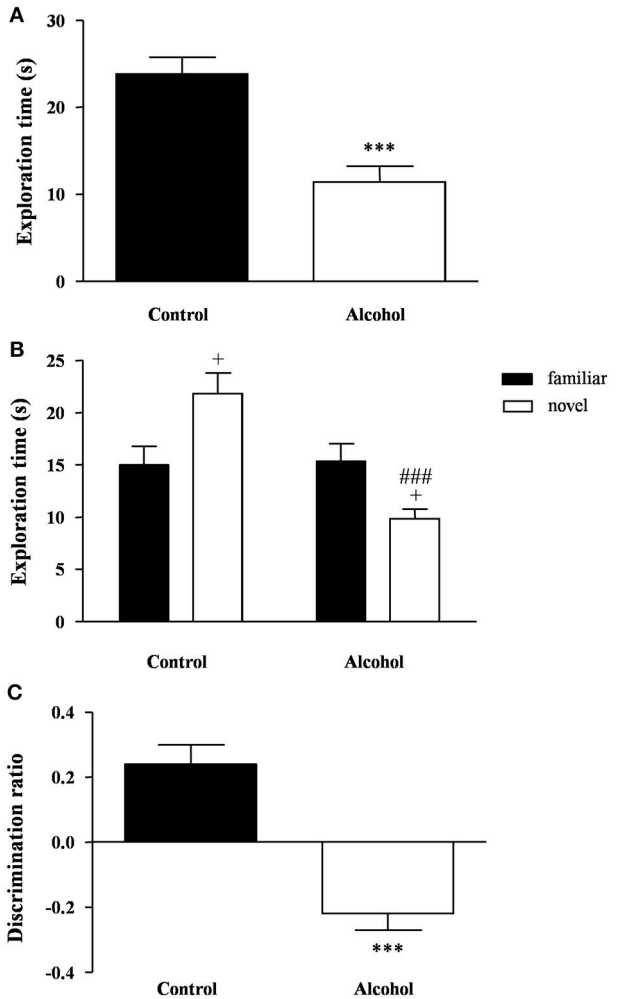


FIGURE 4 | Recognition memory in adult rats exposed to intermittent adolescent alcohol. Exploration time **(A)** during the familiarization phase, exploration time for both familiar and novel object **(B)** during the test phase and discrimination ration **(C)** of the novel object. Bars represent the mean ± SEM (8–12 animals per group). Data were analyzed by Student's *t*-test (Welch's correction) and two-way ANOVA. $^{***}p < 0.001$ denotes significant differences compared with the control group. $^{+}p < 0.05$ denotes significant differences compared with time exploring the familiar object; and $^{\#\#\#}p < 0.001$ denotes significant differences compared with time exploring the novel object in the control group.

Amygdala

Similar to the striatum, we found again inhibitory effects in the amygdala (**Figure 5C**). The mRNA levels of *Cnr1* [$t_{(14)} = 4.12$, $p = 0.001$], *Napepld* [$t_{(9)} = 2.69$, $p = 0.025$] and *Dagla* [$t_{(14)} = 3.44$, $p = 0.004$] were significantly decreased in the alcohol group relative to the control group before adjustment for multiple comparisons. The subsequent correction ($q = 0.013$) revealed significant differences in the expression of *Cnr1* and *Dagla* but not in *Napepld*.

Hippocampus

In the hippocampus (**Figure 5D**), the alcohol group displayed a significant decrease in the mRNA levels of *Cnr2* [$t_{(7)} = 3.96$, $p = 0.006$] and a significant increase in both *Dagla* [$t_{(14)} = 2.81$, $p = 0.014$] and *Mgll* [$t_{(9)} = 3.02$, $p = 0.015$] relative to the control group. Consistently, the FDR adjusted significance level ($q = 0.019$) revealed that these differences in the mRNA expression were still significant.

Table S1 summarizes the adjustment for multiple comparisons of the mRNA expression of the ECS in each brain region.

Effects of Intermittent Alcohol Exposure during Adolescence on the Gene Expression of Neuropeptides Linked to Anxiety and Stress

We also evaluated the effect of alcohol exposure during adolescence on the gene expression of neuropeptides linked to anxiety/stress responses and their receptors [neuropeptides: corticotropin-releasing factor (CRF) (*Crh*) and neuropeptides Y (NPY) (*Npy*); and receptors: CRF1R (*Crhr1*), CRF2R (*Crhr2*), NPY1R (*Npy1r*), NPY2R (*Npy2r*) and NPY5R (*Npy5r*)]. Determinations were conducted in the amygdala and hippocampus, two main regions belonging to the circuit that modulates emotional responses.

Amygdala

As shown in **Figure 6A**, the statistical analysis indicated no differences in the mRNA expression of *Crh* and its receptors but there were significant decreases in the expression of *Npy* [$t_{(9)} = 2.40$, $p = 0.040$] and *Npy2r* [$t_{(14)} = 3.07$, $p = 0.007$] in the alcohol group compared with the control group. However, the FDR adjusted significance level ($q = 0.007$) only remained significant differences between groups for *Npy2r*.

Hippocampus

In the hippocampus (**Figure 6B**), repeated *t*-tests showed significant differences in the mRNA levels of *Crh*, *Npy*, *Npy2r,* and *Npy5r* between groups. Thus, the alcohol group displayed higher *Crh* mRNA expression [$t_{(14)} = 3.76$, $p = 0.002$] and lower mRNA expression of *Npy* [$t_{(14)} = 2.22$, $p = 0.043$], *Npy2r* [$t_{(14)} = 6.60$, $p < 0.001$] and *Npy5r* [$t_{(14)} = 2.46$, $p = 0.027$] than the control group. After adjusting for multiple comparisons ($q = 0.014$) in the hippocampus, the significant differences were only observed in *Crh* and *Npy2r*.

Table S2 summarizes the adjustment for multiple comparisons of the mRNA expression of neuropeptides and receptors in the amygdala and hippocampus.

Effects of Intermittent Alcohol Exposure during Adolescence on the Gene Expression of Neuroinflammatory Factors

Finally, we examined the effect of alcohol exposure during adolescence on the gene expression of relevant inflammatory-related markers [pro-inflammatory mediators: tumor necrosis factor-α (TNF-α) (*Tnf*), TLR4 (*Tlr4*), COX-2 (*Ptgs2*) and NF-κβ (*Rela*); and glia-activating factors: glial fibrillary acidic protein (GFAP) (*Gfap*) and allograft inflammatory factor-1 or microglia response factor (MRF-1) (*Aif1*)] following 2 weeks after the last alcohol exposure.

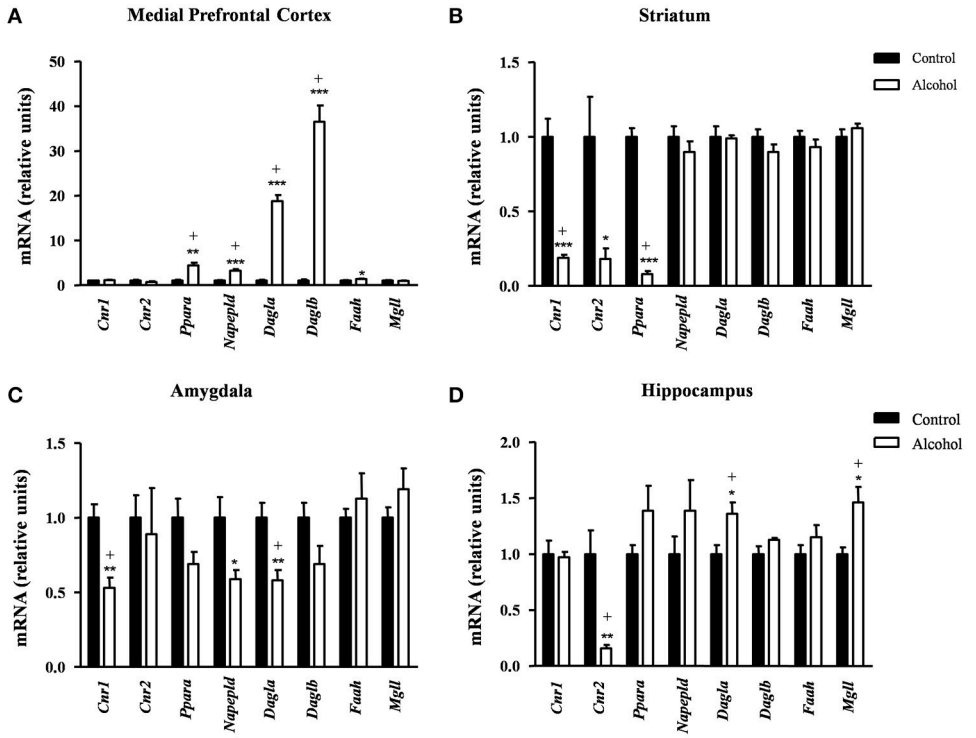


FIGURE 5 | Relative mRNA expression of endocannabinoid signaling-related genes in different brain regions of adult rats exposed to intermittent adolescent alcohol. Relative mRNA expression of *Cnr1*, *Cnr2*, *Ppara, Napepld, Dagla, Daglb, Faah* and *Mgll* in the medial prefrontal cortex **(A)**, striatum **(B)**, amygdala **(C)**, and hippocampus **(D)**. Bars represent the mean ± SEM (8 animals per group). Data were analyzed by Student's *t*-test (Welch's correction) and Benjamini-Holchberg false discovery rate procedure for multiple comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ denote significant differences compared with the control group. (+) denotes significant differences compared with the control group after adjustment for multiple comparisons in each brain region.

Medial Prefrontal Cortex

In the mPFC (**Figure 7A**), the mRNA levels of *Tnf* [$t_{(9)} = 3.55$, $p = 0.006$], *Tlr4* [$t_{(14)} = 12.50$, $p < 0.001$], *Ptgs2* [$t_{(14)} = 2.67$, $p = 0.018$] and *Gfap* [$t_{(7)} = 9.08$, $p < 0.001$] were significantly increased in the alcohol group in comparison with the control group. By contrast, the mRNA expression of *Rela* was significantly decreased in the alcohol group [$t_{(14)} = 4.17$, $p < 0.001$]. The adjustment for multiple comparisons ($q = 0.042$) confirmed all the significant differences found after repeated *t*-tests.

Striatum

In the striatum (**Figure 7B**), the statistical analysis showed a significant decrease in the mRNA levels of *Ptgs2* [$t_{(14)} = 4.34$, $p < 0.001$], *Rela* [$t_{(9)} = 5.91$, $p < 0.001$], *Gfap* [$t_{(14)} = 2.48$, $p = 0.027$] and *Aif1* [$t_{(7)} = 2.51$, $p = 0.041$] in the alcohol group compared with the control group. However, the FDR adjusted significance level ($q = 0.018$) remained significant differences only in the expression of *Ptgs2* and *Rela*.

Amygdala

As shown in **Figure 7C**, the alcohol group only displayed lower *Aif1* mRNA levels compared with the control group [$t_{(8)} = 3.45$, $p = 0.008$] and this difference was still observed after the correction for multiple comparisons ($q = 0.008$).

Hippocampus

Regarding the hippocampus (**Figure 7D**), the statistical analysis indicated that the alcohol group displayed lower mRNA levels of *Ptgs2* [$t_{(14)} = 2.72$, $p = 0.017$] and *Gfap* [$t_{(14)} = 2.74$, $p = 0.016$] and higher *Aif1* mRNA levels [$t_{(8)} = 2.84$, $p = 0.022$] compared with the control group. The adjustment for multiple comparisons ($q = 0.025$) confirmed the significance of all differences found using repeated *t*-tests.

Table S3 summarizes the adjustment for multiple comparisons of the mRNA expression of neuroinflammation-related factors in each brain region.

DISCUSSION

The present study in adult rats investigated the effects of intermittent alcohol intoxication on emotional behavior, cognition and the relative mRNA expression of genes from the endocannabinoid system (ECS), neuropeptides regulating anxiety- and alcohol-related behavior and mediators of neuroinflammation and plasticity. We must keep in mind that the social isolation of the animals in their home-cages throughout the experiments can affect the basal values in emotional behaviors (e.g., isolation induces anxiety-like behaviors and impairs fear extinction) and the

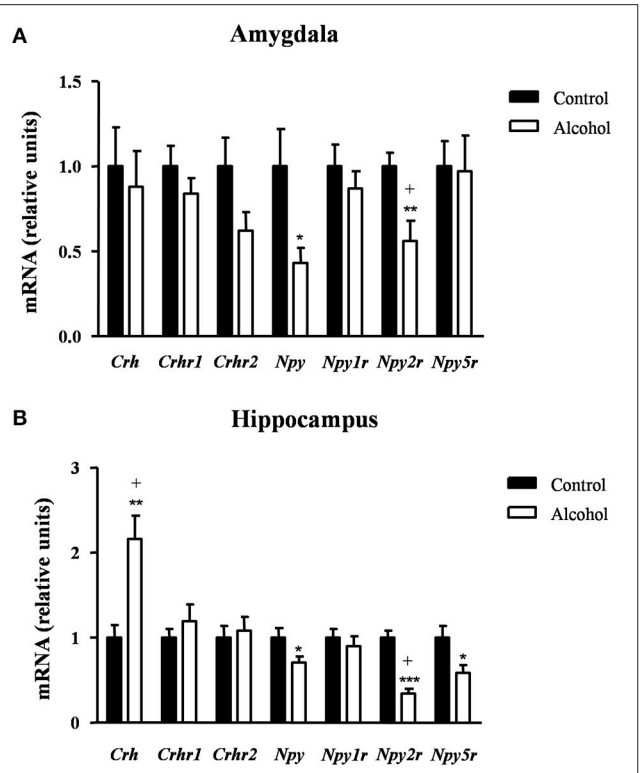


FIGURE 6 | Relative mRNA expression of markers of anxiety in different brain regions of adult rats exposed to intermittent adolescent alcohol. Relative mRNA expression of *Crh, Crhr1, Crhr2, Npy, Npy1r, Npy2r,* and *Npy5r* in the amygdala **(A)** and hippocampus **(B)**. Bars represent the mean ± SEM (8 animals per group). Data were analyzed by Student's *t*-test (Welch's correction) and Benjamini-Holchberg false discovery rate procedure for multiple comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ denote significant differences compared with the control group. (+) denotes significant differences compared with the control group after adjustment for multiple comparisons in each brain region.

expression of signaling systems in the brain (Skelly et al., 2015).

Intermittent Alcohol Intoxication

As expected, the alcohol group showed elevated ethanol levels in the blood. These BECs were about 200 mg/dl and they are comparable to the concentrations reported in other studies conducted in adolescent Wistar rats using a model of binge-like drinking with i.p. injections of 3 g/kg, which reported 150–180 mg/dl 1 h postinjection (Przybycien-Szymanska et al., 2010, 2012). Although our procedure raised plasma alcohol levels in a binge-like manner [binge drinking is indeed defined as heavy episodic drinking within a BEC of 80 mg/dl or higher (NIAAA, 2004)], the alcohol administration was injected and it is possible that our model would yield higher alcohol doses than animals would consume voluntarily as it is expected in a binge drinking pattern (Spanagel, 2000). However, both patterns of excessive alcohol consumption are characterized by high BECs, and this can be critical in the effects of adolescent alcohol on behavior and expression of signaling systems in the brain.

During the alcohol exposure, we monitored the feeding behavior of adolescent rats by measuring body weight and food intake. Thus, the alcohol group displayed a lower body weight gain accompanied by a reduction in food intake during the alcohol exposure. These observations agree with previous studies reporting a significant decrease in body weight after i.p. administration of ethanol (Luz et al., 1996; Iwaniec and Turner, 2013). In fact, Lauing and colleagues reported decreased weight gain after modeling binge drinking using i.p. alcohol administration delivered on 2 consecutive days followed by 5 days of abstinence (Lauing et al., 2008). Several factors such as local inflammation and pain related to repeated injections, repeated acute ethanol intoxications with subsequent physical discomfort... could participate in this negative energy balance. However, there are studies reporting no differences in the final body weight after an intermittent binge-drinking exposure (Przybycien-Szymanska et al., 2010) although the total duration of this protocol was only 8 days.

Effects on Emotional Behaviors and Cognitive Responses

After the alcohol exposure, locomotor activity, anxiety-like behavior and cognitive responses were assessed using a behavioral test battery consisting of the open field, elevated plus maze and novel object recognition task.

One week after the last alcohol session, we observed no differences in locomotor activity in the open field but there was an anxiogenic-like response to the test in the alcohol group compared with the control group, which could be related to the effects of alcohol deprivation on emotional behavior. These results are consistent with previous studies in rodents exposed to repeated binge-drinking during adolescence, which reported long-term anxiety disorders (Gilpin et al., 2012; Montesinos et al., 2016). One week later, the same rats were assessed in the elevated plus-maze and the anxiogenic-like behavior was also detected in the alcohol group. Ethanol withdrawal in rodents is associated with a negative affective state, including an enhanced anxiety-like behavior. Following a period of abstinence, rats spent significantly less time in the open arms of the elevated-plus maze than control animals (File et al., 1993; Moy et al., 1997; Wilson et al., 1998; Pandey et al., 2003). This behavioral profile can persist for as long as 28 days (Rasmussen et al., 2001). In our animals, the elevated anxiety in the alcohol group persisted unchanged for at least 2 weeks following the last alcohol exposure, suggesting enduring changes on emotionality.

A novel object recognition task was used to detect the existence of cognitive impairments because this paradigm relies on the innate preference that rodents display for exploring novel rather than familiar objects (Ennaceur and Delacour, 1988). To perform the task, we used a 1 h delay interval between the familiarization and novelty phases to consolidate a short-term memory that depends on the integrity of the hippocampal formation (García-Moreno et al., 2002). The results showed that rats exposed to alcohol displayed a decreased preference for the novel object over the familiar object but similar exploration time of the familiar object. This attracted our attention, because this

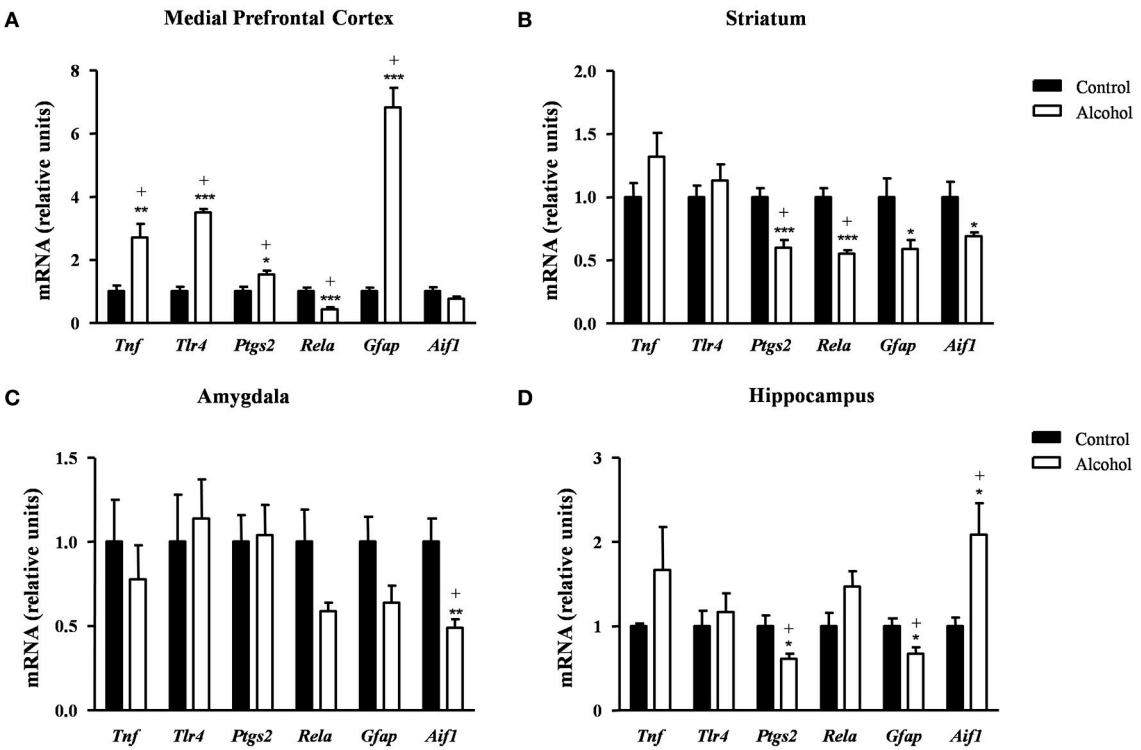


FIGURE 7 | Relative mRNA expression of neuroinflammatory factors in different brain regions of adult rats exposed to intermittent adolescent alcohol. Relative mRNA expression of *Tnf, Tlr4, Ptgs2, Rela, Gfap* and *Aif1* mRNA in the medial prefrontal cortex **(A)**, striatum **(B)**, amygdala **(C)**, and hippocampus **(D)**. Bars represent the mean ± SEM (8 animals per group). Data were analyzed by Student's *t*-test (Welch's correction) and Benjamini–Holchberg false discovery rate procedure for multiple comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ denote significant differences compared with the control group. (+) denotes significant differences compared with the control group after adjustment for multiple comparisons in each brain region.

less exploration time of the novel object was not associated with a locomotor impairment in this task, which was in agreement with the open field observations. In fact, the alcohol group displayed less exploration time during the familiarization phase when both identical objects were considered novel. Thus, the reduced preference for the novel object exhibited by the rats exposed to alcohol might be associated with the development of neophobia-induced anxiety and cognitive deficit, which is thought to prevent exploratory behavior (Myhrer, 1988).

Effects on Endocannabinoid System Components

The brain is undergoing extensive maturation during adolescence. Similarly, the ECS is also undergoing maturational changes and some alteration of these processes of maturation can produces long-term alterations, including deficits in emotional behavior and cognition.

Several lines of evidence indicate that alcohol leads to neuroadaptations in endocannabinoid signaling mechanisms (Basavarajappa and Hungund, 1999; Basavarajappa et al., 2003). The present results showed that rats exposed to adolescent alcohol displayed alterations in the expression of endocannabinoid enzymes and receptors that appear to be reliant on the brain region, which is consistent with previous studies reporting regionally distinct effects on brain endocannabinoid levels after alcohol exposure (González et al., 2002, 2004; Rubio et al., 2007).

The mPFC is a key region for multiple cognitive functions, including executive function or anxiety. In addition, the ECS is involved in the regulation of emotional behaviors in this brain region (Rubino et al., 2008; McLaughlin et al., 2014; Morena et al., 2016). Thus, the negative impact of the alcohol to the adolescent mPFC may be associated to the observed alterations in cognition and behavior. We found that rats exposed to adolescent alcohol had a strong increase in the mRNA expression of *Napepld* and *Dagl* in this region. These observations suggest that the ECS is activated in response to the negative affective state associated to alcohol withdrawal. Additionally, the alcohol group displayed an increase in the mRNA expression of *Faah* and the receptor *Ppara*. It has been described that localized infusion of the FAAH inhibitor URB597 into the mPFC increases ethanol consumption by rats (Hansson et al., 2007). Also, a recent study has reported that pretreatment with an inhibitor of FAAH prevents against oxidative stress caused by binge ethanol consumption in the mPFC of adolescent rats (Pelição et al., 2016). Furthermore, PPAR-α is involved in the actions of other N-acylethanolamines with no endocannabinoid activity (e.g., oleoylethanolamide and palmitoylethanolamide) that have been reported to exhibit neuroprotective effects (Fu et al., 2003; Scuderi et al., 2012).

Collectively these findings suggest a strengthening of the ECS that may reflect a homeostatic mechanism to prevent the neurotoxic effects induced by alcohol with a relevant role of other non-cannabinoid congeners in the alcohol exposure.

The ECS is an important modulator of neuroplasticity in the striatum. Overall, we observed that alcohol-exposed rats displayed a decreased mRNA expression of *Cnr1* and *Ppara*, as well as a decreasing trend in *Cnr2*. These results agree with preclinical studies that indicate a decrease in *Cnr1* mRNA levels after alcohol exposure in mice (Vinod et al., 2006) and rats (Adermark et al., 2011). Regarding the *Cnr2* mRNA levels, it is known that alcohol consumption alters the *Cnr2* expression in the brain and that an increased ethanol preference is associated with a reduced *Cnr2* expression in the striatum (Onaivi et al., 2008).

The amygdala is a crucial subcortical area that integrates reward, emotions, and conditioned learning. Previous studies have demonstrated drug-induced alterations in amygdalar endocannabinoid function (Orio et al., 2009; Kamprath et al., 2011; Schmidt et al., 2011). In the present study, we observed a decreasing trend in the receptors and synthesis enzymes but not in the degradative enzymes. Consistently, we have reported that chronic alcohol treatment alters the expression of different cannabinoid signaling-related genes in the amygdala (Serrano et al., 2012).

The hippocampus is a brain region involved in learning which cooperates with the amygdala to modulate emotion and belongs to the reward circuit. Numerous studies have shown that alcohol exposure alters hippocampal function (for review see Kutlu and Gould, 2016). Regarding the expression of the endocannabinoid signaling in the hippocampus, we found a dramatic decrease in the expression of *Cnr2*. In the hippocampus, such cannabinoid receptor is involved in the modulation of excitatory synapses (García-Gutiérrez et al., 2013; Kim and Li, 2015). A recent study has demonstrated a role of the hippocampal CB_2 to modulate cognitive functions in mice (Li and Kim, 2016). Thus, the effects on *Cnr2* mRNA expression in our alcohol-exposed rats may be associated to the adverse effects of alcohol on synaptic plasticity (basically long-term potentiation and long-term depression processes) and the consequent defects in learning and memory (Lovinger and Roberto, 2013).

Effects on Neuropeptides Linked to Emotional Behaviors

As mentioned above, the presence of anxiety is one of the most consistent features of alcohol withdrawal. Since the amygdala and hippocampus are key brain regions in the modulation of emotional behaviors and the ECS is also involved in the regulation of emotionality (Hill and Gorzalka, 2009), the present findings suggest that the differential changes observed in the ECS after adolescent alcohol exposure may be associated with different responses to anxiety. In this regard, to get a better understanding of the potential mechanisms underlying the anxiety effects that were observed, we analyzed the CRF and NPY signals in these regions. Furthermore, both neuropeptides are clearly involved in alcohol-related behaviors and binge alcohol drinking (Pleil et al., 2015).

Interestingly, we found an increase in the hippocampal expression of *Crh* mRNA in the alcohol group with no changes in the CRF receptors. In line with this, previous studies have associated CRF with binge drinking and alcohol dependence (Lowery et al., 2010; Gilpin et al., 2012; Pleil et al., 2015).

Regarding the NPY signal, in both brain areas the alcohol group displayed a down-regulation of this system with a strong decrease in the mRNA levels of *Npy2r* that may be associated with the anxiogenic-like behavior observed in this group. These findings are in agreement with a recent study showing that a decrease in the expression of receptors for NPY are associated with an increase in the anxiety-like behavior in adolescent rats exposed to repeated binge-like alcohol drinking (McClintick et al., 2016).

Effects on Neuroinflammatory Signals

Since alcohol increases neuroinflammation through its ability of activating natural immunity, we evaluated the gene expression of factors involved in pro-inflammatory signaling pathways but also factors associated with plasticity and neurodevelopment.

The data indicate a region-specific susceptibility to the alcohol regulation of the mRNA expression of these factors, being these alterations more prominent in the mPFC. In line with this, binge drinking has been reported to produce alcohol-induced inflammatory PFC damage and this can be accompanied by reduced executive functions and compulsive behavior (Crews et al., 2000). Thus, we observed that adult rats exposed to adolescent alcohol displayed an overall increase in the mRNA expression of the neuroinflammation-related factors *Tlr4, Tnf* and *Ptgs2* in the mPFC. Several evidence indicates that alcohol induces the TLR4 signaling in the PFC (Vetreno and Crews, 2012; Pascual et al., 2014), triggering the induction of a cascade of pro-inflammatory mediators that affect, among others, cognitive impairments and anxiety-like behaviors associated with the alcohol abuse (Montesinos et al., 2015; Pascual et al., 2015).

TLR4 receptors are mediators of alcohol-induced inflammatory damage in the adolescent and adult brain (Alfonso-Loeches et al., 2010). In fact, Crews and colleagues have reported an increased *Tlr4* expression in the brain of alcoholics as well as in mice treated chronically with ethanol (Crews et al., 2013). In addition to TLR4, there was an up-regulation of *Tnf* and *Ptgs2* mRNA levels in alcohol-exposed rats, which is consistent with previous studies reporting that ethanol binge drinking is associated with increased cortical levels of TNF-α and COX-2 (Knapp and Crews, 1999; Antón et al., 2016). By contrast, the gene expression of various pro-inflammatory mediators were down-regulated (e.g., COX-2 mRNA levels were decreased in the striatum and hippocampus and NF-κβ mRNA levels were decreased in the mPFC and striatum), which appears to be incongruent with literature since alcohol exposure activates COX-2 and NF-κβ by triggering cytokine and chemokine release and neuroinflammation. In the mPFC, the decreased gene expression of *Rela* may be associated with the up-regulation of *Ppara* receptor observed in this

brain region because it has been described that the activation of this nuclear receptor inhibits *Rela* (Stahel et al., 2008). Nevertheless, we have to keep in mind that changes in gene expression are often not concordant with protein expression (Vogel and Marcotte, 2012).

In addition to these factors and receptors, the inflammatory response is associated with astrocytes activation (Ransohoff and Brown, 2012). In fact, previous studies have demonstrated that *Gfap* levels are affected by alcohol exposure (Miguel-Hidalgo et al., 2006; Udomuksorn et al., 2011). Consistently, the gene expression of GFAP and MRF-1 were found up-regulated in the mPFC and hippocampus. Similar to COX-2 and NF-κβ, the gene expression of both astrocyte marker *Gfap* and microglial marker *Aif1* were also down-regulated in other brain regions (hippocampus and amygdala, respectively) and this was only reported in human alcoholic brain in conjunction with astrocytic loss (Lewohl et al., 2005).

Summary

In summary, adult rats exposed to intermittent alcohol exposure during adolescence displayed anxiety-like behaviors and cognitive deficits related to recognition memory. Furthermore, the mRNA expression of some components of signaling systems involved in behavior, neuroinflammation and plasticity were found altered in these animals, with brain region-dependent changes.

Although adult rats exposed to adolescent alcohol had no alterations in locomotor activity, they exhibited a strong anxiogenic-like behavior. In addition to the anxiety-like response, we observed a clear cognitive deficit with a reduced preference for the novelty. These behavioral and cognitive effects in adulthood were accompanied by molecular changes in the brain.

Alterations in the mRNA expression of genes of the ECS appear to be reliant on the brain region. However, we observed an overall decreasing trend in the mRNA expression of the cannabinoid receptors in the alcohol group. This down-regulation can be associated to the adverse effects of alcohol on synaptic plasticity with cognitive consequences in adulthood such as deficits in learning and memory.

Whereas, the ECS is also associated with emotionality, neuropeptides such as CRF and NPY are clearly involved in alcohol-related behaviors and binge alcohol drinking. Thus, we observed changes in both signals that were linked to anxiogenic-like behaviors, in particular a decrease in the NPY signaling expression.

Regarding neuroinflammatory-related factors, our data indicated a brain region-specific susceptibility in the adult rats exposed to intermittent alcohol. These alterations were more prominent in the mPFC with an up-regulation of the mRNA levels of *Tlr4, Tnf, Ptgs2,* and *Gfap* and a decrease of *Rela*. The activation of PPAR-α inhibits NF-κβ and the mRNA expression of *Ppara* was increased in the mPFC, a key region for multiple cognitive functions. Interestingly, *Ppara* is involved in the anti-inflammatory and neuroprotective actions of endocannabinoid congeners. In contrast to the mPFC, the mRNA expression of *Ptgs2* and *Gfap* were down-regulated in the rest of brain regions.

Limitations and Perspectives

We are aware of the limitations of our study, mainly because of the exploratory nature of the present investigation and the necessity of performing future studies that include female subjects and alcohol exposure during adulthood. Furthermore, the protein expression and function of signaling systems and factors involved in behavior, neuroinflammation and neuroplasticity will open new lines of research using pharmacological and genetic approaches to characterize the association and regulation of these systems with emotional behavior and cognition in the context of adolescent alcohol and its long-term consequences.

The existence of sex differences in the response to alcohol and other drugs has been extensively reported (Becker and Koob, 2016) but we have conducted our experiments only in male rats for clarity. Studies using female rats will reveal whether the estrous cycle and gonadal hormones influence the behavioral and molecular alterations that we have observed here.

Although our data indicate changes in the mRNA expression of signaling systems in the brain and alterations in both emotional and cognitive responses of adult rats exposed to adolescent alcohol, we cannot link these molecular and behavioral effects exclusively to an alcohol exposure during adolescence because we have no rats exposed to alcohol during adulthood for comparison. Consequently, the specificity of the effects associated with intermittent alcohol exposure on these biological substrates will have to be elucidated.

Finally, additional studies will have to resolve the lack of the protein expression of these enzymes, factors and receptors because changes in gene expression level are frequently not reflected at the protein level. In fact, post-transcriptional, translational and degradation regulation must also be taken into account in the determination of protein concentrations because contribute at least as much as transcriptional itself (Vogel and Marcotte, 2012). Also, functional assays will be required to elucidate the role of these signaling systems in the appearance of emotional behaviors and addictive behaviors related to an adolescent alcohol exposure.

AUTHOR CONTRIBUTIONS

All authors had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analyzes. Study concept and design: FR, AS. Acquisition of data: LS, FP, JD, JS, AG, EC. Analysis and interpretation of data: FP, FR, AS. Drafting of the manuscript: FR, AS. Critical revision of the manuscript for important intellectual content, obtained funding and study supervision: FP, FR, AS.

FUNDING

The present study has been supported by the following institutions and grants: Instituto de Salud Carlos III (ISCIII) and European Union-European Regional Development Fund (EU-ERDF) (Subprograma RETICS Red de Trastornos Adictivos RD12/0028/0001); ISCIII and Ministerio de Economía y Competitividad (PI13/02261); Junta de Andalucía-Consejería de Igualdad, Salud y Políticas Sociales (PI-0823-2012 and

PI-0228-2013); Ministerio de Sanidad, Servicios Sociales e Igualdad and Plan Nacional sobre Drogas (PNSD2015/047). FP (CP14/00212), JS (CP12/03109), and AS (CP14/00173) are recipients of a research contract from Miguel Servet Program of ISCIII and EU-ERDF.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnbeh.2017.00015/full#supplementary-material

REFERENCES

Adermark, L., Jonsson, S., Ericson, M., and Söderpalm, B. (2011). Intermittent ethanol consumption depresses endocannabinoid-signaling in the dorsolateral striatum of rat. *Neuropharmacology* 61, 1160–1165. doi: 10.1016/j.neuropharm.2011.01.014

Alfonso-Loeches, S., Pascual-Lucas, M., Blanco, A. M., Sanchez-Vera, I., and Guerri, C. (2010). Pivotal role of TLR4 receptors in alcohol-induced neuroinflammation and brain damage. *J. Neurosci.* 30, 8285–8295. doi: 10.1523/JNEUROSCI.0976-10.2010

Antón, M., Alén, F., Gómez de Heras, R., Serrano, A., Pavón, F. J., Leza, J. C. et al. (2016). Oleoylethanolamide prevents neuroimmune HMGB1/TLR4/NF-kB danger signaling in rat frontal cortex and depressive-like behavior induced by ethanol binge administration. *Addict. Biol.* doi: 10.1111/adb.12365. [Epub ahead of print].

Basavarajappa, B. S., and Hungund, B. L. (1999). Chronic ethanol increases the cannabinoid receptor agonist anandamide and its precursor N-arachidonoylphosphatidylethanolamine in SK-N-SH cells. *J. Neurochem.* 72, 522–528. doi: 10.1046/j.1471-4159.1999.0720522.x

Basavarajappa, B. S., Saito, M., Cooper, T. B., and Hungund, B. L. (2003). Chronic ethanol inhibits the anandamide transport and increases extracellular anandamide levels in cerebellar granule neurons. *Eur. J. Pharmacol.* 466, 73–83. doi: 10.1016/S0014-2999(03)01557-7

Becker, J. B., and Koob, G. F. (2016). Sex Differences in animal models: focus on addiction. *Pharmacol. Rev.* 68, 242–263. doi: 10.1124/pr.115.011163

Benjamini, Y., Drai, D., Elmer, G., Kafkafi, N., and Golani, I. (2001). Controlling the false discovery rate in behavior genetics research. *Behav. Brain Res.* 125, 279–284. doi: 10.1016/S0166-4328(01)00297-2

Bisogno, T., Howell, F., Williams, G., Minassi, A., Cascio, M. G., Ligresti, A., et al. (2003). Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J. Cell Biol.* 163, 463–468. doi: 10.1083/jcb.200305129

Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., and Gilula, N. B. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384, 83–87. doi: 10.1038/384083a0

Crews, F., Nixon, K., Kim, D., Joseph, J., Shukitt-Hale, B., Qin, L., et al. (2006). BHT blocks NF-*k*B activation and ethanol-induced brain damage. *Alcohol. Clin. Exp. Res.* 30, 1938–1949. doi: 10.1111/j.1530-0277.2006.00239.x

Crews, F. T., Braun, C. J., Hoplight, B., Switzer, R. C. III, and Knapp, D. J. (2000). Binge ethanol consumption causes differential brain damage in young adolescent rats compared with adult rats. *Alcohol. Clin. Exp. Res.* 24, 1712–1723. doi: 10.1111/j.1530-0277.2000.tb01973.x

Crews, F. T., Qin, L., Sheedy, D., Vetreno, R. P., and Zou, J. (2013). High mobility group box 1/Toll-like receptor danger signaling increases brain neuroimmune activation in alcohol dependence. *Biol. Psychiatry* 73, 602–612. doi: 10.1016/j.biopsych.2012.09.030

Dawson, D. A., Goldstein, R. B., Chou, S. P., Ruan, W. J., and Grant, B. F. (2008). Age at first drink and the first incidence of adult-onset DSM-IV alcohol use disorders. *Alcohol. Clin. Exp. Res.* 32, 2149–2160. doi: 10.1111/j.1530-0277.2008.00806.x

Dinh, T. P., Carpenter, D., Leslie, F. M., Freund, T. F., Katona, I., Sensi, S. L., et al. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10819–10824. doi: 10.1073/pnas.152334899

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. *Behav. Brain Res.* 31, 47–59. doi: 10.1016/0166-4328(88)90157-X

Fernandez-Lizarbe, S., Pascual, M., and Guerri, C. (2009). Critical role of TLR4 response in the activation of microglia induced by ethanol. *J. Immunol.* 183, 4733–4744. doi: 10.4049/jimmunol.0803590

File, S. E., Andrews, N., and Al-Farhan, M. (1993). Anxiogenic responses of rats on withdrawal from chronic ethanol treatment: effects of tianeptine. *Alcohol. Alcohol.* 28, 281–286.

Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodriguez De Fonseca, F., et al. (2003). Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature* 425, 90–93. doi: 10.1038/nature01921

García-Gutiérrez, M. S., Ortega-Alvaro, A., Busquets-Garcia, A., Perez-Ortiz, J. M., Caltana, L., Ricatti, M. J., et al. (2013). Synaptic plasticity alterations associated with memory impairment induced by deletion of CB2 cannabinoid receptors. *Neuropharmacology* 73, 388–396. doi: 10.1016/j.neuropharm.2013.05.034

García-Moreno, L. M., Conejo, N. M., Capilla, A., Garcia-Sanchez, O., Senderek, K., and Arias, J. L. (2002). Chronic ethanol intake and object recognition in young and adult rats. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 26, 831–837. doi: 10.1016/S0278-5846(01)00327-X

Gilpin, N. W., Karanikas, C. A., and Richardson, H. N. (2012). Adolescent binge drinking leads to changes in alcohol drinking, anxiety, and amygdalar corticotropin releasing factor cells in adulthood in male rats. *PLoS ONE* 7:e31466. doi: 10.1371/journal.pone.0031466

González, S., Cascio, M. G., Fernandez-Ruiz, J., Fezza, F., Di Marzo, V., and Ramos, J. A. (2002). Changes in endocannabinoid contents in the brain of rats chronically exposed to nicotine, ethanol or cocaine. *Brain Res.* 954, 73–81. doi: 10.1016/S0006-8993(02)03344-9

González, S., Valenti, M., De Miguel, R., Fezza, F., Fernandez-Ruiz, J., Di Marzo, V., et al. (2004). Changes in endocannabinoid contents in reward-related brain regions of alcohol-exposed rats, and their possible relevance to alcohol relapse. *Br. J. Pharmacol.* 143, 455–464. doi: 10.1038/sj.bjp.0705963

Goral, J., Karavitis, J., and Kovacs, E. J. (2008). Exposure-dependent effects of ethanol on the innate immune system. *Alcohol* 42, 237–247. doi: 10.1016/j.alcohol.2008.02.003

Guerri, C., Bazinet, A., and Riley, E. P. (2009). Foetal alcohol spectrum disorders and alterations in brain and behaviour. *Alcohol Alcohol.* 44, 108–114. doi: 10.1093/alcalc/agn105

Hansson, A. C., Bermudez-Silva, F. J., Malinen, H., Hyytia, P., Sanchez-Vera, I., Rimondini, R., et al. (2007). Genetic impairment of frontocortical endocannabinoid degradation and high alcohol preference. *Neuropsychopharmacology* 32, 117–126. doi: 10.1038/sj.npp.1301034

Hill, M. N., and Gorzalka, B. B. (2009). The endocannabinoid system and the treatment of mood and anxiety disorders. *CNS Neurol. Disord. Drug Targets* 8, 451–458. doi: 10.2174/187152709789824624

Howlett, A. C., Bidaut-Russell, M., Devane, W. A., Melvin, L. S., Johnson, M. R., and Herkenham, M. (1990). The cannabinoid receptor: biochemical, anatomical and behavioral characterization. *Trends Neurosci.* 13, 420–423. doi: 10.1016/0166-2236(90)90124-S

Iwaniec, U. T., and Turner, R. T. (2013). Intraperitoneal injection of ethanol results in drastic changes in bone metabolism not observed when ethanol is administered by oral gavage. *Alcohol. Clin. Exp. Res.* 37, 1271–1277. doi: 10.1111/acer.12105

Kalivas, P. W., and O'Brien, C. (2008). Drug addiction as a pathology of staged neuroplasticity. *Neuropsychopharmacology* 33, 166–180. doi: 10.1038/sj.npp.1301564

Kamprath, K., Romo-Parra, H., Haring, M., Gaburro, S., Doengi, M., Lutz, B., et al. (2011). Short-term adaptation of conditioned fear responses through endocannabinoid signaling in the central amygdala. *Neuropsychopharmacology* 36, 652–663. doi: 10.1038/npp.2010.196

Kim, J., and Li, Y. (2015). Chronic activation of CB2 cannabinoid receptors in the hippocampus increases excitatory synaptic transmission. *J. Physiol.* 593, 871–886. doi: 10.1113/jphysiol.2014.286633

Knapp, D. J., and Crews, F. T. (1999). Induction of cyclooxygenase-2 in brain during acute and chronic ethanol treatment and ethanol withdrawal. *Alcohol. Clin. Exp. Res.* 23, 633–643. doi: 10.1111/j.1530-0277.1999.tb04165.x

Koob, G. F. (2008). A role for brain stress systems in addiction. *Neuron* 59, 11–34. doi: 10.1016/j.neuron.2008.06.012

Koob, G. F., Roberts, A. J., Schulteis, G., Parsons, L. H., Heyser, C. J., Hyytia, P., et al. (1998). Neurocircuitry targets in ethanol reward and dependence. *Alcohol. Clin. Exp. Res.* 22, 3–9. doi: 10.1111/j.1530-0277.1998.tb03611.x

Kovacs, E. J., and Messingham, K. A. (2002). Influence of alcohol and gender on immune response. *Alcohol Res. Health* 26, 257–263.

Kutlu, M. G., and Gould, T. J. (2016). Effects of drugs of abuse on hippocampal plasticity and hippocampus-dependent learning and memory: contributions to development and maintenance of addiction. *Learn. Mem.* 23, 515–533. doi: 10.1101/lm.042192.116

Lauing, K., Himes, R., Rachwalski, M., Strotman, P., and Callaci, J. J. (2008). Binge alcohol treatment of adolescent rats followed by alcohol abstinence is associated with site-specific differences in bone loss and incomplete recovery of bone mass and strength. *Alcohol* 42, 649–656. doi: 10.1016/j.alcohol.2008.08.005

Lewohl, J. M., Wixey, J., Harper, C. G., and Dodd, P. R. (2005). Expression of MBP, PLP, MAG, CNP, and GFAP in the human alcoholic brain. *Alcohol. Clin. Exp. Res.* 29, 1698–1705. doi: 10.1097/01.alc.0000179406.98868.59

Li, Y., and Kim, J. (2016). CB2 cannabinoid receptor knockout in mice impairs contextual long-term memory and enhances spatial working memory. *Neural Plast.* 2016:9817089. doi: 10.1155/2016/9817089

Lovinger, D. M., and Roberto, M. (2013). Synaptic effects induced by alcohol. *Curr. Top. Behav. Neurosci.* 13, 31–86. doi: 10.1007/978-3-642-28720-6_143

Lowery, E. G., Spanos, M., Navarro, M., Lyons, A. M., Hodge, C. W., and Thiele, T. E. (2010). CRF-1 antagonist and CRF-2 agonist decrease binge-like ethanol drinking in C57BL/6J mice independent of the HPA axis. *Neuropsychopharmacology* 35, 1241–1252. doi: 10.1038/npp.2009.209

Luz, J., Griggio, M. A., Plapler, H., De-Meo-Bancher, M., and Carvalho-Kosmiskas, J. V. (1996). Effects of ethanol on energy balance of rats and the inappropriateness of intraperitoneal injection. *Alcohol* 13, 575–580. doi: 10.1016/S0741-8329(96)00070-5

McClintick, J. N., McBride, W. J., Bell, R. L., Ding, Z. M., Liu, Y., Xuei, X., et al. (2016). Gene expression changes in glutamate and GABA-A receptors, neuropeptides, ion channels, and cholesterol synthesis in the periaqueductal gray following binge-like alcohol drinking by adolescent alcohol-preferring (P) rats. *Alcohol. Clin. Exp. Res.* 40, 955–968. doi: 10.1111/acer.13056

McLaughlin, R. J., Hill, M. N., and Gorzalka, B. B. (2014). A critical role for prefrontocortical endocannabinoid signaling in the regulation of stress and emotional behavior. *Neurosci. Biobehav. Rev.* 42, 116–131. doi: 10.1016/j.neubiorev.2014.02.006

Miguel-Hidalgo, J. J., Overholser, J. C., Meltzer, H. Y., Stockmeier, C. A., and Rajkowska, G. (2006). Reduced glial and neuronal packing density in the orbitofrontal cortex in alcohol dependence and its relationship with suicide and duration of alcohol dependence. *Alcohol. Clin. Exp. Res.* 30, 1845–1855. doi: 10.1111/j.1530-0277.2006.00221.x

Montesinos, J., Pascual, M., Pla, A., Maldonado, C., Rodriguez-Arias, M., Minarro, J., et al. (2015). TLR4 elimination prevents synaptic and myelin alterations and long-term cognitive dysfunctions in adolescent mice with intermittent ethanol treatment. *Brain Behav. Immun.* 45, 233–244. doi: 10.1016/j.bbi.2014.11.015

Montesinos, J., Pascual, M., Rodriguez-Arias, M., Minarro, J., and Guerri, C. (2016). Involvement of TLR4 in the long-term epigenetic changes, rewarding and anxiety effects induced by intermittent ethanol treatment in adolescence. *Brain Behav. Immun.* 53, 159–171. doi: 10.1016/j.bbi.2015.12.006

Morena, M., Patel, S., Bains, J. S., and Hill, M. N. (2016). Neurobiological interactions between stress and the endocannabinoid system. *Neuropsychopharmacology* 41, 80–102. doi: 10.1038/npp.2015.166

Moy, S. S., Knapp, D. J., Criswell, H. E., and Breese, G. R. (1997). Flumazenil blockade of anxiety following ethanol withdrawal in rats. *Psychopharmacology (Berl).* 131, 354–360. doi: 10.1007/s002130050303

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365, 61–65. doi: 10.1038/365061a0

Myhrer, T. (1988). Exploratory behavior and reaction to novelty in rats with hippocampal perforant path systems disrupted. *Behav. Neurosci.* 102, 356–362. doi: 10.1037/0735-7044.102.3.356

Nagel, B. J., Schweinsburg, A. D., Phan, V., and Tapert, S. F. (2005). Reduced hippocampal volume among adolescents with alcohol use disorders without psychiatric comorbidity. *Psychiatry Res.* 139, 181–190. doi: 10.1016/j.pscychresns.2005.05.008

NIAAA (2004). *NIAAA Council Approves Definition of Binge Drinking*. Bethesda: National Institutes of Health NIAAA Newsletter.

Obernier, J. A., Bouldin, T. W., and Crews, F. T. (2002a). Binge ethanol exposure in adult rats causes necrotic cell death. *Alcohol. Clin. Exp. Res.* 26, 547–557. doi: 10.1111/j.1530-0277.2002.tb02573.x

Obernier, J. A., White, A. M., Swartzwelder, H. S., and Crews, F. T. (2002b). Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. *Pharmacol. Biochem. Behav.* 72, 521–532. doi: 10.1016/S0091-3057(02)00715-3

Oesterle, S., Hill, K. G., Hawkins, J. D., Guo, J., Catalano, R. F., and Abbott, R. D. (2004). Adolescent heavy episodic drinking trajectories and health in young adulthood. *J. Stud. Alcohol.* 65, 204–212. doi: 10.15288/jsa.2004.65.204

Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T., and Ueda, N. (2004). Molecular characterization of a phospholipase D generating anandamide and its congeners. *J. Biol. Chem.* 279, 5298–5305. doi: 10.1074/jbc.M306642200

Onaivi, E. S., Ishiguro, H., Gong, J. P., Patel, S., Meozzi, P. A., Myers, L., et al. (2008). Functional expression of brain neuronal CB2 cannabinoid receptors are involved in the effects of drugs of abuse and in depression. *Ann. N.Y Acad. Sci.* 1139, 434–449. doi: 10.1196/annals.1432.036

Orio, L., Edwards, S., George, O., Parsons, L. H., and Koob, G. F. (2009). A role for the endocannabinoid system in the increased motivation for cocaine in extended-access conditions. *J. Neurosci.* 29, 4846–4857. doi: 10.1523/JNEUROSCI.0563-09.2009

Pandey, S. C., Roy, A., and Zhang, H. (2003). The decreased phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein in the central amygdala acts as a molecular substrate for anxiety related to ethanol withdrawal in rats. *Alcohol. Clin. Exp. Res.* 27, 396–409. doi: 10.1097/01.ALC.0000056616.81971.49

Pascual, M., Balino, P., Alfonso-Loeches, S., Aragon, C. M., and Guerri, C. (2011). Impact of TLR4 on behavioral and cognitive dysfunctions associated with alcohol-induced neuroinflammatory damage. *Brain Behav Immun.* 25(Suppl. 1), S80–S91. doi: 10.1016/j.bbi.2011.02.012

Pascual, M., Balino, P., Aragon, C. M., and Guerri, C. (2015). Cytokines and chemokines as biomarkers of ethanol-induced neuroinflammation and anxiety-related behavior: role of TLR4 and TLR2. *Neuropharmacology* 89, 352–359. doi: 10.1016/j.neuropharm.2014.10.014

Pascual, M., Blanco, A. M., Cauli, O., Minarro, J., and Guerri, C. (2007). Intermittent ethanol exposure induces inflammatory brain damage and causes long-term behavioural alterations in adolescent rats. *Eur. J. Neurosci.* 25, 541–550. doi: 10.1111/j.1460-9568.2006.05298.x

Pascual, M., Pla, A., Minarro, J., and Guerri, C. (2014). Neuroimmune activation and myelin changes in adolescent rats exposed to high-dose alcohol and associated cognitive dysfunction: a review with reference to human adolescent drinking. *Alcohol. Alcohol.* 49, 187–192. doi: 10.1093/alcalc/agt164

Paxinos, G., and Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates*. New York, NY: Academic Press; Spiral Bound.

Pelição, R., Santos, M. C., Freitas-Lima, L. C., Meyrelles, S. S., Vasquez, E. C., Nakamura-Palacios, E. M., et al. (2016). URB597 inhibits oxidative stress induced by alcohol binging in the prefrontal cortex of adolescent rats. *Neurosci. Lett.* 624, 17–22. doi: 10.1016/j.neulet.2016.04.068

Pleil, K. E., Rinker, J. A., Lowery-Gionta, E. G., Mazzone, C. M., McCall, N. M., Kendra, A. M., et al. (2015). NPY signaling inhibits extended amygdala CRF neurons to suppress binge alcohol drinking. *Nat. Neurosci.* 18, 545–552. doi: 10.1038/nn.3972

Przybycien-Szymanska, M. M., Gillespie, R. A., and Pak, T. R. (2012). 17beta-Estradiol is required for the sexually dimorphic effects of repeated binge-pattern alcohol exposure on the HPA axis during adolescence. *PLoS ONE* 7:e32263. doi: 10.1371/journal.pone.0032263

Przybycien-Szymanska, M. M., Rao, Y. S., and Pak, T. R. (2010). Binge-pattern alcohol exposure during puberty induces sexually dimorphic changes in genes regulating the HPA axis. *Am. J. Physiol. Endocrinol. Metab.* 298, E320–E328. doi: 10.1152/ajpendo.00615.2009

Ransohoff, R. M., and Brown, M. A. (2012). Innate immunity in the central nervous system. *J. Clin. Invest.* 122, 1164–1171. doi: 10.1172/JCI58644

Rasmussen, D. D., Mitton, D. R., Green, J., and Puchalski, S. (2001). Chronic daily ethanol and withdrawal: 2. Behavioral changes during prolonged abstinence. *Alcohol. Clin. Exp. Res.* 25, 999–1005. doi: 10.1111/j.1530-0277.2001.tb02308.x

Read, J. P., Beattie, M., Chamberlain, R., and Merrill, J. E. (2008). Beyond the "Binge" threshold: heavy drinking patterns and their association with alcohol involvement indices in college students. *Addict. Behav.* 33, 225–234. doi: 10.1016/j.addbeh.2007.09.001

Rubino, T., Realini, N., Castiglioni, C., Guidali, C., Vigano, D., Marras, E., et al. (2008). Role in anxiety behavior of the endocannabinoid system in the prefrontal cortex. *Cereb. Cortex* 18, 1292–1301. doi: 10.1093/cercor/bhm161

Rubio, M., Mchugh, D., Fernandez-Ruiz, J., Bradshaw, H., and Walker, J. M. (2007). Short-term exposure to alcohol in rats affects brain levels of anandamide, other N-acylethanolamines and 2-arachidonoyl-glycerol. *Neurosci. Lett.* 421, 270–274. doi: 10.1016/j.neulet.2007.05.052

Schmidt, K., Krishnan, B., Xia, Y., Sun, A., Orozco-Cabal, L., Pollandt, S., et al. (2011). Cocaine withdrawal reduces group I mGluR-mediated long-term potentiation via decreased GABAergic transmission in the amygdala. *Eur. J. Neurosci.* 34, 177–189. doi: 10.1111/j.1460-9568.2011.07769.x

Scuderi, C., Valenza, M., Stecca, C., Esposito, G., Carratu, M. R., and Steardo, L. (2012). Palmitoylethanolamide exerts neuroprotective effects in mixed neuroglial cultures and organotypic hippocampal slices via peroxisome proliferator-activated receptor-alpha. *J. Neuroinflammation* 9:49. doi: 10.1186/1742-2094-9-49

Serrano, A., and Parsons, L. H. (2011). Endocannabinoid influence in drug reinforcement, dependence and addiction-related behaviors. *Pharmacol. Ther.* 132, 215–241. doi: 10.1016/j.pharmthera.2011.06.005

Serrano, A., Rivera, P., Pavon, F. J., Decara, J., Suárez, J., Rodriguez de Fonseca, F., et al. (2012). Differential effects of single versus repeated alcohol withdrawal on the expression of endocannabinoid system-related genes in the rat amygdala. *Alcohol Clin. Exp. Res.* 36, 984–894. doi: 10.1111/j.1530-0277.2011.01686.x

Skelly, M. J., Chappell, A. E., Carter, E., and Weiner, J. L. (2015). Adolescent social isolation increases anxiety-like behavior and ethanol intake and impairs fear extinction in adulthood: possible role of disrupted noradrenergic signaling. *Neuropharmacology* 97, 149–159. doi: 10.1016/j.neuropharm.2015.05.025

Spanagel, R. (2000). Recent animal models of alcoholism. *Alcohol Res. Health* 24, 124–131.

Stahel, P. F., Smith, W. R., Bruchis, J., and Rabb, C. H. (2008). Peroxisome proliferator-activated receptors: "key" regulators of neuroinflammation after traumatic brain injury. *PPAR Res.* 2008:538141. doi: 10.1155/2008/538141

Tajuddin, N., Moon, K. H., Marshall, S. A., Nixon, K., Neafsey, E. J., Kim, H. Y., et al. (2014). Neuroinflammation and neurodegeneration in adult rat brain from binge ethanol exposure: abrogation by docosahexaenoic acid. *PLoS ONE* 9:e101223. doi: 10.1371/journal.pone.0101223

Udomuksorn, W., Mukem, S., Kumarnsit, E., Vongvatcharanon, S., and Vongvatcharanon, U. (2011). Effects of alcohol administration during adulthood on parvalbumin and glial fibrillary acidic protein immunoreactivity in the rat cerebral cortex. *Acta Histochem.* 113, 283–289. doi: 10.1016/j.acthis.2009.11.001

Vetreno, R. P., and Crews, F. T. (2012). Adolescent binge drinking increases expression of the danger signal receptor agonist HMGB1 and Toll-like receptors in the adult prefrontal cortex. *Neuroscience* 226, 475–488. doi: 10.1016/j.neuroscience.2012.08.046

Vinod, K. Y., Yalamanchili, R., Xie, S., Cooper, T. B., and Hungund, B. L. (2006). Effect of chronic ethanol exposure and its withdrawal on the endocannabinoid system. *Neurochem. Int.* 49, 619–625. doi: 10.1016/j.neuint.2006.05.002

Vogel, C., and Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat. Rev. Genet.* 13, 227–232. doi: 10.1038/nrg3185

Wilson, J., Watson, W. P., and Little, H. J. (1998). CCK(B) antagonists protect against anxiety-related behaviour produced by ethanol withdrawal, measured using the elevated plus maze. *Psychopharmacology* 137, 120–131. doi: 10.1007/s002130050601

Zeigler, D. W., Wang, C. C., Yoast, R. A., Dickinson, B. D., Mccaffree, M. A., Robinowitz, C. B., et al. (2005). The neurocognitive effects of alcohol on adolescents and college students. *Prev. Med.* 40, 23–32. doi: 10.1016/j.ypmed.2004.04.044

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: 28 November 2017
doi: 10.3389/fnbeh.2017.00233

Long-Term Effects of Intermittent Adolescent Alcohol Exposure in Male and Female Rats

Eva M. Marco[1], Sara Peñasco[1,2], María-Donina Hernández[1,2], Anabel Gil[3], Erika Borcel[1], Marta Moya[1,2], Elena Giné[4], José Antonio López-Moreno[5], Consuelo Guerri[3], Meritxell López-Gallardo[2], Fernando Rodríguez de Fonseca[6]*

[1]Departamento de Fisiología (Fisiología Animal II), Facultad de Ciencias Biológicas, Universidad Complutense, Madrid, Spain, [2]Departamento de Fisiología Humana, Facultad de Medicina, Universidad Complutense, Madrid, Spain, [3]Centro de Investigación Príncipe Felipe, Valencia, Spain, [4]Departamento de Biología Celular, Facultad de Medicina, Universidad Complutense, Madrid, Spain, [5]Departamento de Psicobiología, Facultad de Psicología, Universidad Complutense, Madrid, Spain, [6]Unidad Gestión Clínica de Salud Mental, Instituto de Investigación Biomédica de Málaga (IBIMA), Hospital Regional Universitario de Málaga-Universidad de Málaga, Málaga, Spain

Alcohol is a serious public health concern that has a differential impact on individuals depending upon age and sex. Patterns of alcohol consumption have recently changed: heavy episodic drinking—known as binge-drinking—has become most popular among the youth. Herein, we aimed to investigate the consequences of intermittent adolescent alcohol consumption in male and female animals. Thus, *Wistar* rats were given free access to ethanol (20% in drinking water) or tap water for 2-h sessions during 3 days, and for an additional 4-h session on the 4th day; every week during adolescence, from postnatal day (pnd) 28–52. During this period, animals consumed a moderate amount of alcohol despite blood ethanol concentration (BEC) did not achieve binge-drinking levels. No withdrawal signs were observed: no changes were observed regarding anxiety-like responses in the elevated plus-maze or plasma corticosterone levels (pnd 53–54). In the novel object recognition (NOR) test (pnd 63), a significant deficit in recognition memory was observed in both male and female rats. Western Blot analyses resulted in an increase in the expression of synaptophysin in the frontal cortex (FC) of male and female animals, together with a decrease in the expression of the CB2R in the same brain region. In addition, adolescent alcohol induced, exclusively among females, a decrease in several markers of dopaminergic and serotonergic neurotransmission, in which epigenetic mechanisms, i.e., histone acetylation, might be involved. Taken together, further research is still needed to specifically correlate sex-specific brain and behavioral consequences of adolescent alcohol exposure.

Keywords: alcohol, adolescence, drinking-in-the-dark, sex differences, cognitive function, neural plasticity, hippocampal formation, frontal cortex

OPEN ACCESS

Edited by:
Mary M. Torregrossa, University of Pittsburgh, United States

Reviewed by:
Olga Valverde, Pompeu Fabra University, Spain
Walter Adriani, Istituto Superiore di Sanità, Italy

****Correspondence:***
Eva M. Marco
emmarco@bio.ucm.es

Received: *11 July 2017*
Accepted: *10 November 2017*
Published: *28 November 2017*

Citation:
Marco EM, Peñasco S, Hernández M-D, Gil A, Borcel E, Moya M, Giné E, López-Moreno JA, Guerri C, López-Gallardo M and Rodríguez de Fonseca F (2017) Long-Term Effects of Intermittent Adolescent Alcohol Exposure in Male and Female Rats. Front. Behav. Neurosci. 11:233. doi: 10.3389/fnbeh.2017.00233

INTRODUCTION

Alcohol is a serious public health concern and age and sex have been reported as main factors affecting alcohol consumption and alcohol-related harm (WHO, 2014). In particular, the use of alcohol frequently initiates during adolescence; and the adolescent brain is still undergoing maturation and reorganization programs, and responds to alcohol differently

than adults. Drinking rates are often reported to be higher at adolescence as compared to adulthood (consult reviews from: Crews et al., 2016; Spear, 2016b), and alcohol use at adolescence may also increase the risk for mental illness and substance abuse disorders in adulthood (Crews et al., 2016). It is worth analyzing sex differences since an increased vulnerability of females to alcohol-related harm has been reported (Wilsnack et al., 2013).

Notably, in the last decades, alcohol consumption patterns have substantially changed. Nowadays, heavy episodic drinking—also known as binge-drinking—has become popular among the young population (WHO, 2014). Consequently, new preclinical approaches have emerged to more closely mimic the currently adopted pattern of adolescent alcohol consumption. Among those, drinking in the dark (DID), has emerged as a valuable tool in both mice (Crabbe et al., 2011) and rats (Holgate et al., 2017). DID was selected because this approach considers: (1) ethanol self-administration, closer to voluntary alcohol intake in humans; (2) intermittent access to ethanol, providing a cycle of consumption-withdrawal that has been previously related to escalating patterns of consumption; and (3) DID has been adapted to the adolescent period (see Carnicella et al., 2014; Crews et al., 2016; Spear, 2016a for review). Despite not devoid of limitations, DID has arisen as the most suitable schedule in rodents to investigate the consequences of adolescent alcohol administration.

Therefore, in the present study we have investigated the consequences of adolescent alcohol exposure, by using the DID method, in which animals are given access to ethanol (or tap water) for 2-h sessions during 3 days, and for an additional 4-h session on the 4th day.

Alcohol withdrawal signs include heightened anxiety together with a dysregulation of the hypothalamic-pituitary-adrenal axis activity and the release of glucocorticoids (Rasmussen et al., 2000, 2001; Mons and Beracochea, 2016; Somkuwar et al., 2017). Thus, short after alcohol cessation, anxiety-like responses were evaluated in the elevated plus maze (EPM) and plasma corticosterone levels measured. Adolescent alcohol has extensively been related with impairments in cognitive function later in life (Guerri and Pascual, 2010; Alfonso-Loeches and Guerri, 2011) as measured in several paradigms including the novel object recognition (NOR) test (Sanchez-Marin et al., 2017). Therefore, we evaluated animals' recognition memory in the NOR and focused on two brain regions largely involved in recognition memory, frontal cortex (FC) and the hippocampal formation (HF) (Squire et al., 2007; Morici et al., 2015).

Although the underlying mechanisms of adolescent alcohol exposure are not completely understood, several molecular targets have been identified. In the first place, FC and HF have been frequently described as particularly vulnerable to alcohol effect (Alfonso-Loeches and Guerri, 2011; Ozsoy et al., 2013; Oliveira et al., 2015; Staples and Mandyam, 2016). In addition, changes in glial cells (Evrard et al., 2006; Kane et al., 2014; Oliveira et al., 2015) and activation of the immune response (Montesinos et al., 2016) have been reported after adolescent alcohol exposure. Alterations in the neurogenesis and/or several players of brain plasticity have also been described (Briones and Woods, 2013), as well as modifications in several neurotransmitter systems such as the serotonergic and dopaminergic systems (Crews et al., 2016). The endocannabioid system has also been given a role in the consequences of adolescent alcohol (Sanchez-Marin et al., 2017). Therefore, we scanned for several neurobiological markers related to astrogliosis (anti-glial fibrillary acidic protein, GFAP), neural plasticity (brain derived neurotrophic factor, BDNF; and pre-synaptic proteins such as synaptophysin, SYN and SNAP25), and neurotransmitter signaling systems such as dopaminergic, serotonergic, cannabinoid and other neurotransmitter systems in these two brain areas.

Epigenetic modifications occur during the developing brain and are associated with plasticity and behavior (Fagiolini et al., 2009; Roth, 2013). Indeed, epigenetic changes have been reported to underlie some effects of alcohol on brain and behavior (see Ponomarev, 2013 for review) and our previous results have demonstrated an association between behavioral changes and changes in histone acetylation (H3 and H4), in preFC of adolescent mice with binge ethanol treatment in adolescence (Pascual et al., 2012; Montesinos et al., 2016). Therefore, in the present study, acetylation changes of H3 and H4 were also investigated.

MATERIALS AND METHODS

Animals

The animals employed were the offspring of adult *Wistar* rats purchased from Harlan Laboratories® (Milan, Italy). Following 15 days of habituation animals were mated (one male with two females) for ten consecutive days, then, pregnant females were isolated and daily observed for delivery control. At birth (postnatal day, pnd 0), litters were culled and sex balanced—no cross-fostering allowed—up to eight pups per dam (4 males and 4 females); then, litters were left undisturbed until weaning (pnd 22) when rats were housed in pairs of siblings of the same sex.

Animals were housed in plastic cages (50 × 25 × 17.5 cm) at the animal facilities in the Faculty of Biological Sciences at the Complutense University of Madrid (EX08-UCS). Animals were maintained at constant conditions (temperature, 21 ± 1°C and humidity, 60 ± 10%), under a 12 h light-dark inversed cycle (light son at 20.00). Food (2018 Global Diet; Harlan Laboratories®) and water were provided *ad libitum* except during exposure to alcohol.

This study was carried out in accordance with European Directive 2010/63/EU and in compliance with the Spanish Royal Decree 53/2013 on the protection of animals used for research and other scientific purposes. The protocol was approved by the "*Comité de Experimentación Animal (CEA)*" of the *Universidad Complutense de Madrid* (Madrid, Spain).

Alcohol Exposure during Adolescence

As shown in **Figure 1**, animals were exposed to alcohol for the whole adolescence period, from pnd 28 to pnd 52 (Spear, 2000). A modified drinking in the dark administration (DID) protocol based on Crabbe et al. (2011) was employed; each week, animals

were exposed for 2 h to a single bottle of an ethanol solution (20%, v/v) for three consecutive days, and for a 4 h session on the 4th day, for the following 3 days animals had no access to alcohol. The ethanol solution was prepared from ethanol 96° (Alcoholes Aroca S.L., Madrid, Spain) in tap water. For the drinking sessions animals were moved to similar plastic cages, singly housed and placed in an adjacent room. Control animals were submitted to the same manipulation although in their cages the single bottle contained tap water. Water and ethanol solutions were daily replenished.

Alcohol (or water) consumption was daily calculated by weighting bottles before and after exposure to the drinking. Body weight and food intake in the home-cage (data not shown) were also controlled throughout the administration protocol. Additional bottles with the ethanol solution and tap water were included to control for spillage and evaporation during the test sessions.

Experimental Design

We have employed a "within-litter design", in which all the experimental groups are represented within the same litter (Festing, 2006). A total of 12 litters were submitted to the present protocol; and within each litter, the two animals housed together were assigned to the same drug condition (control *vs*. alcohol). In the present study we will present data from the 12 litters regarding alcohol intake; four litters remained intact and were devoted to a different study. The other eight litters were used for the behavioral analysis, blood samples were collected, and at sacrifice, half of the brains were collected and employed in this study for western blot analysis, while the remaining animals were dedicated to another study which results have been already published (Pavón et al., 2016). Therefore, these neurobiological data come from animals previously submitted to behavioral analysis.

Alcohol withdrawal signs were investigated short after alcohol cessation: plasma corticosterone levels were analyzed 24 h after the last alcohol exposure session (pnd 53), and anxiety-like responses were evaluated in the elevated plus-maze (EPM) 48 h after the last alcohol session (pnd 54). Cognitive function was evaluated in the long-term, and recognition memory was evaluated in the novel object recognition (NOR) test in young adult animals (pnd 63). Then (pnd 68), animals were sacrificed by rapid decapitation. Brains were rapidly extracted and dissected on ice. Frontal cortex (FC) and Hippocampal Formation (HF) were stored at −30°C until further analysis (see **Figure 1**).

Behavioral Assessment

Elevated Plus-Maze

The elevated plus-maze (EPM) is formed by two open arms (50 cm × 10 cm) and two equally sized enclosed arms with 40 cm high walls, arranged so that the arms of the same type are opposite one another. The junction of the four arms formed a central square area (10 cm × 10 cm). The apparatus was made of opaque black polyvinyl chloride (PVC) elevated to a height of 62 cm. On the test day, animals were placed in the central platform of the apparatus facing one of the enclosed arms, and they were allowed to freely explore the maze for 5 min under red light conditions. Whenever an animal entered an arm with all four limbs, it was considered a visit, and the frequency and duration of the visits to the open and closed arm were recorded. Some animals fell from the plus-maze and were excluded from the analysis. The percentage of open arm entries and the percentage of time spent in the open arms, considered the most relevant parameters related to anxiety, were calculated upon the total entries into any arm and upon the total time spent in both arms. Instead, the frequency of closed arm entries was considered as an index of general motor activity (Pellow et al., 1985). Between animals, the apparatus was carefully cleaned with water to remove possible odors.

Novel Object Recognition Test (NOR)

The NOR was performed in a square arena (60 cm × 60 cm × 45 cm) with matte-painted metallic walls and a plastic-covered wooden floor divided by white painted lines into 36 squares (10 cm × 10 cm). Animals were exposed to a 3 days habituation period to the arena, followed by the training and test session on the 4th day (pnd 63) as previously described (Ennaceur and Delacour, 1988; Mateos et al., 2011) with some minor modifications.

During the habituation period, animals were allowed to freely explore the arena, under dim light conditions, for 5 min. On the first day, the behavior of the animal in the arena was video recorded for subsequent behavioral evaluation. On the test day, *training session*, rats were first exposed to two identical objects (two plastic boxes) for them to explore the objects for at least 30 s or for a maximum period of 4 min. After a 4 h inter-trial interval, *test session*, rats were exposed to one of the previously encountered objects (familiar object, F1 or F2) and to a novel, unfamiliar object (metallic colored box, N) for 3 min. The objects were not bigger than twice the size of a rat and they were located in contiguous corners, at a distance of 10 cm from the walls. At the beginning of each session, the animals were placed in the center of the apparatus facing the wall opposite to the objects. For each animal, the position of the objects was not changed between the training and the test session. However, the objects' position was changed between animals in order to avoid spatial preference. The apparatus and the objects were carefully cleaned between tests on different animals with a 20% (v/v) ethanol solution. Both training and test sessions were video recorded (Sony DCR-DVD310E) and the animals' behavior was later evaluated by an experienced observer by means of event-recorder software (Observer®, Noldus, Netherlands). Exploration of an object was considered whenever animals pointed their nose toward an object at a distance ≤1 cm, while turning around, climbing and/or biting the objects was not considered as exploration. The time animals' spent exploring the objects during the two sessions was registered, and the discrimination index (DI) was calculated as the difference between the time spent exploring the novel object (N) and the familiar one (F1 or F2) in relation to the total time spent exploring the objects ((N − F)/(N + F)). Animals that explored for less than 20 s during the training session and those exclusively exploring only one of the

objects during the test session were excluded from the statistical analyses.

Blood Ethanol Concentration (BEC)

Blood samples were collected from the tail vein 90 min after the 4 h session of alcohol exposure on the first and last week of alcohol exposure, pnd 31 and 52, respectively (McClain et al., 2011). Blood samples were collected into capillary tubes that contained EDTA dipotassium salt (Microvette CB 300 K2E, Sarstedt, Germany); blood was then centrifuged at 1500 rpm for 15 min at 4°C, and the plasma was stored at −20°C. BEC was determined using the EnzyChrom ethanol assay kit following the protocol recommended by the manufacturer (Bioassay Systems, Hayward, CA, USA). All measurements were performed in duplicate.

Corticosterone Measurements

Blood samples were collected, between 10.00 and 13.00, into capillary tubes containing EDTA dipotassium salt (Microvette CB 300 K2E, Sarstedt, Germany); blood was then centrifuged at 1500 rpm for 15 min at 4°C, and the plasma was stored at −20°C. Corticosterone was measured using a solid phase ^{125}I radioimmunoassay (Immuchem^{TM} Corticosterone kit, MP Biomedicals, Orangeburg, NY, USA). The detection limit was 7.7 ng/ml and the intra-assay and inter-assay coefficients of variation were less than 10%. All samples were run in duplicate and plasma corticosterone levels were calculated from the standard curve.

Western Blot Analysis

Only one FC and HF from each animal was randomly assigned to evaluate protein expression levels of GFAP, CB1R and CB2R cannabinoid receptors and synaptic plasticity markers in the Faculty of Medicine of the Universidad Complutense de Madrid. Neurotransmitter and epigenetic markers were measured in the Research Center "Príncipe Felipe" in Valencia.

GFAP, Cannabinoid Receptors and Synaptic Plasticity Markers

The tissue samples were homogenized at a ratio of 1:3 (w/v) in ice-cold lysis-buffer (Hepes 10 mM pH = 7.5; EGTA 10 mM; EDTA 10 mM; NaCl 150 mM; CHAPs 2.5%) with protease inhibitors (Roche) and PMSF 0.1 M. After homogenization, samples were centrifuged at 14,000 rpm for 20 min at 4°C. Supernatants were transferred to a new tube and the protein concentration was estimated by Bradford protein assay (Bio-Rad, Hercules, CA, USA), measure by Multiskan FC (Thermo Fisher Technologies) and analyzed by software Skanlt (Multiskan FC, version 2.5).

In each assay the same amount of protein was loaded in all wells (20 or 30 μg) depending on the protein to be detected and resolved by using 7.5%–12% SDS-acrylamide gels. After electrophoresis proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences, UK) and transfer efficiency was determined by Ponceau red dyeing. Membranes were then blocked with Tris-buffered saline (TBS: NaCl; Tris, pH 7.5 1 M) containing 5% (w/v) non-fat dried milk (Sveltesse, Nestle, Spain) and 0.1% Tween-20; and incubated with the appropriate primary antibody. The antibodies employed included anti-glial fibrillary acidic protein (GFAP; BD Pharmingen, San Jose, CA, USA) was used at a concentration of 1:750, anti-cannabinoid receptor type 1 (CB1; Sigma-Aldrich, St. Louis, MO, USA) and 2 (CB2; Sigma-Aldrich, St. Louis, MO, USA) were used at a concentration of 1:500, anti-brain-derived neurotrophic factor (BDNF; Santa Cruz biotechnology, Dallas, TX, USA) and anti-synaptophysin (Sigma-Aldrich, St. Louis, MO, USA) were used at a concentration of 1:300, anti-Synaptosomal-associated protein 25 (SNAP25; AbD Serotec, Raleigh, NC, USA) was used at a concentration of 1:1000. Membranes were subsequently washed and incubated with the corresponding secondary antibody conjugated with peroxidase. Bound peroxidase activity was visualized by chemiluminescence and quantified by densitometry using ImageJ software 1.43× (NIH, New York, NY, USA). All blots were rehybridized with actin to normalize each sample for gel loading variability. All data are normalized to control values on each gel.

Neurotransmitter and Epigenetic Markers

The tissue samples were homogenized in 250 mg tissue/0.5 ml cold lysis buffer (1% NP-40, 20 mM, Tris–HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 g/ml aprotinin, 10 g/ml leupeptin, 10 mM DTT, 1 mM Na_3VO_4 and 1 mM PMSF). Brain homogenates were kept on ice for 30 min, centrifuged at maximum speed for 15 min, and the supernatant was collected to determine the proteins levels using the BCA Assay (Thermo Fisher Scientific Inc., Waltham, MA, USA). Lysates were separated by SDS-PAGE gels and transferred to PVDF membranes following standard techniques.

Membranes were blocked with 5% BSA in TBS containing 0.1% Tween-20 (TBS/T), and were then incubated overnight with the following primary antibodies: anti-SR2A (1:500; Santa Cruz Biotechnology), anti-DRD1 (1:2000; Santa Cruz Biotechnology), anti-DRD2 (1:1000; Santa Cruz Biotechnology), anti-pNMDAR2B (1:1000; Abcam plc.), anti-NMDAR2B (1:1000, Abcam), anti-EAAT1 (1:1000, Abcam), anti-Lys^9-acetyl-histone H3 (1:500; Cell Signaling Technology, Hertfordshire, UK), anti-Lys^5-acetyl-histone H4 (1:500; Cell Signaling Technology). After washing with TBS/T, blots were incubated with appropriate HRP-conjugated secondary antibody. Proteins were visualized either with alkaline phosphatase conjugate (Sigma-Aldrich) or an enhanced chemiluminescence system (ECL Plus; Thermo Fisher Scientific Inc.). To make the loading control some membranes were stripped 30 min at room temperature, washed and incubated with anti-GAPDH mAb (1:5000; Chemicon, Hampshire, UK) or anti-tubuline (1:1000, Sigma-Aldrich). The intensity of the bands was quantified with the Alpha-Ease FC program image analysis program (Alpha Innotech Corporation).

Statistical Analyses

In general, data were analyzed by using a two-way analysis of variance (ANOVA), considering sex (Male or Female) and adolescent intermittent exposure (Control, Co or Ethanol, EtOH)

as independent factors. Shapiro-Wilk and Levene tests were used to confirm normality and homocedasticity. Ethanol intake data were analyzed by a repeated measures one-way ANOVA, considering sex (male or female) as the independent factor. Additional one-way ANOVAs were employed when needed. *Post hoc* comparisons (Bonferroni or DMS) were performed in case of significant interaction between factors. *T* test comparisons were also employed in some cases. Significance level was set at $p < 0.05$. Statistical analyses were performed by the SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA).

RESULTS

Alcohol Intake during Adolescence

Alcohol intake values are shown in **Figure 2**. No sex differences in weekly alcohol consumption were found (**Figure 2A**). However, a trend for a sexual dimorphism arose by the 3rd and 4th week ($p = 0.063$; and $p = 0.057$, respectively). By the end of the alcohol exposure procedure female animals seem to consume higher amounts of alcohol than their sibling males. Actually, a significant effect of sex was observed on the last 4 h session, on pnd 52 ($F_{(1,46)} = 4.38$; $p = 0.042$; **Figure 2B**). By the end of the alcohol administration protocol female rats seemed to consume higher amounts of alcohol than male animals; despite this profile was the opposite at the beginning of the administration protocol: males drinking more alcohol than females during the first week. This inversion in the pattern of alcohol consumption is in agreement with previous literature showing that at adolescence, males consume more ethanol than females, whereas adult females generally exhibit higher ethanol intake than their male counterparts (Cailhol and Mormède, 2001; Vetter-O'Hagen et al., 2009).

Data from weekly alcohol (and water) consumption during the drinking sessions were also analyzed. Both male and female animals preferred water to alcohol during the exposure protocol (Amount of fluid intake (mL/kg) during the 2 h sessions in males: water: 44.88 ± 4.21 mL/kg, and alcohol: 30.43 ± 3.44 mL/kg; and in females: water: 51.72 ± 5.03 mL/kg and alcohol: 31.35 ± 3.10 mL/kg).

Ethanol intake data could also be influenced by the fact that food was not available during the DID session (2–4 h); thus, animals could be prompted to drink because of the caloric power of ethanol. However, if food was available drinking could have been enhanced through eating. Future experiments should take all these factors into account to better understand animal models of human alcohol consumption.

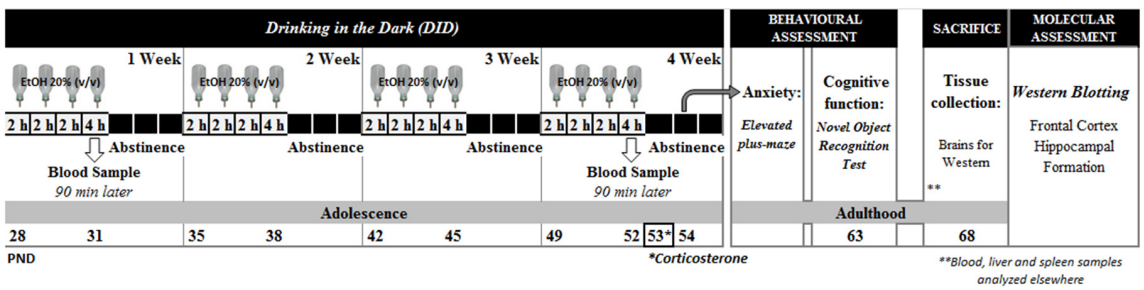


FIGURE 1 | Experimental design.

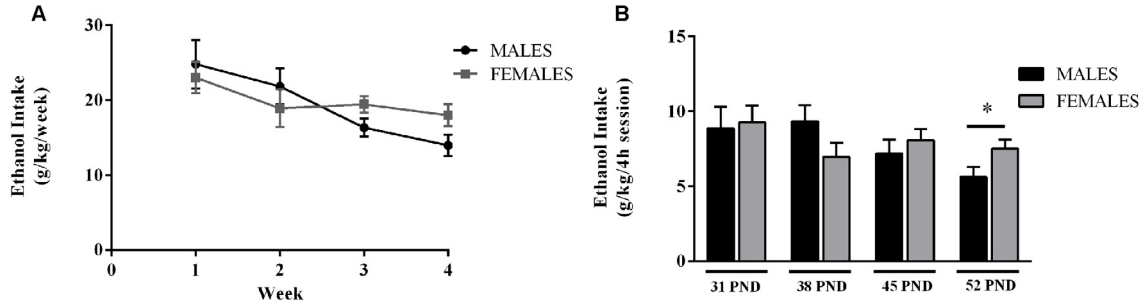


FIGURE 2 | Ethanol consumption during adolescence. Data are presented as mean ± SEM. **(A)** Weekly ethanol intake (g/kg/week). **(B)** Average amount of ethanol consumed during the last 4 h session, on the 4th day of each week (g/kg/4 h session). *Wistar* rats were given access to ethanol (20% in drinking water) or tap water for 2-h sessions during 3 days, and for an additional 4-h session on the 4th day, every week during adolescence, from postnatal days (pnd) 28–52. $n = 24$ animals per experimental group, coming from 12 litters. $p < 0.05$; *effect of sex.

Blood Ethanol Concentration (BEC)

After the last 4 h alcohol exposure session (pnd 52) BEC did not differ between male and female animals, values were 24.66 ± 1.80 mg/dL in male rats and 22.77 ± 2.01 mg/dL in female rats.

Evaluation of Alcohol Withdrawal Signs following Alcohol Cessation

On pnd 53, 24 h after last alcohol exposure, no effects of adolescent alcohol were observed on plasma corticosterone levels ($F_{(1,24)}$ = 1.02; n.s.), but a significant effect of sex was revealed ($F_{(1,24)}$ = 26.65; $p < 0.001$). As expected, females showed higher plasma corticosterone levels than males (Control: males 295.42 ± 52.74 vs. females 473.66 ± 71.46; Alcohol: males 190.31 ± 8.61 vs. females 521.31 ± 77.98). Similarly, on the EPM, no changes in anxiety-like responses were observed due to the adolescent alcohol exposure (**Table 1**).

Cognitive Function

As young adults, animals were evaluated for their recognition memory in the NOR test. During the training phase, no differences in total exploration times were observed (data not shown). During the test phase, the two-way ANOVA revealed a significant main effect of the alcohol condition on the DI ($F_{(1,51)}$ = 6.20; $p < 0.05$). The animals exposed to the alcohol DID protocol during the adolescent period exhibited diminished discrimination values, thus indicating an impaired ability to recognize the novel object. No effects of sex was found ($F_{(1,51)}$ = 0.43; n.s.), nor a significant interaction between factors ($F_{(1,51)}$ = 0.01; n.s.). The total time animals devoted to the exploration of both objects did not differ between groups (**Figure 3**).

GFAP Expression Levels

In the FC, a significant interaction between sex and alcohol exposure was found ($F_{(1,20)}$ = 6.59; $p < 0.05$); a basal sexual dimorphism was revealed with females showing higher levels of GFAP than males, and alcohol seemed to exclusively affect female animals decreasing its expression levels (**Figure 4A**). In the HF a significant interaction between factors was also reported ($F_{(1,20)}$ = 20.97; $p < 0.005$). In this occasion, females showed lower GFAP levels than males, and alcohol induced an opposite effect on male and female animals: GFAP expression levels were increased among alcohol-exposed males while diminished among females (**Figure 4B**).

CB1R and CB2R Expression

In the FC no significant effects on CB1R expression were found (**Figure 5A**). However, there was a significant effect of alcohol adolescent exposure on CB2R expression ($F_{(1,20)}$ = 5.46; $p < 0.05$), with CB2R levels diminished in the alcohol

TABLE 1 | Elevated plus maze.

	MALES		FEMALES	
	Control	Ethanol	Control	Ethanol
Open arm entries (%)	31.27 ± 5.77	22.63 ± 5.69	27.64 ± 4.92	29.99 ± 6.55
Time in open arms (%)	31.63 ± 7.29	21.76 ± 6.19	27.83 ± 5.51	31.80 ± 7.74
Closed arm entries (nr.)	7.86 ± 0.77	10.09 ± 0.72	8.46 ± 0.69	8.83 ± 0.75

Wistar rats were given access to ethanol (20% in drinking water) or tap water for 2-h sessions during 3 days, and for an additional 4-h session on the 4th day, every week during adolescence, from pnd 28–52. After (pnd 54), animals were submitted to the elevated plus maze, 5 min session. Data are presented as mean ± SEM. n = 13–15 animals per experimental group, coming from eight litters.

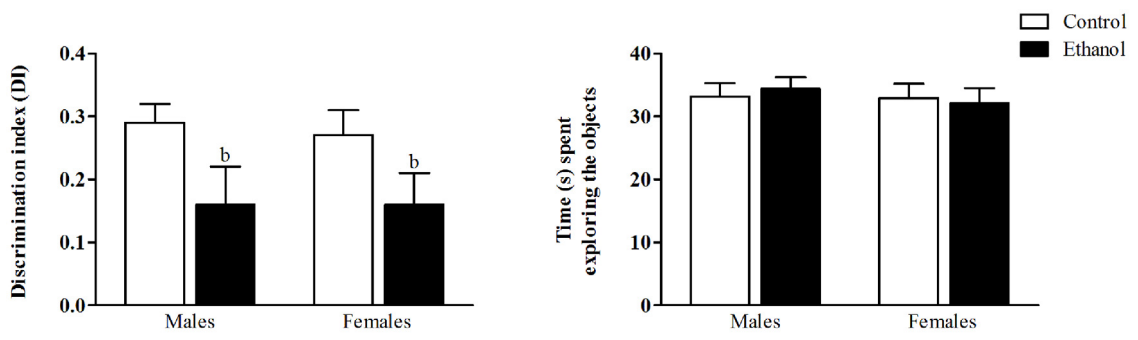


FIGURE 3 | Novel Object Recognition test (NOR). *Wistar* rats were given access to ethanol (20% in drinking water) or tap water for 2-h sessions during 3 days, and for an additional 4-h session on the 4th day, every week during adolescence, from pnd 28–52. At adulthood (pnd 63), animals were submitted to the NOR test. The discrimination index (DI) was calculated as the difference between the time spent exploring the novel object (N) and the familiar one (F1 or F2) in relation to the total time spent exploring the objects ((N − F)/(N + F)). Data are presented as mean ± SEM. Analysis of variance (ANOVA), $^{b}p < 0.05$, general effect of treatment. *n* = 13–15 animals per experimental group, coming from eight litters.

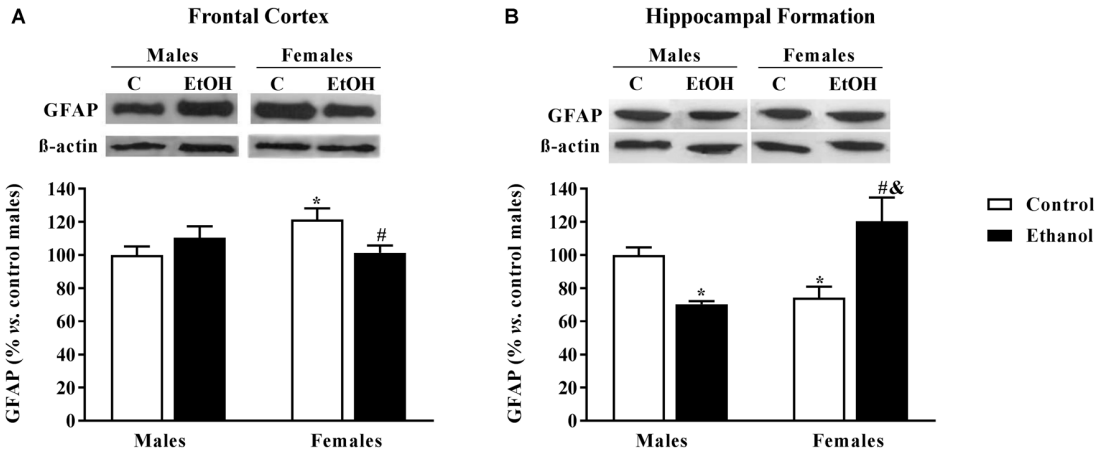


FIGURE 4 | Glial Fibrillary Acid Protein (*GFAP*) expression, as an astrocyte marker. Protein expression levels within the **(A)** frontal cortex (FC) and **(B)** Hippocampal formation (HF) of adult male and female rats that were exposed to a ethanol (20% in drinking water) or tap water for 2-h sessions during 3 days and for an additional 4-h session on the 4th day, every week during adolescence, from pnd 28–52. Histograms (mean ± SEM) represent the protein levels expressed as values of optical density calculated as changes from the control male group (%); representative western blotting bands are presented above each histogram. ANOVA, $^{*}p < 0.05$ vs. control male; $^{\#}p < 0.05$ vs. control female; $^{\&}p < 0.05$ vs. alcohol male. $n = 6$ per experimental group, coming from four litters.

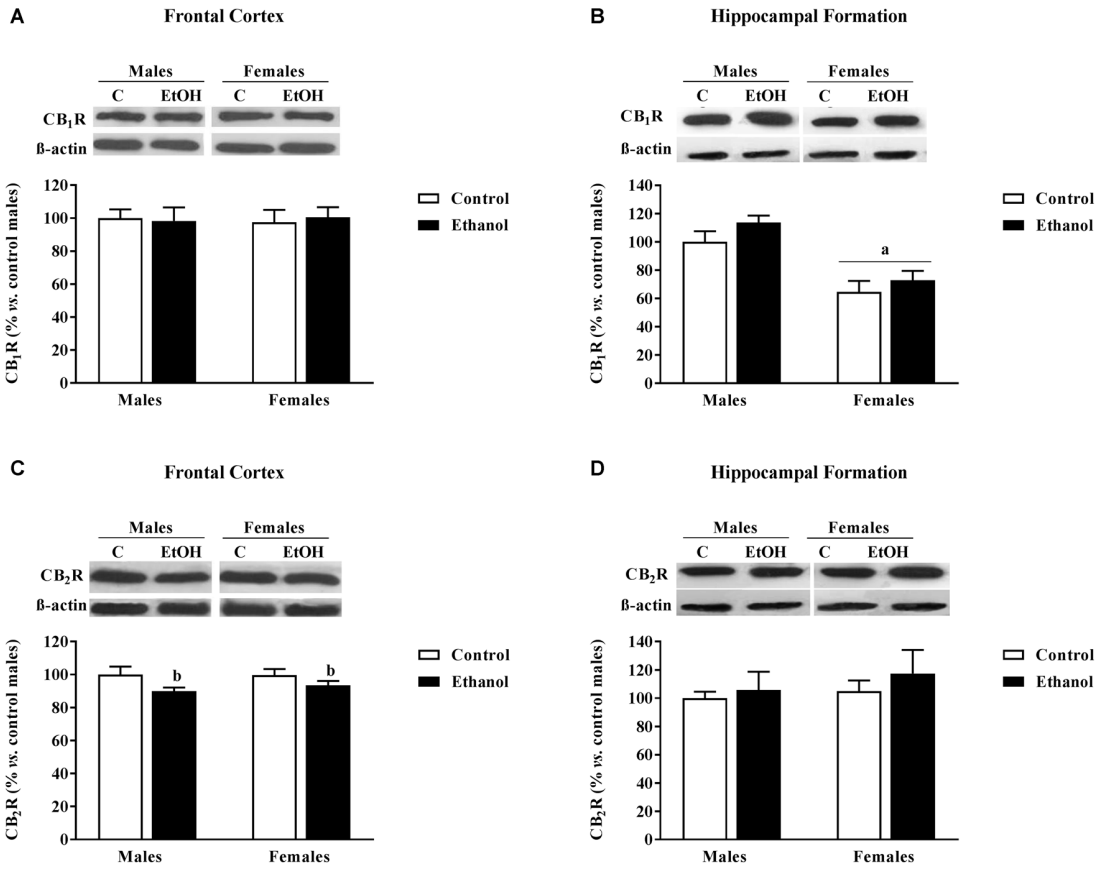


FIGURE 5 | Cannabinoid Receptors (CB1R and CB2R) expression. Protein expression levels within the **(A,C)** FC and **(B,D)** HF of adult male and female rats that were exposed to a ethanol (20% in drinking water) or tap water for 2-h sessions during 3 days, and for an additional 4-h session on the 4th day, every week during adolescence, from pnd 28–52. Histograms (mean ± SEM) represent the protein levels expressed as values of optical density calculated as changes from the control male group (%); representative western blotting bands are presented above each histogram. ANOVA, $^{a}p < 0.05$ general effect of sex; $^{b}p < 0.05$ general effect of treatment. $n = 6$ per experimental group, coming from four litters.

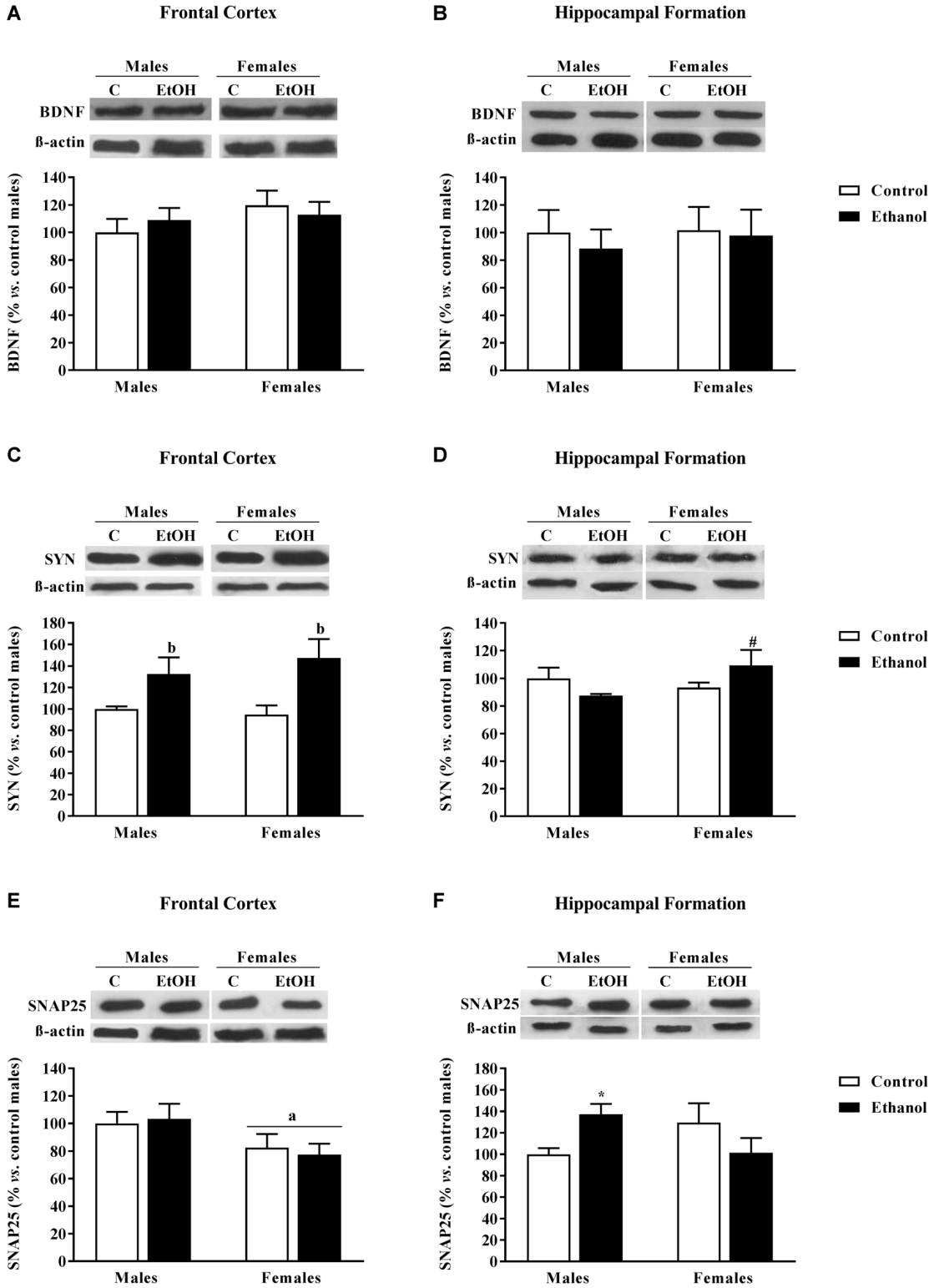


FIGURE 6 | Expression of some markers of synaptic plasticity. Protein expression levels of **(A,B)** brain derived neurotrophic factor (BDNF); **(C,D)** synaptophysin (SYN); and **(E,F)** SNAP25 within the frontal cortex (left panels, **A,C,E**) and the Hippocampal Formation (right panels, **B,D,F**) of adult male and female rats that were exposed to ethanol (20% in drinking water) or tap water for 2-h sessions during 3 days, and for an additional 4-h session on the 4th day, every week during adolescence, from postnatal days (pnd) 28–52. Histograms (mean ± SEM) represent the protein levels expressed as values of optical density calculated as changes from the control male group (%). ANOVA, $^{a}p < 0.05$ general effect of sex; $^{b}p < 0.05$ general effect of treatment. $^{*}p < 0.05$ vs. control male; $^{\#}p < 0.05$ vs. control female. $n = 6$ per experimental group, coming from four litters.

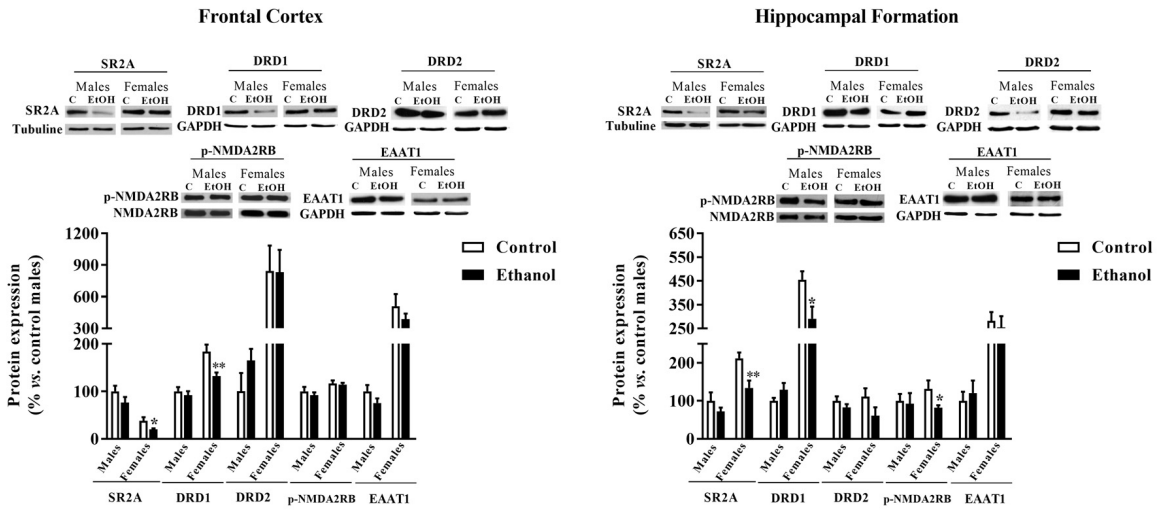


FIGURE 7 | Expression of some markers of serotoninergic, dopaminergic and glutamatergic neurotransmission. Protein expression levels of serotoninergic receptors (5HT2A y 5HT2B), dopaminergic receptors (D1R and D2R), pNMDA2RB and EAAT1 in the FC (left panels) and the HF (right panels) of adult male and female rats that were exposed to a ethanol (20% in drinking water) or tap water for 2-h sessions during 3 days, and for an additional 4-h session on the 4th day, every week during adolescence, from pnd 28–52. Histograms (mean ± SEM) represent the protein levels expressed as values of optical density calculated as changes from the control male group (%). *t*-Student, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the corresponding control group. n = 5–8 per experimental group, coming from four litters.

exposed groups compared to their corresponding counterparts (**Figure 5C**).

In the HF, a significant main effect of sex was found on CB1R expression ($F_{(1,20)} = 31.47$; $p < 0.005$) with females showing lower expression levels than males (**Figure 5B**). No significant effects were observed on CB2R expression (**Figure 5D**).

Synaptic Plasticity Markers

No changes in BDNF expression levels were detected in the FC (**Figure 6A**) or in the HF (**Figure 6B**).

Synaptophysin (SYN) expression levels were affected by adolescent alcohol exposure. In the FC, the ANOVA rendered a significant effect of alcohol exposure ($F_{(1,20)} = 11.80$; $p < 0.005$); adolescent alcohol increased SYN levels in both male and female animals (**Figure 6C**). In the HF, a significant interaction between factors was observed ($F_{(1,20)} = 4.08$; $p < 0.06$), only among alcohol-exposed females were SYN levels augmented (**Figure 6D**).

Regarding SNAP25, in the FC, a significant effect of sex was found ($F_{(1,20)} = 5.81$; $p < 0.05$); females exhibited lower SNAP25 levels than male animals (**Figure 6E**). In the HF a significant interaction between sex and alcohol exposure was found ($F_{(1,20)} = 5.87$; $p < 0.05$), in this case, alcohol exposed males demonstrated an increase in SNAP25 expression levels (**Figure 6F**).

Neurotransmitter Markers

In the FC (**Figure 7**), significant decreases in both the serotonergic SR2A and the dopaminergic DRD1 expression were observed among female animals ($p = 0.040$ and $p = 0.009$, respectively) as a consequence of adolescence alcohol exposure.

A similar effect of adolescent alcohol administration was observed within the HF (**Figure 7**). A significant decreased in the expression of SR2A ($p = 0.007$) and DRD1 ($p = 0.028$) was observed. In the HF a significant difference between adolescent alcohol exposed females was also reported for the p-NMDAR2B expression compared to control females ($p = 0.036$).

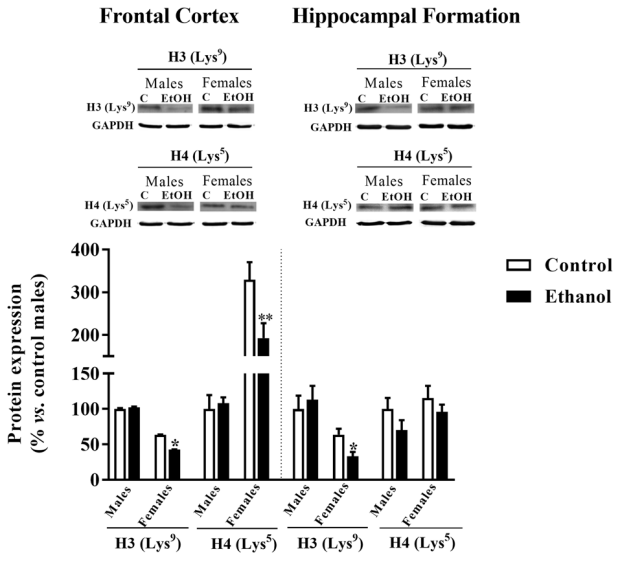


FIGURE 8 | Epigenetics markers. Protein expression levels of H3 (Lys^{9}) and H4 (Lys^{5}) in the FC (left panels) and the HF (right panels) of adult male and female rats that were exposed to a ethanol (20% in drinking water) or tap water for 2-h sessions during 3 days, and for an additional 4-h session on the 4th day, every week during adolescence, from pnd 28–52. Histograms (mean ± SEM) represent the protein levels expressed as values of optical density calculated as changes from the control male group (%). *t* Student, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the corresponding control group. n = 5–8 per experimental group, coming from four litters.

Epigenetic Markers

A reduction in H3 (Lys^9) was observed between alcohol-exposed females and their corresponding control group within the FC ($p = 0.0004$) and the HF ($p = 0.031$). Similarly, a decrease in H4 (Lys^5) expression was only observed due to adolescent alcohol exposure among females in the FC ($p = 0.021$) but not in the HF (**Figure 8**).

DISCUSSION

In the present study, by using the DID schedule, we aimed to mimic the most common pattern of alcohol consumption at adolescence, the so-called "binge drinking". Alcohol consumption data are highly variable as previously demonstrated in a similar paradigm (Momeni and Roman, 2014). We achieved alcohol consumption data similar to those presented in previous studies using a similar DID schedule in adult *Wistar* rats: consumption data of about 3.6 g/kg of ethanol for an access time of 2 h has been previously reported (George et al., 2012), although Holgate et al. (2017) reported a lower mean value of 1.83 g/kg/4 h session and 8.94 g/kg/week. Other studies in which access to alcohol was allowed for 24 h reported alcohol consumption of around 5.2 g/kg ethanol (Cippitelli et al., 2012) or 5.8 g/kg (Simms et al., 2008), data that can also be taken into account since previous data have suggested that "*rats intermittently exposed to the 20% alcohol solution voluntarily drank in bouts of sufficient size and frequency particularly during the first hour following the onset of the exposure*" (Cippitelli et al., 2012). Based upon BEC levels, our animals did not achieve the criterion (BECs at or above 80 mg/dL). Recently, Holgate et al. (2017) have proposed alternative binge criteria more specific for rodents; they consider that, if one standard drink contains 14 g of pure alcohol and the average body weight of an American adult is 80.7 kg, then, an average adult male rat would need to consume around 0.87 g/kg of ethanol in 2 h to meet the criteria of binge drinking. Taking this new proposal into consideration, our rats did consume alcohol at a binge-drinking rate, although BEC could not give support to this assumption. Controversial data may result from the higher metabolic rate and smaller body size of rats (compared to humans) would make it difficult for the rats to consume enough ethanol to reach the BEC of 80 mg/dL defined for humans (Holgate et al., 2017). Moreover, it has been reported that *Wistar* rats need to consume higher amounts of ethanol than other strains (e.g., Long–Evans rats) to reach the same BECs (Simms et al., 2008; Carnicella et al., 2014). In addition, BEC were evaluated 90 min after last alcohol session based upon literature (McClain et al., 2011), although this point may not reflect the maximal peak of alcohol absorption in this alcohol drinking paradigm; other studies have rather suggested a 30 min time to be more appropriate for blood sampling (Cippitelli et al., 2012). Moreover, each animal may have followed a specific pattern of alcohol consumption during the 4 h session, thus increasing the variability of BEC data. A more exhaustive analysis of alcohol consumption during the last alcohol session might be needed to be able to characterize the time course analysis of BEC in male and female adolescent animals (possibly different to that of adults), among which, pharmacodynamic and metabolic differences might be present.

No signs of alcohol withdrawal were observed in our adolescent animals following intermittent access to alcohol. In line with our findings, a previous study reported no signs of elevated anxiety at adolescence short after a single large dose of ethanol, while an acute withdrawal reaction was observed among adults (Doremus et al., 2003). Indeed, adolescent rats have been described as less vulnerable than adults to some acute effects of alcohol such as sedation, motor alteration and acute withdrawal (Little et al., 1996; Silveri and Spear, 1998; White et al., 2002; Varlinskaya and Spear, 2004). The anxiety-related symptoms observed among adults during hangover may serve as a deterrent for alcohol drinking, instead, its lack at adolescence may increase the risk for alcohol drinking behaviors at this age, thus facilitating the perpetuation of cycle of drinking that may lead to dependency and possibly alcohol-related problems at adulthood. In addition, a higher sensibility to ethanol-induced rewarding properties have been described among adolescents (Doremus-Fitzwater et al., 2010). Therefore, adolescents might be at great risk for alcohol consumption. Is it worth mentioning that among adult animals, a similar alcohol DID schedule rendered no effects on the elevated plus-maze (Cippitelli et al., 2012). Besides, controversial results have been reported following repeated binge-drinking during adolescence: increased anxiety levels have been reported, yet in the long-term (Rasmussen et al., 2001; Montesinos et al., 2016; Sanchez-Marin et al., 2017), and also decreased anxiety or increased impulsivity has also been reported (Gilpin et al., 2012). Our data may reflect moderate alcohol consumption during adolescence, a dampen effect of alcohol when administered by a DID protocol, or even resilience to the anxiogenic-like effects of alcohol at adolescence. Further studies are needed to clarify this point.

According to previous literature by using the same (Sanchez-Marin et al., 2017) or other behavioral paradigms (Coleman et al., 2014; Oliveira et al., 2015), our results further support the detrimental effects of adolescent alcohol in cognitive function (Guerri and Pascual, 2010; Alfonso-Loeches and Guerri, 2011). Alcohol-induced deficits in cognitive function have been related to changes in the volume of the HF, the orbitoFC, cerebellum and thalamus in animals (Coleman et al., 2014; Oliveira et al., 2015) and humans (De Bellis et al., 2000; Ozsoy et al., 2013), though decreases in hippocampal volume have also been related to alcohol consumption vulnerability (Nagel et al., 2005).

In the present study, the alcohol-induced deficit in cognitive function could be related to the observed increase in SYN expression within the FC. Despite in the short-term alcohol may decrease SYN expression due to its neurotoxic effects, possibly related to neuronal excitotoxic damage resulting from repeated alcohol withdrawal episodes (Alfonso-Loeches and Guerri, 2011), a compensatory up-regulation may arise if alcohol is continued and/or administered at adolescence. Changes in presynaptic markers have also been reported in the HF: a similar increase in SYN was observed among females, although SNAP25 seems to play this role in males. We found no changes in BDNF expression, although in the literature controversial results have been reported (decreases (Briones and Woods, 2013) and

increases (Tapia-Arancibia et al., 2001) in the hippocampus). Discrepancies may rely on the alcohol paradigm used, age of the brain examined, and timing of measurement during the withdrawal period. In general, repeated alcohol exposure during adolescence seems to interfere with presynaptic proteins inducing a long-lasting compensatory up-regulation that may critically affect synaptic function and the establishment of adult neural circuitries.

The endocannabioid system has also been given a role in alcohol effects (Basavarajappa and Hungund, 2002). We observed no changes in CB1R expression, but a significant decrease in CB2R expression within the FC was observed in both male and female animals. A recent study has reported a decrease in the mRNA expression of *Cnr2* in the hippocampus following adolescent alcohol administration (Sanchez-Marin et al., 2017) that has been demonstrated to modulate cognitive functions in mice (Li and Kim, 2016). On the other hand, existing literature also suggests that a reduction in CB2R function may promote alcohol preference and consumption (Onaivi et al., 2008; Ortega-Álvaro et al., 2015). Therefore, the alcohol-induced decrease in CB2R may underlay the alcohol-induced cognitive impairment, but may also be related to the reported increase for adult alcohol consumption following adolescent exposure (Guerri and Pascual, 2010).

Adolescent exposure to alcohol also impacts glial cells, and important changes in GFAP expression have been previously reported. An increase in GFAP levels have been reported short after alcohol exposure during adolescence, although in the long-term, after abstinence, levels tended to return to normality (Evrard et al., 2006; Kane et al., 2014). The effects of adolescent alcohol on glial cells remains controversial and further studies are needed to better understand alcohol sex-dependent effects, and the possible relevance of the sex and brain-region dependent maturational program of astrocytes (Koss et al., 2012).

Remarkably, intermittent access to ethanol during adolescence seems to particularly affect female animals consistently reducing molecular markers of serotoninergic, dopaminergic and glutamatergic signaling. Such an effects may reflect an increase in neuronal apoptosis, as recently described for the hippocampus (Oliveira et al., 2015) or it may reflect specific effects of the different neurotransmitter systems. Adolescent alcohol indices a loss in 5-HT immunoreactive neurons within the dorsal raphe nucleus (DRN; Evrard et al., 2006; Vetreno et al., 2017) together with persistent alterations in terminal field projections, i.e., hypothalamus, amygdala and hippocampus (Vetreno et al., 2017). Adolescent alcohol also impacts the dopaminergic system by decreasing the expression of its receptors, at least, in the medial pre-FC (Pascual et al., 2009; Trantham-Davidson and Chandler, 2015; Crews et al., 2016).

Epigenetic changes have been associated with behavior during development (Roth, 2013) and epigenetics mechanisms may also underlying the sex-specific consequences of adolescent alcohol. In the present study, exclusively among females, a decrease in H3 acetylation in the two brain regions analyzed has been evidenced, but a decrease in H4 acetylation only within the FC. However, previous studies showed that alcohol administration during adolescence increases H3 and H4 acetylation in the preFC of mice (Pascual et al., 2012; Montesinos et al., 2016). Actually, in those studies acetylation changes were related with changes in the expression of specific genes such as bdnf but also cFos, Cdk5 and FosB. Moreover, adolescent alcohol was reported to also up-regulate histone acetyl transferase (HAT) activity in the preFC. In these studies, these epigenetic changes were mainly related to alcohol-induced anxiety and to the rewarding effects of alcohol. Therefore, discrepancies with our present results may rely on differences on drug doses and routes, on the animal specie employed, but also to the fact that no changes in anxiety were observed in our hands in the present study, and alcohol-related rewarding properties were not analyzed in this study. Further research is still needed to specifically correlate alcohol-induced behavioral and epigenetic changes.

Further research is urgently needed to better understand the underlying molecular mechanisms for adolescent alcohol consequence, mainly for the possible resilience to alcohol negative effects, including withdrawal, for the long-lasting cognitive impairment, as well as for the increased vulnerability of females to alcohol (Barron et al., 2005; Spear, 2015). The consequences of alcohol seem to critically depend on the ethanol dose, administration route and schedule, peak BEC, treatment paradigm (including length of treatment and the presence or absence of a withdrawal period following ethanol administration), sex and the age of the animal (reviewed in Drew and Kane, 2013). Thus, the development of better and more consistent translational methods for the evaluation of adolescent alcohol detrimental effects is of great medical and societal concern.

In spite of the fact that prevention and alcohol control policies are yet effective tools in the reduction of excessive alcohol consumption, a better knowledge of the mechanisms involved in alcohol effects may provide new tools for the identification of vulnerability populations, and may open new horizons in the pharmacology of alcohol abuse disorders.

AUTHOR CONTRIBUTIONS

All authors had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analyses. Design of the work: FRF, ML-G and EMM. Acquisition of data: SP, M-DH, AG, EB, EG, JAL-M and EMM. Analysis of data: SP, M-DH, EB, MM, AG, EG and EMM. Interpretation of data: CG, FRF, ML-G and EMM. Drafting of the manuscript: ML-G and EMM. Critical revision of the manuscript for important intellectual content, obtained funding and study supervision: ML-G, EMM, JAL-M, CG and FRF. All authors revised the manuscript critically for intellectual content; and gave their final approval of the version to be published.

ACKNOWLEDGMENTS

This work was supported by funded by Instituto de Salud Carlos III (ISC-III) and European Regional Development Funds-European Union (ERDF-EU) under the RETICS program Red de Trastornos Adictivos (programs RD12/0028 and

RD16/0017) under the following grants: EMM to grant no. RD2012/0028/0021; CG to grant no. RD12/0028/0007. JAL-M to grant no. RD16/0017/0008; FRF to RD16/0017/0001. Additional funds came from Ministerio de Economía y Competitividad and ISC-III (PI16/01689) and ML-G to grant no. UCM-BSCH, ref. UCM951579.

REFERENCES

Alfonso-Loeches, S., and Guerri, C. (2011). Molecular and behavioral aspects of the actions of alcohol on the adult and developing brain. *Crit. Rev. Clin. Lab. Sci.* 48, 19–47. doi: 10.3109/10408363.2011.580567

Barron, S., White, A., Swartzwelder, H. S., Bell, R. L., Rodd, Z. A., Slawecki, C. J., et al. (2005). Adolescent vulnerabilities to chronic alcohol or nicotine exposure: findings from rodent models. *Alcohol. Clin. Exp. Res.* 29, 1720–1725. doi: 10.1097/01.alc.0000179220.79356.e5

Basavarajappa, B. S., and Hungund, B. L. (2002). Neuromodulatory role of the endocannabinoid signaling system in alcoholism: an overview. *Prostaglandins Leukot. Essent. Fatty Acids* 66, 287–299. doi: 10.1054/plef.2001.0352

Briones, T., and Woods, J. (2013). Chronic binge-like alcohol consumption in adolescence causes depression-like symptoms possibly mediated by the effects of BDNF on neurogenesis. *Neuroscience* 254, 324–334. doi: 10.1016/j.neuroscience.2013.09.031

Cailhol, S., and Mormède, P. (2001). Sex and strain differences in ethanol drinking: effects of gonadectomy. *Alcohol. Clin. Exp. Res.* 25, 594–599. doi: 10.1097/00000374-200104000-00017

Carnicella, S., Ron, D., and Barak, S. (2014). Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse. *Alcohol* 48, 243–252. doi: 10.1016/j.alcohol.2014.01.006

Cippitelli, A., Damadzic, R., Singley, E., Thorsell, A., Ciccocioppo, R., Eskay, R. L., et al. (2012). Pharmacological blockade of corticotropin-releasing hormone receptor 1 (CRH1R) reduces voluntary consumption of high alcohol concentrations in non-dependent Wistar rats. *Pharmacol. Biochem. Behav.* 100, 522–529. doi: 10.1016/j.pbb.2011.10.016

Coleman, L. G., Liu, W., Oguza, I., Styner, M., and Crews, F. T. (2014). Adolescent binge ethanol treatment alters adult brain regional volumes, cortical extracellular matrix protein and behavioral flexibility. *Pharmacol. Biochem. Behav.* 116, 142–151. doi: 10.1016/j.pbb.2013.11.021

Crabbe, J. C., Spence, S. E., Brown, L. L., and Metten, P. (2011). Alcohol preference drinking in a mouse line selectively bred for high drinking in the dark. *Alcohol* 45, 427–440. doi: 10.1016/j.alcohol.2010.12.001

Crews, F. T., Vetreno, R. P., Broadwater, M. A., and Robinson, D. L. (2016). Adolescent alcohol exposure persistently impacts adult neurobiology and behavior. *Pharmacol. Rev.* 68, 1074–1109. doi: 10.1124/pr.115.012138

De Bellis, M. D., Clark, D. B., Beers, S. R., Soloff, P. H., Boring, A. M., Hall, J., et al. (2000). Hippocampal volume in adolescent-onset alcohol use disorders. *Am. J. Psychiatry* 157, 737–744. doi: 10.1176/appi.ajp.157.5.737

Doremus, T. L., Brunell, S. C., Varlinskaya, E. I., and Spear, L. P. (2003). Anxiogenic effects during withdrawal from acute ethanol in adolescent and adult rats. *Pharmacol. Biochem. Behav.* 75, 411–418. doi: 10.1016/s0091-3057(03)00134-5

Doremus-Fitzwater, T. L., Varlinskaya, E. I., and Spear, L. P. (2010). Motivational systems in adolescence: possible implications for age differences in substance abuse and other risk-taking behaviors. *Brain Cogn.* 72, 114–123. doi: 10.1016/j.bandc.2009.08.008

Drew, P. D., and Kane, C. J. M. (2013). "Neuroimmune mechanisms of glia and their interplay with alcohol exposure across the lifespan," in *Neural-Immune Interactions in Brain Function and Alcohol Related Disorders*, eds C. Cui, L. Grandison and A. Noronha Cui (New York, NY: Springer Science + Business Media), 359-386.

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. *Behav. Brain Res.* 31, 47–59. doi: 10.1016/0166-4328(88)90157-x

Evrard, S., Duhalde-Vega, M., Tagliaferro, P., Mirochnic, S., Caltana, L., and Brusco, A. (2006). A low chronic ethanol exposure induces morphological changes in the adolescent rat brain that are not fully recovered even after a long abstinence: an immunohistochemical study. *Exp. Neurol.* 200, 438–459. doi: 10.1016/j.expneurol.2006.03.001

Fagiolini, M., Jensen, C. L., and Champagne, F. A. (2009). Epigenetic influences on brain development and plasticity. *Curr. Opin. Neurobiol.* 19, 207–212. doi: 10.1016/j.conb.2009.05.009

Festing, M. F. W. (2006). Design and statistical methods in studies using animal models of development. *ILAR J.* 47, 5–14. doi: 10.1093/ilar.47.1.5

George, O., Sanders, C., Freiling, J., Grigoryan, E., Vu, S., Allen, C. D., et al. (2012). Recruitment of medial prefrontal cortex neurons during alcohol withdrawal predicts cognitive impairment and excessive alcohol drinking. *Proc. Natl. Acad. Sci. U S A* 109, 18156–18161. doi: 10.1073/pnas.1116523109

Gilpin, N. W., Karanikas, C. A., and Richardson, H. N. (2012). Adolescent binge drinking leads to changes in alcohol drinking, anxiety, and amygdalar corticotropin releasing factor cells in adulthood in male rats. *PLoS One* 7:e31466. doi: 10.1371/journal.pone.0031466

Guerri, C., and Pascual, M. (2010). Mechanisms involved in the neurotoxic, cognitive, and neurobehavioral effects of alcohol consumption during adolescence. *Alcohol* 44, 15–26. doi: 10.1016/j.alcohol.2009.10.003

Holgate, J. Y., Shariff, M., Mu, E. W. H., and Bartlett, S. (2017). A rat drinking in the dark model for studying ethanol and sucrose consumption. *Front. Behav. Neurosci.* 11:29. doi: 10.3389/fnbeh.2017.00029

Kane, C. J. M., Phelan, K. D., Douglas, J. C., Wagoner, G., Johnson, J. W., Xu, J., et al. (2014). Effects of ethanol on immune response in the brain: region-specific changes in adolescent versus adult mice. *Alcohol. Clin. Exp. Res.* 38, 384–391. doi: 10.1111/acer.12244

Koss, W., Sadowski, R., Sherrill, L., Gulley, J., and Juraska, J. (2012). Effects of ethanol during adolescence on the number of neurons and glia in the medial prefrontal cortex and basolateral amygdala of adult male and female rats. *Brain Res.* 1466, 24–32. doi: 10.1016/j.brainres.2012.05.023

Li, Y., and Kim, J. (2016). CB2 Cannabinoid receptor knockout in mice impairs contextual long-term memory and enhances spatial working memory. *Neural Plast.* 2016:9817089. doi: 10.1155/2016/9817089

Little, P. J., Kuhn, C. M., Wilson, W. A., and Swartzwelder, H. S. (1996). Differential effects of ethanol in adolescent and adult rats. *Alcohol. Clin. Exp. Res.* 20, 1346–1351. doi: 10.1111//j.1530-0277.1996.tb01133.x

Mateos, B., Borcel, E., Loriga, R., Luesu, W., Bini, V., Llorente, R., et al. (2011). Adolescent exposure to nicotine and/or the cannabinoid agonist CP 55,940 induces gender-dependent long-lasting memory impairments and changes in brain nicotinic and CB_1 cannabinoid receptors. *J. Psychopharmacol.* 25, 1676–1690. doi: 10.1177/0269881110370503

McClain, J. A., Morris, S. A., Deeny, M. A., Marshall, S. A., Hayes, D. M., Kiser, Z. M., et al. (2011). Adolescent binge alcohol exposure induces long-lasting partial activation of microglia. *Brain Behav. Immun.* 25, S120–S128. doi: 10.1016/j.bbi.2011.01.006

Momeni, S., and Roman, E. (2014). Subgroup-dependent effects of voluntary alcohol intake on behavioral profiles in outbred Wistar rats. *Behav. Brain Res.* 275, 288–296. doi: 10.1016/j.bbr.2014.08.058

Mons, N., and Beracochea, D. (2016). Behavioral neuroadaptation to alcohol: from glucocorticoids to histone acetylation. *Front. Psychiatry* 7:165. doi: 10.3389/fpsyt.2016.00165

Montesinos, J., Pascual, M., Rodríguez-Arias, M., Miñarro, J., and Guerri, C. (2016). Involvement of TLR4 in the long-term epigenetic changes, rewarding and anxiety effects induced by intermittent ethanol treatment in adolescence. *Brain Behav. Immun.* 53, 159–171. doi: 10.1016/j.bbi.2015.12.006

Morici, J. F., Bekinschtein, P., and Weisstaub, N. V. (2015). Medial prefrontal cortex role in recognition memory in rodents. *Behav. Brain Res.* 292, 241–251. doi: 10.1016/j.bbr.2015.06.030

Nagel, B. J., Schweinsburg, A. D., Phan, V., and Tapert, S. F. (2005). Reduced hippocampal volume among adolescents with alcohol use disorders without psychiatric comorbidity. *Psychiatry Res.* 139, 181–190. doi: 10.1016/j.pscychresns.2005.05.008

Oliveira, A. C., Pereira, M. C., Santana, L. N. D. S., Fernandes, R. M., Teixeira, F. B., Oliveira, G. B., et al. (2015). Chronic ethanol exposure during adolescence through early adulthood in female rats induces emotional and memory deficits associated with morphological and molecular alterations in hippocampus. *J. Psychopharmacol.* 29, 712–724. doi: 10.1177/0269881115581960

Onaivi, E. S., Ishiguro, H., Gong, J.-P., Patel, S., Meozzi, P. A., Myers, L., et al. (2008). Brain neuronal CB2 cannabinoid receptors in drug abuse and depression: from mice to human subjects. *PLoS One* 3:e1640. doi: 10.1371/journal.pone.0001640

Ortega-Álvaro, A., Ternianov, A., Aracil-Fernández, A., Navarrete, F., García-Gutiérrez, M. S., and Manzanares, J. (2015). Role of cannabinoid CB2 receptor in the reinforcing actions of ethanol. *Addict. Biol.* 20, 43–55. doi: 10.1111/adb.12076

Ozsoy, S., Durak, A., and Esel, E. (2013). Hippocampal volumes and cognitive functions in adult alcoholic patients with adolescent-onset. *Alcohol* 47, 9–14. doi: 10.1016/j.alcohol.2012.09.002

Pascual, M., Boix, J., Felipo, V., and Guerri, C. (2009). Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. *J. Neurochem.* 108, 920–931. doi: 10.1111/j.1471-4159.2008.05835.x

Pascual, M., Do Couto, B. R., Alfonso-Loeches, S., Aguilar, M. A., Rodriguez-Arias, M., and Guerri, C. (2012). Changes in histone acetylation in the prefrontal cortex of ethanol-exposed adolescent rats are associated with ethanol-induced place conditioning. *Neuropharmacology* 62, 2309–2319. doi: 10.1016/j.neuropharm.2012.01.011

Pavón, F. J., Marco, E. M., Vázquez, M., Sánchez, L., Rivera, P., Gavito, A., et al. (2016). Effects of adolescent intermittent alcohol exposure on the expression of endocannabinoid signaling-related proteins in the spleen of young adult rats. *PLoS One* 11:e0163752. doi: 10.1371/journal.pone.0163752

Pellow, S., Chopin, P., File, S. E., and Briley, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods* 14, 149–167. doi: 10.1016/0165-0270(85)90031-7

Ponomarev, I. (2013). Epigenetic control of gene expression in the alcoholic brain. *Alcohol Res.* 35, 69–76.

Rasmussen, D. D., Boldt, B. M., Bryant, C. A., Mitton, D. R., Larsen, S. A., and Wilkinson, C. W. (2000). Chronic daily ethanol and withdrawal: 1. Long-term changes in the hypothalamo-pituitary-adrenal axis. *Alcohol. Clin. Exp. Res.* 24, 1836–1849. doi: 10.1097/00000374-200012000-00013

Rasmussen, D. D., Mitton, D. R., Green, J., and Puchalski, S. (2001). Chronic daily ethanol and withdrawal: 2. Behavioral changes during prolonged abstinence. *Alcohol. Clin. Exp. Res.* 25, 999–1005. doi: 10.1097/00000374-200107000-00008

Roth, T. L. (2013). Epigenetic mechanisms in the development of behavior: advances, challenges, and future promises of a new field. *Dev. Psychopathol.* 25, 1279–1291. doi: 10.1017/s0954579413000618

Sanchez-Marin, L., Pavon, F. J., Decara, J., Suarez, J., Gavito, A., Castilla-Ortega, E., et al. (2017). Effects of intermittent alcohol exposure on emotion and cognition: a potential role for the endogenous cannabinoid system and neuroinflammation. *Front. Behav. Neurosci.* 11:15. doi: 10.3389/fnbeh.2017.00015

Silveri, M. M., and Spear, L. P. (1998). Decreased sensitivity to the hypnotic effects of ethanol early in ontogeny. *Alcohol. Clin. Exp. Res.* 22, 670–676. doi: 10.1097/00000374-199805000-00019

Simms, J. A., Steensland, P., Medina, B., Abernathy, K. E., Chandler, L. J., Wise, R., et al. (2008). Intermittent access to 20% ethanol induces high ethanol consumption in long-evans and wistar rats. *Alcohol. Clin. Exp. Res.* 32, 1816–1823. doi: 10.1111/j.1530-0277.2008.00753.x

Somkuwar, S. S., Vendruscolo, L. F., Fannon, M. J., Schmeichel, B. E., Nguyen, T. B., Guevara, J., et al. (2017). Abstinence from prolonged ethanol exposure affects plasma corticosterone, glucocorticoid receptor signaling and stress-related behaviors. *Psychoneuroendocrinology* 84, 17–31. doi: 10.1016/j.psyneuen.2017.06.006

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* 24, 417–463. doi: 10.1016/s0149-7634(00)00014-2

Spear, L. P. (2015). Adolescent alcohol exposure: are there separable vulnerable periods within adolescence? *Physiol. Behav.* 148, 122–130. doi: 10.1016/j.physbeh.2015.01.027

Spear, L. P. (2016a). Alcohol consumption in adolescence: a translational perspective. *Curr. Addict. Rep.* 3, 50–61. doi: 10.1007/s40429-016-0088-9

Spear, L. P. (2016b). Consequences of adolescent use of alcohol and other drugs: studies using rodent models. *Neurosci. Biobehav. Rev.* 70, 228–243. doi: 10.1016/j.neubiorev.2016.07.026

Squire, L. R., Wixted, J. T., and Clark, R. E. (2007). Recognition memory and the medial temporal lobe: a new perspective. *Nat. Rev. Neurosci.* 8, 872–883. doi: 10.1038/nrn2154

Staples, M. C., and Mandyam, C. D. (2016). Thinking after drinking: impaired hippocampal-dependent cognition in human alcoholics and animal models of alcohol dependence. *Front. Psychiatry* 7:162. doi: 10.3389/fpsyt.2016.00162

Tapia-Arancibia, L., Rage, F., Givalois, L., Dingeon, P., Arancibia, S., and Beaugé, F. (2001). Effects of alcohol on brain-derived neurotrophic factor mRNA expression in discrete regions of the rat hippocampus and hypothalamus. *J. Neurosci. Res.* 63, 200–208. doi: 10.1002/1097-4547(20010115)63:2<200::aid-jnr1012>3.0.co;2-q

Trantham-Davidson, H., and Chandler, L. (2015). Alcohol-induced alterations in dopamine modulation of prefrontal activity. *Alcohol* 49, 773–779. doi: 10.1016/j.alcohol.2015.09.001

Varlinskaya, E. I., and Spear, L. P. (2004). Acute ethanol withdrawal (hangover) and social behavior in adolescent and adult male and female sprague-dawley rats. *Alcohol. Clin. Exp. Res.* 28, 40–50. doi: 10.1097/01.alc.0000108655.51087.df

Vetreno, R. P., Patel, Y., Patel, U., Walter, T. J., and Crews, F. T. (2017). Adolescent intermittent ethanol reduces serotonin expression in the adult raphe nucleus and upregulates innate immune expression that is prevented by exercise. *Brain Behav. Immun.* 60, 333–345. doi: 10.1016/j.bbi.2016.09.018

Vetter-O'Hagen, C., Varlinskaya, E., and Spear, L. (2009). Sex differences in ethanol intake and sensitivity to aversive effects during adolescence and adulthood. *Alcohol Alcohol.* 44, 547–554. doi: 10.1093/alcalc/agp048

White, A. M., Truesdale, M. C., Bae, J. G., Ahmad, S., Wilson, W. A., Best, P. J., et al. (2002). Differential effects of ethanol on motor coordination in adolescent and adult rats. *Pharmacol. Biochem. Behav.* 73, 673–677. doi: 10.1016/s0091-3057(02)00860-2

WHO. (2014). *Global Status Report on Alcohol and Health.* Geneva: World Health Organization.

Wilsnack, S. C., Wilsnack, R. W., and Kantor, L. W. (2013). Focus on: women and the costs of alcohol use. *Alcohol Res.* 35, 219–228.

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 July 2017
doi: 10.3389/fnbeh.2017.00134

Maladaptive Decision Making in Adults with a History of Adolescent Alcohol use, in a Preclinical Model, Is Attributable to the Compromised Assignment of Incentive Value during Stimulus-Reward Learning

*Lauren C. Kruse[1], Abigail G. Schindler[1,2], Rapheal G. Williams[3], Sophia J. Weber[1] and Jeremy J. Clark[1]**

[1]Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, WA, United States, [2]Geriatric Research Education and Clinical Center, VA Puget Sound Health Care System, Seattle, WA, United States, [3]Graduate Program in Neuroscience, University of Washington, Seattle, WA, United States

According to recent WHO reports, alcohol remains the number one substance used and abused by adolescents, despite public health efforts to curb its use. Adolescence is a critical period of biological maturation where brain development, particularly the mesocorticolimbic dopamine system, undergoes substantial remodeling. These circuits are implicated in complex decision making, incentive learning and reinforcement during substance use and abuse. An appealing theoretical approach has been to suggest that alcohol alters the normal development of these processes to promote deficits in reinforcement learning and decision making, which together make individuals vulnerable to developing substance use disorders in adulthood. Previously we have used a preclinical model of voluntary alcohol intake in rats to show that use in adolescence promotes risky decision making in adulthood that is mirrored by selective perturbations in dopamine network dynamics. Further, we have demonstrated that incentive learning processes in adulthood are also altered by adolescent alcohol use, again mirrored by changes in cue-evoked dopamine signaling. Indeed, we have proposed that these two processes, risk-based decision making and incentive learning, are fundamentally linked through dysfunction of midbrain circuitry where inputs to the dopamine system are disrupted by adolescent alcohol use. Here, we test the behavioral predictions of this model in rats and present the findings in the context of the prevailing literature with reference to the long-term consequences of early-life substance use on the vulnerability to develop substance use disorders. We utilize an impulsive choice task to assess the selectivity of alcohol's effect on decision-making profiles and conditioned reinforcement to parse out the effect of incentive value attribution, one mechanism of incentive learning. Finally, we use the differential reinforcement of low rates of responding (DRL) task to examine the degree to which behavioral disinhibition may contribute to an overall decision-making profile. The findings presented here support the proposition that early life alcohol use selectively alters risk-based choice behavior

OPEN ACCESS

Edited by:
Jacqueline M. Barker, Medical University of South Carolina, United States

Reviewed by:
Jocelyn M. Richard, Johns Hopkins University, United States
Alicia Izquierdo, University of California, Los Angeles, United States

***Correspondence:**
Jeremy J. Clark
jjc1@uw.edu

Received: 29 April 2017
Accepted: 07 July 2017
Published: 25 July 2017

Citation:
Kruse LC, Schindler AG, Williams RG, Weber SJ and Clark JJ (2017) Maladaptive Decision Making in Adults with a History of Adolescent Alcohol use, in a Preclinical Model, Is Attributable to the Compromised Assignment of Incentive Value during Stimulus-Reward Learning. Front. Behav. Neurosci. 11:134. doi: 10.3389/fnbeh.2017.00134

through modulation of incentive learning processes, both of which may be inexorably linked through perturbations in mesolimbic circuitry and may serve as fundamental vulnerabilities to the development of substance use disorders.

Keywords: adolescent, alcohol, decision making, incentive learning, risk taking, impulsivity

INTRODUCTION

Adolescence is characterized by risky and impulsive behaviors, often including initial experimentation with drugs of abuse and most commonly, alcohol (Casey and Jones, 2010). During development, the adolescent brain undergoes substantial maturation in cortical and limbic regions involved in impulsive behaviors, decision making and reward processing (Spear, 2000; Chambers et al., 2003; Crews et al., 2007; Bava and Tapert, 2010; Marinelli and McCutcheon, 2014). Given the high vulnerability of these developing regions to the damaging effects of alcohol (Chambers et al., 2003; Pascual et al., 2009; Bava and Tapert, 2010), exposure during this critical time period can produce long-term neurobiological and behavioral changes that increase risk for chronic alcohol problems later in adulthood. In clinical work, alcohol use has been associated with deficits in adaptive decision making strategies, impulsivity and reward valuation (Goudriaan et al., 2007; Johnson et al., 2008; Brevers et al., 2014).

Using a probability discounting task coupled with a preclinical model of voluntary adolescent alcohol use, our laboratory previously demonstrated that rats with a history of adolescent alcohol use opt for more risky decision-making strategies when they are adults, consistent with findings in humans (Brevers et al., 2014; Schindler et al., 2014). This increase in risk preference is specific to adolescent alcohol use as adult animals with identical alcohol exposure do not differ in the probability discounting task (Schindler et al., 2014). Subsequent reports have demonstrated that this behavioral phenotype following adolescent alcohol use is robust (Nasrallah et al., 2011; Clark et al., 2012; Schindler et al., 2014), reproducible (Boutros et al., 2014; McMurray et al., 2014), and translates to the human condition (Brevers et al., 2014). These three core features: robust, reproducible and translational make up the fundamental elements of a successful pre-clinical model of attributes that may correspond to vulnerabilities to the development of substance abuse and alcohol use disorders (Collins and Tabak, 2014).

In addition, we have identified a neural correlate of maladaptive decision making in the mesolimbic dopamine system that is promoted by early life alcohol use. Adult animals with a history of alcohol use in adolescence show increased phasic dopamine release in the nucleus accumbens core in response to risky options and pavlovian incentive cues when compared to controls (Nasrallah et al., 2011; Spoelder et al., 2015). The mesolimbic dopamine system is implicated in reinforcement learning, goal-directed behavior and motivational processes, and may be particularly critical when these behaviors are associated with the craving and seeking of abused substances (Kelley, 2004; Everitt and Robbins, 2005; Salamone and Correa, 2012). Further examination of this mesolimbic circuit revealed a potentiation in the excitatory drive on ventral tegmental area dopamine neurons from the pedunculopontine nucleus (PPT) in animals with a history of adolescent alcohol use (Schindler et al., 2016). This is particularly striking given the important role that this structure is thought to play in both incentive-based reinforcement learning (Pan and Hyland, 2005) and choice behavior under uncertainty (Leblond et al., 2014). Importantly, we were able to demonstrate that pharmacological mitigation of this increased PPT drive was capable of completely rescuing maladaptive risk preference in animals with a history of adolescent alcohol use (Schindler et al., 2016). We have therefore hypothesized that maladaptive decision making under risk as a result of adolescent alcohol use perturbing the mesolimbic dopamine system may result from a selective deficit in incentive learning (Clark et al., 2012; Spoelder et al., 2015).

However, both risk-seeking and risk-taking behaviors have been previously attributed to impulsivity, which is another potential underlying mechanism influencing altered decision making. The concept of impulsivity incorporates various behaviors, but is commonly separated into two important features: impulsive choice and impulsive action, both of which are linked to addiction (Evenden, 1999; Olmstead, 2006; Winstanley et al., 2010). Indeed, preclinical and clinical studies have long identified impulsivity and risky decision making as behaviors contributing to the development and perpetuation of substance use disorders, including alcohol (Littlefield and Sher, 2010; Brevers et al., 2014; Jupp and Dalley, 2014a,b). Delay discounting is commonly used as a measure of impulsive decision making and performance on this task has historically been linked with performance on probability discounting tasks (Richards et al., 1999). In addition to the temporal delay of rewards influencing decision making, the amount of effort required to receive a reward can also influence choice. Several studies have connected dopamine signaling with mediating choice in the effort discounting task, but the role of alcohol, and particularly adolescent alcohol use, on effort discounting remains unclear (Floresco et al., 2008; Day et al., 2010; Ghods-Sharifi and Floresco, 2010). Interestingly, recent reports propose that performance on delay discounting and probability discounting require different decision-making processes with separate underlying neurobiological mechanisms (van Gaalen et al., 2006; Olson et al., 2007; St Onge and Floresco, 2009; Green and Myerson, 2013). In the present studies, we examine the effect of voluntary adolescent alcohol intake on impulsivity and incentive attribution to differentiate the potential role impulsivity may play in risk preference from the role incentive learning may play.

We examined differences in incentive attribution as a result of adolescent alcohol intake using Pavlovian conditioning and conditioned reinforcement, the gold standard for examining

acquired motivational properties through learning. We also examined the two primary features of impulsivity, impulsive choice and impulsive action, using delay-discounting and the differential reinforcement of low rates of responding (DRL) tasks, respectively. The purpose of these studies is to further test our hypothesis that incentive based learning and assignment of motivational value through reinforcement learning, not impulsivity, is the underlying psychological mechanism of maladaptive decision making after early life alcohol use. Our results show that adults exposed to alcohol during adolescence exhibit enhanced attribution of value to reward-predictive cues compared to controls. Alternatively, we find no significant difference between alcohol-exposed and control animals on either measure of impulsivity (impulsive choice and action). Therefore, we provide further compelling evidence that adolescent alcohol use promotes disadvantageous decision making by altering a learning-based incentive attribution process and not through a general mechanism of impulsivity.

MATERIALS AND METHODS

Animals and Housing

Male Sprague-Dawley rats (Charles River, Hollister, CA, USA) were weaned at postnatal day (PND) 21 and aged PND 27 at the start of experiments. All animals were housed individually in polycarbonate tubs on a 12-h light/dark cycle (lights on at 06:00). Animals were housed individually for accurate assessment of alcohol gel consumption. Water and Teklad (Harlan, Kent, WA, USA) rodent chow was available *ad libitum* except as noted. Rats were weighed and handled daily throughout the course of the experiment. All work in this manuscript was conducted under the guidance and permission of the Institutional Animal Care and Use Committee at the University of Washington and pursuant to federal regulations regarding work with animals.

Alcohol Preparation, Administration and Withdrawal

Alcohol was presented to adolescent (PND 30–49) rats in a gel matrix consisting of distilled water, Knox gelatin, polycose (10%) and 190-proof ethanol (10%). Control gels had ethanol replaced with distilled water. Preparation was as previously described (Rowland et al., 2005; Nasrallah et al., 2011; Schindler et al., 2014). The gels were made available 24 h/day, unless otherwise noted, in addition to *ad libitum* water and chow. Alcohol gel intake levels were monitored daily and expressed in g/kg of body weight using individual gel consumption and body weights measured daily. Rats failing to consume gel during the control gel pre-exposure, exhibiting three consecutive days of no consumption, or burying of the gel in bedding once the alcohol gel exposure began were excluded from the study. Experiments began with 3 days of pre-exposure to control gel. Subsequently, adolescent rats were each split into alcohol gel and control gel groups matched by weight and baseline intake. Twenty days of 24 h/day gel exposure followed. Upon completion of the 20-day exposure to ethanol rats were monitored daily for withdrawal symptoms (e.g., seizures, weight loss and anxious behavior) for the following 20 days. No overt signs of withdrawal were observed.

Instrumental Training

All behavioral experiments were conducted in standard Med Associates chambers (St. Albans, VT, USA). For all tasks, prior to the beginning of training, animals were food restricted to maintain them at $\sim$90 $\pm$ 2% free-feeding weight. Free-feeding weight was based on pre-restriction weights. The rewards for instrumental responses and during Pavlovian conditioning were 45-mg sucrose pellets (Bio Serve, Prospect, CT, USA). Rats underwent magazine training before all tasks where 10 sucrose pellets were delivered over 15 min. For delay discounting and DRL, rats were trained on a fixed ratio 1 schedule to a criterion of $\geq$24 responses in a 30-min session. For delay discounting, once criterion was met, rats were auto-shaped over the course of 5 days (day 1 of auto-shaping required rats to perform a nosepoke into the food tray for trial initiation; day 2 increased the inter-trial interval (ITI) from 0 s to 15 s; day 3 reduced the time to perform trial-initiating nosepoke to 10 s; day 4 increased the ITI from 15 s to 30 s; day 5 increased the ITI from 30 s to 45 s).

Pavlovian Conditioned Approach and Conditioned Reinforcement

Following magazine training, rats underwent 7 days of pavlovian training with 25 trials per day. For each training session, a trial consisted of the extension of a lever (left or right, counterbalanced between animals) into the chamber along with illumination of a cue light above the lever (conditioned stimulus, CS) for 8 s, followed immediately by the delivery of two sucrose pellets (unconditioned stimulus, US) into the food tray and illumination of the tray light. The CS-US presentations occurred on a 60 s variable ITI schedule for each session. Lever presses and cup entries during lever presentation were recorded as measures of pavlovian conditioned approach. Total number of lever presses and food tray head entries during lever presentation were recorded but had no programmable response.

After 7 days of pavlovian training, all animals underwent a test for conditioned reinforcement. The chambers were rearranged such that the left and right retractable levers flanking the food tray were replaced with nosepoke ports and the food tray was replaced with a single retractable lever. The conditioned reinforcement session lasted a total of 40 min and the houselight was on for the full duration. During the session, nosepokes into the designated "active" port (located on the opposite side of wall as the lever-CS) resulted in insertion of the illuminated lever for 2 s. Nosepokes into the "inactive" port had no programmable consequences. Total number of lever presses, nosepokes into the active port, and nosepokes into the inactive port were recorded.

Delay Discounting

Following instrumental training as outlined above, animals were tested on a concurrent instrumental task involving the presentation of two levers. Each daily session consisted of 24 forced trials followed by 24 free-choice trials, with a total of

seven testing sessions. At the start of each session, the chamber was in the ITI state, completely dark with no light cues. All trials began with illumination of the house light and a light in the food tray cueing the animal to make a nosepoke into the food tray within 10 s. Failure to nosepoke resulted in trial termination, and the chamber returned to the ITI state. During training, animals were exposed to forced trials wherein a successful nosepoke led to the extension of a single lever, presented pseudo-randomly. These trials served to expose the animal to each option and its associated expected value. During each session, forced choice trials were followed by free-choice trials with the same contingency for each lever. Free-choice trials follow the guidelines described above, but each successful nosepoke resulted in the extension of both levers, and the animal was free to choose between the two levers within 10 s. During both forced- and free-choice trials, one lever was associated with the delivery two sucrose pellets after 1 s and the other lever associated with the delivery of four sucrose pellets after an increasing delay of time (either 1, 2, 4, 8, 16, 32 and 64 s). Increasing delay times were tested in separate testing sessions (days) with a 1 s delay on the first day of testing, a 2 s delay on the second day of testing, and so on for all animals. This method of testing the different delay times on separate days was done in order to be kept consistent with our previous studies using the probability discounting task where the different probabilistic deliveries of reward were also tested on separate, subsequent days. Each session assessed the animal's preference between the two levers. Choice of the high reward lever was recorded during free-choice trials.

DRL

Following instrumental training as described above, animals were tested on the DRL task as a measure of impulsive action. Each 45 min session began with illumination of the house light and lever presentation. Each animal was assigned an active lever (left or right) counterbalanced between rats, which remained extended throughout the duration of the sessions. For the first 5 days, rats were tested using a 5 s DRL interval, in which a lever press resulted in pellet delivery only if at least 5 s elapsed since the previous press. Each lever press that occurred before the required 5 s wait period resulted in a resetting of the 5 s criteria, during which animals would have to wait another 5 s before pressing the lever to receive a pellet. Following five consecutive days of the 5 s DRL, the schedule was then switched to a 10 s DRL interval for 5 days, then to a 20 s DRL interval for 5 days, and finally to a 30 s DRL interval for 5 days. The total number of lever presses and the total number of reinforcers received were recorded. Percent of effective lever presses was also determined by dividing the total number of pellets received by total number of lever presses.

Statistical Analyses

All statistical analyses were conducted using GraphPad Prism 6. Behavioral data for the pavlovian training sessions were binned into five-trial epochs. A response bias score, which is a measure of the relative allocation of behavioral responses between the lever and food tray during pavlovian training, was calculated by subtracting the number of food tray entries from the number of lever presses divided by the sum of both responses: (lever press − food tray entries/(lever presses + food tray entries), resulting in a number ranging from −1 (goal-tracking response) to +1 (sign-tracking response; Meyer et al., 2012). Animals with a response bias >+0.70 were defined as animals with a strong sign-tracking bias and animals with a response bias <−0.70 were defined as having a strong goal-tracking bias, consistent with previous work (Flagel et al., 2011; Meyer et al., 2012; Spoelder et al., 2015). Conditioned responses (lever presses and food tray entries) from all phases of training were analyzed using repeated measures two-way analysis of variance (ANOVA) with treatment (alcohol and control) and trial bin treated as independent variables. Performance on the conditioned reinforcement test was analyzed using a two-way ANOVA with treatment (alcohol vs. control) and port (active vs. inactive) as the independent variables and the number of nosepokes as the dependent variable. A *t*-test was used to analyze total number of lever presses during the conditioned reinforcement test. Performance on the delay-discounting and DRL tasks were analyzed using repeated measures two-way ANOVA with treatment (alcohol vs. control) and delay (1 s, 2 s, 4 s, 8 s, 16 s, 32 s, and 64 s) or DRL interval (5, 10, 20, 30 s) as the independent variables, and preference for the high reward lever or lever presses, reinforcers and effective lever presses, respectively, as the dependent variables. Bonferroni *post hoc* analyses were used and were corrected for multiple comparisons. All data are presented as mean ± SEM and threshold for statistical significance was set at $p < 0.05$.

RESULTS

Adolescent Alcohol Intake

For the conditioned reinforcement, delay-discounting and DRL animals, average alcohol intake across the 20-day exposure period was 11.3 ± 1.6 g/kg, 12.0 ± 2.3 g/kg, and 15.4 ± 1.5 g/kg, respectively, and intake did not differ across behavior groups ($F_{(2,30)\ \text{group}} = 2.1$, NS). Across all three behavioral experiments, a total of 10% of rats did not consume the alcohol gelatin during the 20 exposure days and thus were not run in the behavioral studies. These intake levels are comparable to our previous studies further supporting this method of alcohol intake as a consistent model of moderate/recreational alcohol use (Nasrallah et al., 2011; Schindler et al., 2014; Spoelder et al., 2015). Blood alcohol levels were not obtained but have been assessed previously (Schindler et al., 2014).

The Effect of Adolescent Alcohol Intake on Acquisition of Pavlovian Conditioned Approach Behavior in Adulthood

Consistent with previously published data (Spoelder et al., 2015), pavlovian conditioned responses to either the reward-predicting lever (sign tracking) or the food tray (goal tracking) during CS presentation developed differently for alcohol-exposed and control animals over the 7 days of pavlovian training. Although both groups developed a response bias towards sign-tracking over the course of training ($F_{(34,986)\ \text{trial bin}} = 57.2$

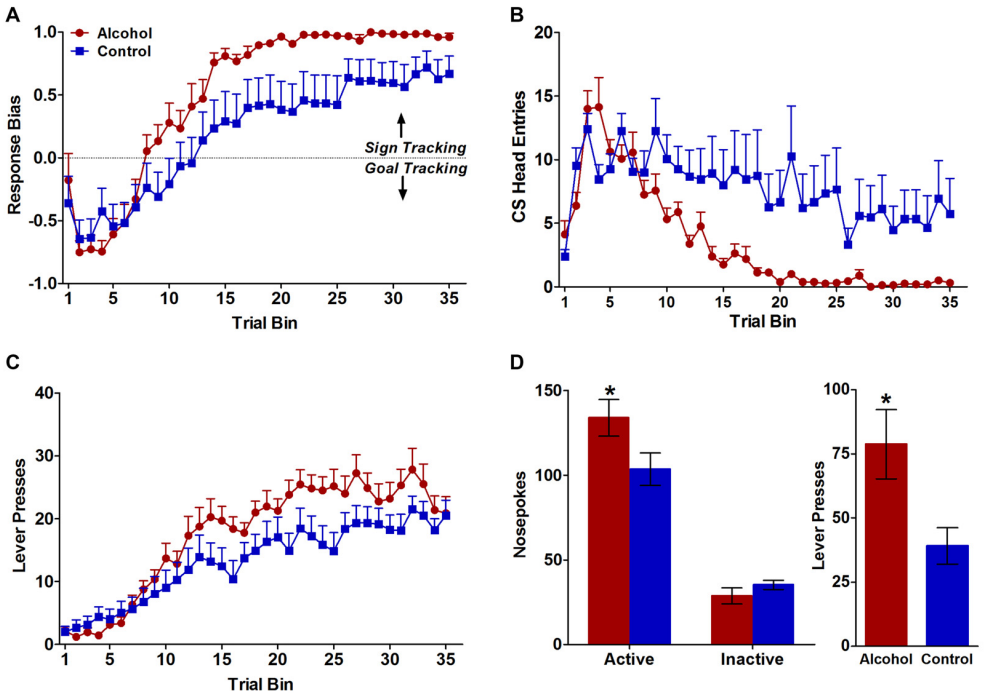


FIGURE 1 | Behavioral responses during pavlovian conditioned approach and test for conditioned reinforcement. **(A)** Alcohol-exposed (n = 16) animals reduced their conditioned responses to the food tray, whereas control (n = 15) animals continued to approach the food tray throughout the course of learning. **(B)** Both alcohol-exposed and control animals increased their conditioned responses to the reward-predicting lever over the course of learning but alcohol-exposed animals demonstrated a stronger conditioned response to the lever throughout the course of learning. **(C)** Both alcohol-exposed and control animals developed a response-bias toward sign tracking over the course of learning, but alcohol-exposed animals developed a stronger sign-tracker response bias. **(D)** In the test for conditioned reinforcement alcohol-exposed animals designated as sign trackers (n = 12) made significantly more active nosepokes compared to controls (n = 9). Alcohol-exposed and control animals did not differ in inactive nosepokes. All data are presented as mean ± SEM. *$P < 0.05$.

$p < 0.0001$; **Figure 1A**), the alcohol-exposed animals showed a stronger sign-tracking bias relative to controls later in the conditioning trials ($F_{(34,986)\ \text{trial bin} \times \text{treatment}} = 2.6$, $p < 0.001$; $F_{(1,986)\ \text{treatment}} = 4.3$, $p < 0.05$; **Figure 1A**). Further, both alcohol-exposed and control animals demonstrated reduced conditioned responding to the food tray over trials ($F_{(34,986)\ \text{trial bin}} = 14.0$, $p < 0.0001$; **Figure 1B**), but alcohol-exposed animals largely stopped responding to the food tray by trial bin 20 ($F_{(34,986)\ \text{trial bin} \times \text{treatment}} = 3.9$, $p < 0.0001$; $F_{(1,986)\ \text{treatment}} = 4.6$, $p < 0.05$ **Figure 1B**). Both alcohol-exposed animals and controls showed increased responses towards the lever over trials ($F_{(34,986)\ \text{trial bin}} = 40.6$, $p < 0.0001$; **Figure 1C**), but a greater number of contacts upon cue presentation in alcohol-exposed animals compared to controls developed over trial bins ($F_{(34,986)\ \text{trial bin} \times \text{treatment}} = 2.0$, $p < 0.001$; **Figure 1C**). Response-bias scores were also calculated as described above in the methods section. Animals with scores greater than +0.70 were designated as sign trackers and animals with scores less than −0.70 were designated as goal trackers. Based on these criteria, 13 out of 16 alcohol-exposed animals were sign trackers and 10 out of 15 control animals were sign trackers. Of the remaining eight animals, three alcohol-exposed and five controls had response bias scores that fell between −0.70 and +0.70 and therefore did not show a strong enough response to be classified as sign or goal trackers.

The Effect of Adolescent Alcohol Intake on Conditioned Reinforcement in Adulthood

All animals underwent the test of conditioned reinforcement following pavlovian training. However, given that this task measures the effectiveness of the lever-CS as a reinforcer (defining a strong sign-tracker bias), we were primarily interested in the behavior of sign-tracking animals. Therefore, only the 23 animals designated as sign trackers based on the above mentioned criterion (>+0.70 response bias score) were kept in the analyses. One sign tracker from each treatment group was dropped because the incorrect active port was assigned for the conditioned reinforcement test yielding n = 12 alcohol sign-trackers and n = 9 control sign-trackers for the final analyses. During the test of conditioned reinforcement, alcohol rats displayed more incentive attribution to the lever-CS compared to control rats. Specifically, both treatment groups made more active than inactive nosepokes ($F_{(1,38)\ \text{port}} = 116.4$, $p < 0.0001$; **Figure 1D**), but sign trackers with a history of adolescent alcohol intake demonstrated significantly more nosepokes in the active port compared to controls ($F_{(1,38)\ \text{treatment} \times \text{port}} = 5.2$, $p < 0.05$; **Figure 1D**), with no difference between alcohol and control animals in the inactive port confirmed by follow-up tests ($p < 0.05$). Further, during the 2-s lever extension period following a poke in the active port, alcohol animals also had significantly more

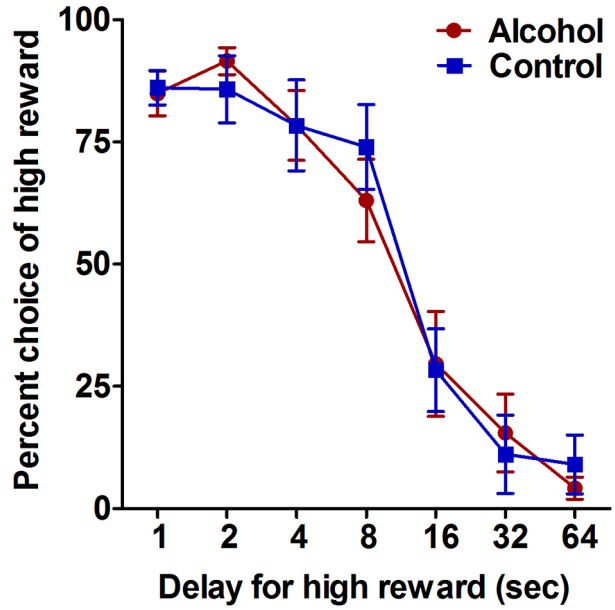


FIGURE 2 | Impulsive choice as measured by performance on the delay-discounting task. Alcohol-exposed (n = 7) and control (n = 6) animals both demonstrated a decrease in choice of the large reward option with increasing delay. Alcohol-exposed and control animals did not differ in choice behavior over delay conditions. All data are presented as mean ± SEM for the percent of trials on which the larger delayed reward was chosen for 1, 2, 4, 8, 16, 32 and 64 s delay intervals.

lever presses than control animals ($t_{(1,19)}$ = 2.4, p < 0.05; **Figure 3**). As expected, the alcohol (n = 3) and control (n = 5) rats that were not classified as sign trackers (response bias scores between −0.70 and +0.70) did not differ in total number of active or inactive nosepokes ($F_{(1,4)\ \text{treatment}}$ = 0.2, NS; $F_{(1,4)\ \text{treatment} \times \text{port}}$ = 0.02, NS; **Figure 1**). Taken together with the results from Pavlovian conditioned approach, our data indicate that the lever-CS acquired both the attractiveness and wanting properties of an incentive stimulus to a greater extent in the alcohol-exposed animals compared to controls.

The Effect of Adolescent Alcohol Intake on Impulsive Choice in Adulthood

The delay-discounting task was used to assess the effect of adolescent alcohol intake on impulsive choice in adulthood. Analysis of choice behavior on the delay-discounting task generated standard discounting curves for choice of the larger but delayed reward option over all conditions, with increasing delay of the large reward resulting in decreased choice of the large reward option for both alcohol-exposed and control animals ($F_{(6,66)\ \text{delay}}$ = 62.1, p < 0.0001; **Figure 2**). Alcohol-exposed and control animals did not differ in choice behavior over any of the delay conditions ($F_{(1,66)\ \text{treatment}}$ = 0.02, NS; $F_{(6,66)\ \text{delay} \times \text{treatment}}$ = 0.4, NS; **Figure 2**). These data indicate that moderate adolescent alcohol intake does not appear to alter impulsive choice in adulthood compared to animals without a history of adolescent alcohol.

The Effect of Adolescent Alcohol Intake on Impulsive Action in Adulthood

The DRL task was used to assess the effect of adolescent alcohol intake on impulsive action in adulthood, as measured by the ability of the animals to withhold responding for reinforcers during 5, 10, 20, and 30 s DRL intervals. Alcohol-exposed animals did not differ from controls on total number of lever presses ($F_{(3,54)\ \text{DRL} \times \text{treatment}}$ = 0.6, NS; $F_{(1,54)\ \text{treatment}}$ = 0.01, NS; **Figure 3A**) or total number of reinforces received ($F_{(3,54)\ \text{DRL} \times \text{treatment}}$ = 0.4, NS; $F_{(1,54)\ \text{treatment}}$ = 0.02, NS; **Figure 3B**) at any of the DRL intervals. Effective lever presses were also analyzed as a measure of the percent of lever presses that resulted in delivery of the reward. Alcohol treatment during adolescence did not alter the percent of effective lever presses ($F_{(3,54)\ \text{DRL} \times \text{treatment}}$ = 0.7, NS; $F_{(1,54)\ \text{treatment}}$ = 0.01, NS; **Figure 3C**) at any of the DRL intervals. Total lever presses ($F_{(3,54)\ \text{treatment}}$ = 24.9, p < 0.0001; **Figure 3A**), reinforcers ($F_{(3,54)\ \text{treatment}}$ = 246.8, p < 0.0001; **Figure 3B**), and effective

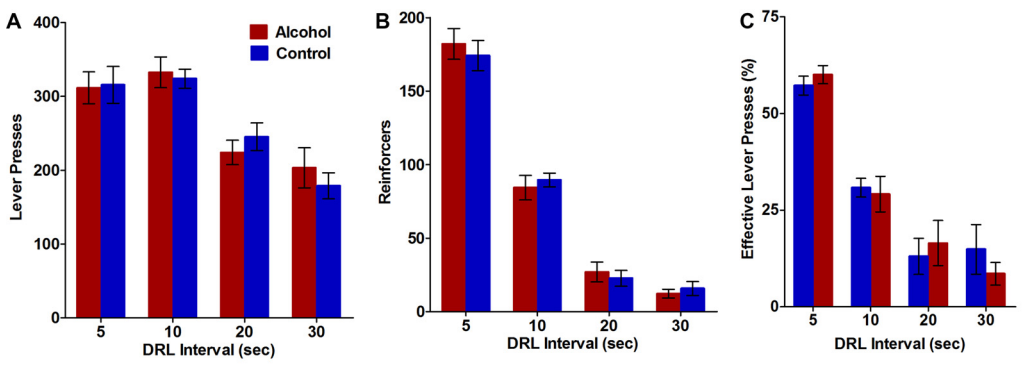


FIGURE 3 | Impulsive action as measured by performance on the differential reinforcement of low rates of responding (DRL) task. Alcohol-exposed (n = 9) animals did not differ from controls (n = 11) on **(A)** the total number of lever presses performed, **(B)** the total number of reinforcers received, or **(C)** the percentage of lever presses that resulted in reward delivery. All data are presented as mean ± SEM averaged across all 5-day blocks for the 5, 10, 20 and 30 s DRL intervals.

lever presses ($F_{(3,54)\ \text{treatment}} = 63.9$, $p < 0.0001$; **Figure 3C**) decreased with increasing DRL intervals, in both groups, suggesting animals in both groups were less able to withhold responding as the time to wait before responding increased. Thus, using our model of voluntary alcohol intake, adolescent alcohol use does not appear to differentially affect the ability of animals to withhold responding for a reward compared to controls.

DISCUSSION

Alcohol is the most commonly abused substance among adolescents and use during this sensitive developmental period is one of the best predictors of risk for development of alcohol use disorder (Grant et al., 2001). Maladaptive decision making strategies and altered reinforcement learning processes are two long-term consequences of adolescent alcohol use that may be contributing factors for alcohol problems later in life. Using a preclinical model of moderate voluntary adolescent alcohol intake, our laboratory has shown that alcohol use during this sensitive developmental period promotes risky decision making and alterations in incentive learning processes in adulthood, both of which are mirrored by perturbations in cue-evoked dopamine signaling (Nasrallah et al., 2011; Schindler et al., 2014; Spoelder et al., 2015). We have proposed that these two processes, risk-based decision making and incentive learning, are fundamentally linked through dysfunction of midbrain circuitry where inputs to the dopamine system are disrupted by adolescent alcohol use; with the latter potentially influencing performance on the former. The present work supports the behavioral predictions of this model. We show that alcohol-exposed animals attribute greater incentive value to a reward-predicting cue, demonstrated by increased approach to the cue and a greater willingness to work to receive the cue even in the absence of reward. Further, we show that animals exposed to alcohol during adolescence did not differ from controls in impulsive choice, impulsive action, or effortful choice. The findings presented here, in combination with previous work from our laboratory, support the proposition that early life alcohol use selectively modulates incentive learning processes and alters risk-based choice behavior (Clark et al., 2012; Schindler et al., 2014; Spoelder et al., 2015), but not other aspects of decision making including impulsive choice and behavioral inhibition.

Cues in an environment that repeatedly predict reward can attain value through a Pavlovian process referred to as stimulus-reward learning. In the realm of addiction, cues associated with drugs often attain enhanced incentive value and play a critical role in promoting drug craving, seeking and relapse following periods of abstinence (O'Brien et al., 1998; Shaham et al., 2003; Milton and Everitt, 2012). In preclinical research, animals classified as sign trackers show enhanced attribution of value to reward predicting cues, are more prone to display behaviors associated with drug-abuse, and are thought to model addiction-vulnerable populations in humans (Flagel et al., 2009, 2010; Spoelder et al., 2015). The value attributed to incentive stimuli can be defined by attractiveness, measured by approach to the stimulus, and wanting, measured by the animal's willingness to work to obtain the stimulus (Berridge et al., 2009). Consistent with our previous work (Spoelder et al., 2015), we confirm that voluntary adolescent alcohol exposure results in adult animals finding the reward-predicting cue more attractive, compared to control animals, as evidenced by a greater sign-tracking bias and increased approach to the lever-CS. Greater sign-tracking behavior in adulthood following a history of adolescent alcohol has also been shown with non-voluntary models of alcohol administration (McClory and Spear, 2014). Thus, exposure to alcohol during adolescence appears to consistently produce a phenotype associated with addiction-vulnerability. Further, we have previously shown that this greater attribution of value to reward-predictive cues may be dopamine dependent (Spoelder et al., 2015). We show that sign trackers with a history of adolescent alcohol use demonstrated a greater wanting of the cue by working harder than control sign trackers to obtain the lever-CS even in the absence of reward delivery. Alcohol-exposed animals also made significantly more lever presses following an active nosepoke during the 40 min conditioned reinforcement test, further demonstrating the greater attraction of the lever-CS for alcohol-exposed animals compared to controls. These data support evidence in both clinical and preclinical literature that alcohol use during adolescence, whether moderate or heavy, leads to alterations in incentive learning processes producing behaviors often apparent in addiction-prone individuals.

These findings further lend support to our hypothesis that maladaptive decision making under risk, a consequence of adolescent alcohol use, is a result of altered incentive learning. However, other underlying constructs such as altered valuation of primary rewards, deficits in reinforcement learning where corrupted value is assigned to specific cues and actions, and/or increased impulsivity, could also influence the risk-seeking phenotype. Thus, in a series of experiments begun in Clark et al., 2012 and completed in the current work we have attempted to parse the specific contribution of these constructs to maladaptive decision making in adults with a history of adolescent alcohol use. First, we asked if increased risk preference was due to altered reward valuation where the shape of an individual's utility curve is thought to determine choice under uncertainty (Clark et al., 2012). Indeed, in economic theory, risk attitude is attributed to this relationship between the objective value of a reward and its desirability (subjective value; Glimcher and Rustichini, 2004). Reward valuation was assessed by conducting progressive ratio experiments at different reward levels to estimate each subject's utility curve, and no differences between alcohol and control groups were found (Clark et al., 2012). We hypothesized, therefore, that increased risk preference can be attributed to a selective deficit in reward learning as probability discounting is a special case where subjects are asked to constantly alter the value they assign to a probabilistic option trial by trial through reinforcement learning. We showed this in a simple computational model and supported this hypothesis in further studies where we demonstrated that alcohol animals, consistent with human alcoholics, are more prone to risky choices in the gain domain and after wins rather than losses (Clark et al., 2012; Brevers et al., 2014; Schindler et al., 2016). Finally, in the current

work, we explore impulsivity as a potential contributor to the risk-seeking phenotype.

Deficits in impulse control are a key feature of alcohol use disorders, with higher levels of impulsivity predisposing to the development of alcohol problems later in life and increased likelihood of relapse following abstinence (Mitchell et al., 2005; Jupp and Dalley, 2014b; Stevens et al., 2014). Impulsive choice reflects a more cognitive aspect of impulsivity thought of as impulsive decision making, whereas impulsive action reflects an inability to inhibit a motor response thought of as behavioral inhibition (Evenden, 1999; Olmstead, 2006; Winstanley et al., 2010). Using delay discounting, we show that voluntary alcohol intake during adolescence had no effect on impulsive choice in adulthood, evidenced by similar discounting curves between alcohol-exposed and control animals across delay sessions. These results are consistent with another study that used a non-voluntary model of alcohol administration and found no effect of chronic intermittent ethanol exposure during adolescence on impulsive choice when tested later in adulthood (Mejia-Toiber et al., 2014). Adolescent alcohol exposure also did not alter the ability to withhold responding for a reward in the DRL task, indicating that moderate adolescent alcohol intake does not alter impulsive action in adulthood. Finally, the amount of work required to obtain a reward could also influence decision making under risk. Less is known about the relationship between effortful decision making and alcohol use disorders, but our data show that moderate alcohol use during adolescence does not appear to influence effortful decision making in adulthood (Supplementary Figure S1).

Previous studies have explored the relationship between probability discounting, delay discounting, incentive learning, sign-tracking behavior and addiction-prone phenotypes with varying results on how these behaviors cluster (Flagel et al., 2010; Lovic et al., 2011). For instance, Lovic et al. (2011) found that sign trackers displayed greater impulsive action, but not choice, compared to goal trackers. (Flagel et al. (2010); Flagel et al. (2011)) found that rats selectively bred for response to novelty, a phenotype associated with addiction, are sign trackers that also show greater impulsive action but not choice, and greater incentive value attribution but no difference in risky decision making. Our work demonstrates that alterations in decision making under risk and incentive value attribution cluster together as a result of alcohol use during adolescence. Given that we have shown the pharmacological attenuation of risky behavior (Schindler et al., 2016), future studies examining if this same drug attenuates the enhanced attribution of value to reward-predicting cues would confirm an overlapping mechanism between these behaviors that appear to cluster. Together with our previous work, we can begin to outline an emerging profile of the selective effects of moderate adolescent alcohol use on decision making, with apparent perturbations in risk-based, but not delay- or effort-based, decision making.

In addition to delay discounting, the probability-discounting task has also been used as a measure of impulsive choice (Flagel et al., 2010), with some researchers attributing performance on tasks involving decision making under risk to features of impulsivity (Richards et al., 1999). However, emerging clinical and preclinical data indicate that performance on probability-discounting and delay-discounting tasks are dissociable neurobiologically (van Gaalen et al., 2006; St Onge and Floresco, 2009), genetically (Flagel et al., 2010) and by age (Olson et al., 2007). Our data are consistent with and add to this emerging literature by showing that moderate adolescent alcohol use alters probability but not delay discounting in adulthood. It is interesting to speculate how these results may support a differential sensitivity of the underlying neurobiological mechanisms of probability and delay discounting, to alcohol during development. The pathway that mediates decision making under risk appears to be sensitive to moderate levels of alcohol during the adolescent time period, whereas the pathway that mediates either feature of impulsivity is not (Schindler et al., 2014, 2016). We should consider that impulsivity later in life could be altered by adolescent exposure, but heavy or excessive alcohol use may be required to see an effect. Nevertheless, the system that mediates risky behavior appears to be highly sensitive to even moderate levels of alcohol during development identifying it as an important target. Future studies examining the role of impulsivity and risk in substance use disorders should therefore treat delay and probability discounting as separable tasks that can provide unique information about the relationship between adolescent drug use and later decision making deficits.

Taken together with our previous work, the findings presented here support our hypothesis that the compromised encoding of Pavlovian cues following alcohol exposure in adolescence may underlie suboptimal decision making strategies in adulthood. Our data additionally support delay- and probability-discounting as dissociable decision-making paradigms, and confirm that impulsivity is not likely an explanation for maladaptive decision making under risk in adulthood seen following adolescent alcohol use. One limitation of the present set of experiments is that only the effect of adolescent alcohol intake, but not adult alcohol intake, on incentive value attribution and impulsivity in adulthood was assessed. Thus, we cannot state that the results are specific to adolescent intake. However, we have previously demonstrated that moderate alcohol intake during adulthood does not alter risky decision making (Schindler et al., 2014). Taken together with our previous work (Nasrallah et al., 2011; Spoelder et al., 2015; Schindler et al., 2016), we believe these effects are a result of alcohol on the developing mesolimbic dopamine system and would not expect those changes to occur with adult alcohol exposure. Aberrant changes in risk-based decision making and incentive learning following adolescent alcohol use are associated with perturbations in mesolimbic dopamine neurotransmission enduring into adulthood and the combination of such neurobiological and behavioral deficits may serve as critical vulnerabilities to the development of substance use disorders later in life.

AUTHOR CONTRIBUTIONS

LCK performed most of the studies, analyzed the data. LCK and JJC wrote the manuscript. JJC, AGS and LCK conceived the studies and edited the manuscript. RGW performed the

delay discounting study. SJW performed analyses for the delay data.

FUNDING

This work was funded by National Institutes of Health (NIH) grants AA021121 (JJC) and AA024599 (JJC). These grants were both awarded by NIAAA to conduct work into adolescent alcohol use and abuse, a major public health concern throughout the world.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnbeh.2017.00134/full#supplementary-material

REFERENCES

Bava, S., and Tapert, S. F. (2010). Adolescent brain development and the risk for alcohol and other drug problems. *Neuropsychol. Rev.* 20, 398–413. doi: 10.1007/s11065-010-9146-6

Berridge, K. C., Robinson, T. E., and Aldridge, J. W. (2009). Dissecting components of reward: 'liking', 'wanting' and learning. *Curr. Opin. Pharmacol.* 9, 65–73. doi: 10.1016/j.coph.2008.12.014

Boutros, N., Semenova, S., Liu, W., Crews, F. T., and Markou, A. (2014). Adolescent intermittent ethanol exposure is associated with increased risky choice and decreased dopaminergic and cholinergic neuron markers in adult rats. *Int. J. Neuropsychopharmacol.* 18:pyu003. doi: 10.1093/ijnp/pyu003

Brevers, D., Bechara, A., Cleeremans, A., Kornreich, C., Verbanck, P., and Noël, X. (2014). Impaired decision-making under risk in individuals with alcohol dependence. *Alcohol. Clin. Exp. Res.* 38, 1924–1931. doi: 10.1111/acer.12447

Casey, B. J., and Jones, R. M. (2010). Neurobiology of the adolescent brain and behavior: implications for substance use disorders. *J. Am. Acad. Child Adolesc. Psychiatry* 49, 1189–1201; quiz 1285. doi: 10.1016/j.jaac.2010.08.017

Chambers, R. A., Taylor, J. R., and Potenza, M. N. (2003). Developmental neurocircuitry of motivation in adolescence: a critical period of addiction vulnerability. *Am. J. Psychiatry* 160, 1041–1052. doi: 10.1176/appi.ajp.160.6.1041

Clark, J. J., Nasrallah, N. A., Hart, A. S., Collins, A. L., Bernstein, I. L., and Phillips, P. E. (2012). Altered risk-based decision making following adolescent alcohol use results from an imbalance in reinforcement learning in rats. *PLoS One* 7:e37357. doi: 10.1371/journal.pone.0037357

Collins, F. S., and Tabak, L. A. (2014). Policy: NIH plans to enhance reproducibility. *Nature* 505, 612–613. doi: 10.1038/505612a

Crews, F., He, J., and Hodge, C. (2007). Adolescent cortical development: a critical period of vulnerability for addiction. *Pharmacol. Biochem. Behav.* 86, 189–199. doi: 10.1016/j.pbb.2006.12.001

Day, J. J., Jones, J. L., Wightman, R. M., and Carelli, R. M. (2010). Phasic nucleus accumbens dopamine release encodes effort- and delay-related costs. *Biol. Psychiatry* 68, 306–309. doi: 10.1016/j.biopsych.2010.03.026

Evenden, J. L. (1999). Varieties of impulsivity. *Psychopharmacology* 146, 348–361. doi: 10.1007/pl00005481

Everitt, B. J., and Robbins, T. W. (2005). Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nat. Neurosci.* 8, 1481–1489. doi: 10.1038/nn1579

Flagel, S. B., Akil, H., and Robinson, T. E. (2009). Individual differences in the attribution of incentive salience to reward-related cues: implications for addiction. *Neuropharmacology* 56, 139–148. doi: 10.1016/j.neuropharm.2008.06.027

Flagel, S. B., Clark, J. J., Robinson, T. E., Mayo, L., Czuj, A., Willuhn, I., et al. (2011). A selective role for dopamine in stimulus-reward learning. *Nature* 469, 53–57. doi: 10.1038/nature09588

Flagel, S. B., Robinson, T. E., Clark, J. J., Clinton, S. M., Watson, S. J., Seeman, P., et al. (2010). An animal model of genetic vulnerability to behavioral disinhibition and responsiveness to reward-related cues: implications for addiction. *Neuropsychopharmacology* 35, 388–400. doi: 10.1038/npp.2009.142

Floresco, S. B., Tse, M. T., and Ghods-Sharifi, S. (2008). Dopaminergic and glutamatergic regulation of effort- and delay-based decision making. *Neuropsychopharmacology* 33, 1966–1979. doi: 10.1038/sj.npp.1301565

Ghods-Sharifi, S., and Floresco, S. B. (2010). Differential effects on effort discounting induced by inactivations of the nucleus accumbens core or shell. *Behav. Neurosci.* 124, 179–191. doi: 10.1037/a0018932

Glimcher, P. W., and Rustichini, A. (2004). Neuroeconomics: the consilience of brain and decision. *Science* 306, 447–452. doi: 10.1126/science.1102566

Goudriaan, A. E., Grekin, E. R., and Sher, K. J. (2007). Decision making and binge drinking: a longitudinal study. *Alcohol. Clin. Exp. Res.* 31, 928–938. doi: 10.1111/j.1530-0277.2007.00378.x

Grant, B. F., Stinson, F. S., and Harford, T. C. (2001). Age at onset of alcohol use and DSM-IV alcohol abuse and dependence: a 12-year follow-up. *J. Subst. Abuse* 13, 493–504. doi: 10.1016/s0899-3289(01)00096-7

Green, L., and Myerson, J. (2013). How many impulsivities? A discounting perspective. *J. Exp. Anal. Behav.* 99, 3–13. doi: 10.1002/jeab.1

Johnson, C. A., Xiao, L., Palmer, P., Sun, P., Wang, Q., Wei, Y., et al. (2008). Affective decision-making deficits, linked to a dysfunctional ventromedial prefrontal cortex, revealed in 10th grade Chinese adolescent binge drinkers. *Neuropsychologia* 46, 714–726. doi: 10.1016/j.neuropsychologia.2007.09.012

Jupp, B., and Dalley, J. W. (2014a). Behavioral endophenotypes of drug addiction: etiological insights from neuroimaging studies. *Neuropharmacology* 76, 487–497. doi: 10.1016/j.neuropharm.2013.05.041

Jupp, B., and Dalley, J. W. (2014b). Convergent pharmacological mechanisms in impulsivity and addiction: insights from rodent models. *Br. J. Pharmacol.* 171, 4729–4766. doi: 10.1111/bph.12787

Kelley, A. E. (2004). Memory and addiction: shared neural circuitry and molecular mechanisms. *Neuron* 44, 161–179. doi: 10.1016/j.neuron.2004.09.016

Leblond, M., Sukharnikova, T., Yu, C., Rossi, M. A., and Yin, H. H. (2014). The role of pedunculopontine nucleus in choice behavior under risk. *Eur. J. Neurosci.* 39, 1664–1670. doi: 10.1111/ejn.12529

Littlefield, A. K., and Sher, K. J. (2010). The multiple, distinct ways that personality contributes to alcohol use disorders. *Soc. Personal. Psychol. Compass* 4, 767–782. doi: 10.1111/j.1751-9004.2010.00296.x

Lovic, V., Saunders, B. T., Yager, L. M., and Robinson, T. E. (2011). Rats prone to attribute incentive salience to reward cues are also prone to impulsive action. *Behav. Brain Res.* 223, 255–261. doi: 10.1016/j.bbr.2011.04.006

Marinelli, M., and McCutcheon, J. E. (2014). Heterogeneity of dopamine neuron activity across traits and states. *Neuroscience* 282, 176–197. doi: 10.1016/j.neuroscience.2014.07.034

McClory, A. J., and Spear, L. P. (2014). Effects of ethanol exposure during adolescence or in adulthood on Pavlovian conditioned approach in Sprague-Dawley rats. *Alcohol* 48, 755–763. doi: 10.1016/j.alcohol.2014.05.006

McMurray, M. S., Amodeo, L. R., and Roitman, J. D. (2014). Effects of voluntary alcohol intake on risk preference and behavioral flexibility during rat adolescence. *PLoS One* 9:e100697. doi: 10.1371/journal.pone.0100697

Mejia-Toiber, J., Boutros, N., Markou, A., and Semenova, S. (2014). Impulsive choice and anxiety-like behavior in adult rats exposed to chronic intermittent ethanol during adolescence and adulthood. *Behav. Brain Res.* 266, 19–28. doi: 10.1016/j.bbr.2014.02.019

Meyer, P. J., Lovic, V., Saunders, B. T., Yager, L. M., Flagel, S. B., Morrow, J. D., et al. (2012). Quantifying individual variation in the propensity to attribute incentive salience to reward cues. *PLoS One* 7:e38987. doi: 10.1371/journal.pone.0038987

Milton, A. L., and Everitt, B. J. (2012). The persistence of maladaptive memory: addiction, drug memories and anti-relapse treatments. *Neurosci. Biobehav. Rev.* 36, 1119–1139. doi: 10.1016/j.neubiorev.2012.01.002

Mitchell, J. M., Fields, H. L., D'Esposito, M., and Boettiger, C. A. (2005). Impulsive responding in alcoholics. *Alcohol. Clin. Exp. Res.* 29, 2158–2169. doi: 10.1097/01.alc.0000191755.63639.4a

Nasrallah, N. A., Clark, J. J., Collins, A. L., Akers, C. A., Phillips, P. E., and Bernstein, I. L. (2011). Risk preference following adolescent alcohol use is

associated with corrupted encoding of costs but not rewards by mesolimbic dopamine. *Proc. Natl. Acad. Sci. U S A* 108, 5466–5471. doi: 10.1073/pnas.1017732108

O'Brien, C. P., Childress, A. R., Ehrman, R., and Robbins, S. J. (1998). Conditioning factors in drug abuse: can they explain compulsion? *J. Psychopharmacol.* 12, 15–22. doi: 10.1177/026988119801200103

Olmstead, M. C. (2006). Animal models of drug addiction: where do we go from here? *Q. J. Exp. Psychol.* 59, 625–653. doi: 10.1080/17470210500356308

Olson, E. A., Hooper, C. J., Collins, P., and Luciana, M. (2007). Adolescents' performance on delay and probability discounting tasks: contributions of age, intelligence, executive functioning, and self-reported externalizing behavior. *Pers. Individ. Dif.* 43, 1886–1897. doi: 10.1016/j.paid.2007.06.016

Pan, W. X., and Hyland, B. I. (2005). Pedunculopontine tegmental nucleus controls conditioned responses of midbrain dopamine neurons in behaving rats. *J. Neurosci.* 25, 4725–4732. doi: 10.1523/JNEUROSCI.0277-05.2005

Pascual, M., Boix, J., Felipo, V., and Guerri, C. (2009). Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. *J. Neurochem.* 108, 920–931. doi: 10.1111/j.1471-4159.2008.05835.x

Richards, J. B., Zhang, L., Mitchell, S. H., and de Wit, H. (1999). Delay or probability discounting in a model of impulsive behavior: effect of alcohol. *J. Exp. Anal. Behav.* 71, 121–143. doi: 10.1901/jeab.1999.71-121

Rowland, N. E., Nasrallah, N., and Robertson, K. L. (2005). Accurate caloric compensation in rats for electively consumed ethanol-beer or ethanol-polycose mixtures. *Pharmacol. Biochem. Behav.* 80, 109–114. doi: 10.1016/j.pbb.2004.10.010

Salamone, J. D., and Correa, M. (2012). The mysterious motivational functions of mesolimbic dopamine. *Neuron* 76, 470–485. doi: 10.1016/j.neuron.2012.10.021

Schindler, A. G., Soden, M. E., Zweifel, L. S., and Clark, J. J. (2016). Reversal of alcohol-induced dysregulation in dopamine network dynamics may rescue maladaptive decision-making. *J. Neurosci.* 36, 3698–3708. doi: 10.1523/JNEUROSCI.4394-15.2016

Schindler, A. G., Tsutsui, K. T., and Clark, J. J. (2014). Chronic alcohol intake during adolescence, but not adulthood, promotes persistent deficits in risk-based decision making. *Alcohol. Clin. Exp. Res.* 38, 1622–1629. doi: 10.1111/acer.12404

Shaham, Y., Shalev, U., Lu, L., De Wit, H., and Stewart, J. (2003). The reinstatement model of drug relapse: history, methodology and major findings. *Psychopharmacology* 168, 3–20. doi: 10.1007/s00213-002-1224-x

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* 24, 417–463. doi: 10.1016/s0149-7634(00)00014-2

Spoelder, M., Tsutsui, K. T., Lesscher, H. M., Vanderschuren, L. J., and Clark, J. J. (2015). Adolescent alcohol exposure amplifies the incentive value of reward-predictive cues through potentiation of phasic dopamine signaling. *Neuropsychopharmacology* 40, 2873–2885. doi: 10.1038/npp.2015.139

Stevens, L., Verdejo-Garcia, A., Goudriaan, A. E., Roeyers, H., Dom, G., and Vanderplasschen, W. (2014). Impulsivity as a vulnerability factor for poor addiction treatment outcomes: a review of neurocognitive findings among individuals with substance use disorders. *J. Subst. Abuse Treat.* 47, 58–72. doi: 10.1016/j.jsat.2014.01.008

St Onge, J. R., and Floresco, S. B. (2009). Dopaminergic modulation of risk-based decision making. *Neuropsychopharmacology* 34, 681–697. doi: 10.1038/npp.2008.121

van Gaalen, M. M., van Koten, R., Schoffelmeer, A. N., and Vanderschuren, L. J. (2006). Critical involvement of dopaminergic neurotransmission in impulsive decision making. *Biol. Psychiatry* 60, 66–73. doi: 10.1016/j.biopsych.2005.06.005

Winstanley, C. A., Olausson, P., Taylor, J. R., and Jentsch, J. D. (2010). Insight into the relationship between impulsivity and substance abuse from studies using animal models. *Alcohol. Clin. Exp. Res.* 34, 1306–1318. doi: 10.1111/j.1530-0277.2010.01215.x

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: 17 August 2017
doi: 10.3389/fnbeh.2017.00153

Chronic Nicotine Exposure Initiated in Adolescence and Unpaired to Behavioral Context Fails to Enhance Sweetened Ethanol Seeking

*Aric C. Madayag[1,2†], Kyle S. Czarnecki[1†], Lynde M. Wangler[1] and Donita L. Robinson[1,3]**

[1]Bowles Center for Alcohol Studies, University of North Carolina, Chapel Hill, NC, United States, [2]Department of Pharmacology, University of North Carolina, Chapel Hill, NC, United States, [3]Department of Psychiatry, University of North Carolina, Chapel Hill, NC, United States

Nicotine use in adolescence is pervasive in the United States and, according to the Gateway Hypothesis, may lead to progression towards other addictive substances. Given the prevalence of nicotine and ethanol comorbidity, it is difficult to ascertain if nicotine is a gateway drug for ethanol. Our study investigated the relationship between adolescent exposure to nicotine and whether this exposure alters subsequent alcohol seeking behavior. We hypothesized that rats exposed to nicotine beginning in adolescence would exhibit greater alcohol seeking behavior than non-exposed siblings. To test our hypothesis, beginning at P28, female rats were initially exposed to once daily nicotine (0.4 mg/kg, SC) or saline for 5 days. Following these five initial injections, animals were trained to nose-poke for sucrose reinforcement (10%, w/v), gradually increasing to sweetened ethanol (10% sucrose; 10% ethanol, w/v) on an FR5 reinforcement schedule. Nicotine injections were administered after the behavioral sessions to minimize acute effects of nicotine on operant self-administration. We measured the effects of nicotine exposure on the following aspects of ethanol seeking: self-administration, naltrexone (NTX)-induced decreases, habit-directed behavior, motivation, extinction and reinstatement. Nicotine exposure did not alter self-administration or the effectiveness of NTX to reduce alcohol seeking. Nicotine exposure blocked habit-directed ethanol seeking. Finally, nicotine did not alter extinction learning or cue-induced reinstatement to sweetened ethanol seeking. Our findings suggest that nicotine exposure outside the behavioral context does not escalate ethanol seeking. Further, the Gateway Hypothesis likely applies to scenarios in which nicotine is either self-administered or physiologically active during the behavioral session.

Keywords: alcohol use disorder, adolescent, addiction, cigarette smoking, tobacco

OPEN ACCESS

Edited by:
Jacqueline M. Barker, Medical University of South Carolina, United States

Reviewed by:
Sheketha R. Hauser, Indiana University School of Medicine, United States
Jee Hyun Kim, Florey Institute of Neuroscience and Mental Health, Australia
Jean-Marie N. Maddux, Lake Forest College, United States

***Correspondence:**
Donita L. Robinson
dlr@unc.edu

†These authors have contributed equally to this work.

***Received:** 30 April 2017*
***Accepted:** 02 August 2017*
***Published:** 17 August 2017*

Citation:
Madayag AC, Czarnecki KS, Wangler LM and Robinson DL (2017) Chronic Nicotine Exposure Initiated in Adolescence and Unpaired to Behavioral Context Fails to Enhance Sweetened Ethanol Seeking. Front. Behav. Neurosci. 11:153. doi: 10.3389/fnbeh.2017.00153

INTRODUCTION

Conventional cigarette use among youth is in steady decline; however, novel modes of consumption have arisen and are more readily available, potentially leading to increased levels of nicotine exposure. In 2016, according to the National Institutes of Health "Monitoring the Future Survey 2016", the percent of youth using conventional cigarettes in the past month among 8th, 10th and 12th grades was 2.6%, 4.9% and 10.5% respectively. Conversely, e-cigarette use encompassed 6.2%,

11.0% and 12.5% at the same grade level. These statistics argue that consumption of nicotine by conventional tobacco sources, e-cigarettes, or smokeless tobacco may pose a health threat to the future of the youth in the United States. In turn, alcohol use among adolescents is widespread, with over 35% of youth grades 8–12 in the United States reporting regular consumption and approximately 15% reporting heavy drinking or binge-drinking episodes (Bachman, 2002). Binge-level alcohol consumption is associated with elevated risk for numerous adverse outcomes including drug abuse susceptibility (Brown et al., 2000). Despite the prevalence of nicotine and ethanol use during adolescence, and their comorbidity, it is unknown if the two substances interact directly or through long-term physiological mechanisms to elicit a synergistic behavioral response. With apparent ease of access to nicotine and ethanol, the aim of this study was to determine if adolescents that are exposed to nicotine are also more susceptible to escalate ethanol seeking.

Preclinical studies suggest that adolescent animals are prone to nicotine and alcohol use. Compared to adults, adolescent rodents exhibit a higher preference for nicotine (Torres et al., 2009; Nesil et al., 2011) and higher rewarding effects of nicotine (Shram and Lê, 2010), though lower operant responding for nicotine (Shram et al., 2008; Schassburger et al., 2016). In turn, adolescent rodents show greater propensity to self-administer ethanol (Tambour et al., 2008; Walker and Ehlers, 2009; Doherty and Gonzales, 2015; Serlin and Torregrossa, 2015) and a higher preference for ethanol over water control (Truxell et al., 2007; Melendez, 2011). These findings collectively suggest adolescents are more prone to substance use and potential abuse, which could render them susceptible to addiction in adulthood. The Common Liability Hypothesis (Palmer et al., 2012) posits that underlying genetic or environmental predispositions are the important factors leading to addiction, and the developmental preference for addictive drugs during adolescence may fall into a "predisposition," albeit developmentally limited. Alternatively, the Gateway Hypothesis (Kandel et al., 1992; Degenhardt et al., 2010) theorizes that drug exposure "primes" the brain in such a way as to promote later addiction to a variety of drugs. Discerning the respective contributions of drug exposure, genetic, and environmental factors has proved to be difficult in human studies due to the inability to manipulate such variables ethically.

Nicotine has been suggested as a potent gateway drug (Kandel and Kandel, 2014). Specifically, animal studies found that nicotine produces molecular, physiological and behavioral changes to render higher susceptibility to later cocaine use (Levine et al., 2011). However, it is not clear whether nicotine use during adolescence—when many people initiate drug use—is associated with more profound "gateway" effects towards ethanol. Studies indicate that animals will escalate ethanol self-administration when it is co-administered with nicotine (Lárraga et al., 2017), suggesting that nicotine exposure escalates ethanol seeking. In Pavlovian-based tasks, when given concurrently with the initiation of a conditioned approach session where ethanol is the unconditioned stimulus, nicotine enhances approach behavior, though not when administered repeatedly in days leading up to the beginning of behavior sessions (Maddux and Chaudhri, 2017). Moreover, we and others have observed that nicotine is sufficient to elevate conditioned responding to cues predicting sucrose or water reward (Olausson et al., 2004a,b; Stringfield et al., 2017), suggesting non-specific elevation of conditioned responding. It is unknown if exposure to nicotine *prior to* ethanol self-administration sessions can lead to such an escalation of ethanol seeking, as would be indicated by the Gateway Hypothesis for nicotine (Kandel and Kandel, 2014).

Consistent with the Gateway Hypothesis for nicotine, we hypothesized that exposure to nicotine in adolescence would increase the rewarding and motivational properties of ethanol. To test this, we exposed adolescent female rats to daily nicotine injections and tested operant responding for sweetened ethanol under multiple conditions to ascertain ethanol seeking, habit-directed behavior, motivation for ethanol and extinction-reinstatement. Nicotine exposure continued through the operant training and testing. Notably, operant sessions and nicotine administration were separated in time so that any changes in behavior between groups would be due to nicotine exposure and associated neuroplasticity rather than acute effects.

MATERIALS AND METHODS

Subjects

Eighteen Sprague-Dawley female rats were bred at the University of North Carolina at Chapel Hill and maintained under a 12:12 h light:dark cycle, lights on from 07:00 to 19:00. Animals were weaned into pair-housed cages on postnatal day (P) 21 with *ad libitum* access to water and standard chow except when indicated. All experimental procedures were performed during the light cycle between 09:00 and 12:00. This study was carried out in accordance with the recommendations of University of North Carolina Division of Laboratory Animal Medicine. The protocol was approved by the Institutional Animal Care and Use Committee of the University of North Carolina. The dataset consisted of four litters grouped into three behavior cohorts. The following is a breakdown of experimental group distribution: cohort 1, 4 saline and 4 nicotine; cohort 2, 2 saline and 4 nicotine; cohort 3, 2 saline and 2 nicotine. Every animal performed the entirety of behavior experiments.

Drugs

Nicotine hydrogen tartrate (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 0.9% saline and pH adjusted to 7.0 $\pm$ 0.2 via NaOH solution. Nicotine was administered via subcutaneous injection at 0.4 mg/kg, calculated using the free base form (Palmatier et al., 2013; Stringfield et al., 2017). Ethanol (95% Decon Labs, King of Prussia, PA, USA) was diluted to concentrations of up to 10% (w/v) in 10% sucrose as previously described (Shnitko and Robinson, 2015). Naltrexone (NTX) HCl (Sigma Aldrich) was dissolved in sterile saline. All injections delivered 1 ml/kg volume.

Operant Acquisition and Maintenance

The experimental timeline is outlined in **Table 1**. Rats were administered saline or nicotine, with cage-mates assigned the

TABLE 1 | Experimental timeline.

Age range at start of epoch	Experimental epoch	
P28	Daily nicotine injections for 5 days	
P33	**Operant training**	
	Sessions 1–3:	*FR1, 10S (water restricted)*
	Session 4:	*FR1, 10S*[a]
	Sessions 5, 6:	*FR1, 10S/2.5E*
	Sessions 7, 8:	*FR1, 10S/5E*
	Session 9:	*FR1, 10S/10E*
	Sessions 10:	*FR3, 10S/10E*
	Sessions 11–21:	*FR5, 10S/10E*
P57–P61	Naltrexone dose-response sessions	
	Maintenance sessions, FR5, 10S/10E	
P76–P90	Satiety-specific devaluation session	
	Maintenance sessions, FR5, 10S/10E	
P84–P95	Progressive ratio session	
P85–P96	12 extinction sessions	
P97–P108	Reinstatement session	

[a] *4/18 rats did not undergo this session (2 saline and 2 nicotine rats).*

same treatment to minimize differences in behavior within a cage after drug exposure. Starting P28, animals received daily saline or nicotine injections for five consecutive days prior to the first behavior session. Behavioral experiments were performed in 12.0″ × 9.5″ × 8.25″ operant chambers (Med Associates, Inc., St. Albans, VT, USA). Beam break detectors were used to measure a nosepoke response. A cue light was located above the reward delivery well to indicate the availability to retrieve the reward. Animals underwent one session per day for 6–7 days per week. To avoid acute effects of nicotine on operant behavior (Chaudhri et al., 2005), nicotine or saline injections were made 2 h after each training session.

To encourage acquisition of operant responding for reward, on P34 animals were water restricted the day prior and concurrently with the three initial days of nosepoke training; one nosepoke resulted in concurrent delivery of a cue light and a tone for 3.5 s, 100 μL of reward solution, and a 20 s time out. Nosepoke training consisted of 100 μL of 10% sucrose reward (10S) on an FR1 schedule of reinforcement; each session lasted 2–3 h, immediately followed by home cage access to water for 1 h. After the third session, animals returned to *ad libitum* water access in their home cages for the duration of the study. Next, each phase of training took place during 20–30 min sessions as described in **Table 1** (reinforcement schedule-reward): FR1-10S; FR1-10S/2.5E (10% sucrose, 2.5% ethanol, both w/v); FR1-10S/5E; FR1-10S/10E; FR3-10S/10E. The age of first exposure to ethanol (10% sucrose, 2.5% ethanol) was P37–38. Following this schedule, animals were maintained on 30-min sessions of an FR5 schedule of reinforcement with 10S/10E reward. Prior to experimental manipulations below, each animal underwent 11 maintenance sessions on FR5-10S/10E. Note that sweetened alcohol was used to promote rapid acquisition of ethanol seeking during the short period of rodent adolescence (approximately P28–P42).

Naltrexone

Each animal received 0, 0.3, 1 and 3 mg/kg (SC) NTX 30 min prior to a standard FR5-10S/10E self-administration session. The doses were counterbalanced such that every animal received every dose, but also equal numbers of rats from each group received the same dose order. Animals underwent two maintenance self-administration sessions between NTX sessions to allow wash-out of the prior NTX dose and to re-establish baseline behavior.

Satiety Specific Devaluation

Next we assessed habitual vs. goal-directed reward seeking with the satiety specific devaluation as previously described (Hay et al., 2013; Shillinglaw et al., 2014), except in the present study we used a between-subjects approach. After the last NTX session, over 3 days leading up to the devaluation session, animal pairs were separated in their home cage by a plastic divider and allowed to drink water from inserted water bottles freely for 60 min prior to standard maintenance sessions; this habituated the rats to the separation and bottle placement. On the test day, animals were separated and allowed to freely drink either 2% maltodextrin solution (control) or 10S/10E solution (devaluation) in a between-subjects design in the 60 min prior to behavioral session. The devaluation test session consisted of a 10-min operant session during which nosepokes were recorded but cue and reward reinforcements were absent.

Progressive Ratio

Following devaluation, animals underwent 4 days of maintenance self-administration sessions to re-establish baseline behavior. Animals were then assessed for motivation for sweetened ethanol with a progressive ratio schedule of reinforcement as previously described (Walker and Koob, 2007). Animals were placed in the operant conditioning boxes and the number of responses required for reinforcement increased according to the schedule of 1, 1, 2, 2, 3, 3, 4, 4, 5, 5, 7, 7, 9, 9, 11, 11, 13, 13, 15, 15, 18, 18, 21, 21, 24, 24, etc. The session ended after an animal failed to respond for 30 min with a maximum session of 3 h. Nosepokes and "breakpoint" was recorded. The "breakpoint" was the last level of required responding achieved by an animal prior to the end of the session.

Extinction and Reinstatement

Following the progressive ratio session, with no maintenance sessions between, animals underwent 12 30-min extinction sessions in which they were placed in the operant boxes and nosepokes were recorded but cues and rewards were not delivered. After the last extinction session, animals underwent a 30-min extinction-reinstatement session that began as an extinction session, where cues and reward reinforcements were not delivered. Ten minutes into the session, a single cue delivery consisting of cue light and tone for 3.5 s was delivered. Thereafter, the conditioned (cue) reinforcements were delivered on an

FR5 schedule but no reward was delivered, similar to previous studies (Bienkowski et al., 2000; Hay et al., 2013).

Statistical Analysis

All figures were generated using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Statistical analyses were performed using Statistica Software (Statistica, Tulsa, OK, USA). Most experiments were analyzed using two-way ANOVA with session, time bin, or dose as repeated measures and exposure history (saline or nicotine) as the between-subjects factor. Satiety-induced reward devaluation analysis was performed as a two-way ANOVA with no repeated measure. Bonferroni corrected *t*-tests were performed for *post hoc* analyses as appropriate. Progressive ratio analyses were performed as independent sample *t*-tests.

RESULTS

Acquisition and Maintenance of Self-Administration of Sweetened Ethanol

We first measured if nicotine exposure beginning during adolescence altered acquisition of operant self-administration of sweetened ethanol. The first four sessions were variable as the rats learned the operant response under water restriction and then were removed from water restriction. Thus, we compared the six sessions in which ethanol was gradually added to the sucrose solution. Nicotine injections 2 h after each behavioral session did not alter acquisition of sweetened ethanol self-administration (**Table 2**). A two-way repeated measures ANOVA yielded a main effect of session ($F_{(1,5)} = 7.35$, $p < 0.001$), but no significant effect of exposure ($F_{(1,5)} = 1.37$, $p = 0.26$) or session by exposure interaction ($F_{(1,5)} = 1.56$, $p = 0.18$). Collapsed across exposure group, *post hoc* analysis revealed that animals made significantly more nosepokes in the second session on FR1-10S/2.5E than in the first session on that schedule, and that animals made significantly fewer nosepokes in the session on FR1-10S10E than in the previous three sessions (Bonferroni *t*-test, $p < 0.05$).

We next measured whether saline and nicotine groups differed in their maintenance of operant behavior. Saline- and nicotine-exposed animals exhibited similar levels of self-administration as measured by nosepokes and sweetened ethanol consumed (**Figure 1**). A two-way ANOVA for nosepokes per session revealed a main effect of session ($F_{(10,160)} = 5.53$, $p < 0.0001$), no main effect of exposure ($F_{(1,16)} = 0.13$, $p = 0.72$) and no significant session by exposure interaction ($F_{(10,160)} = 0.66$, $p = 0.76$). Collapsed across exposure group, *post hoc* analysis showed significant increase in nosepokes in sessions 7 through 10 compared to the first session (Bonferroni *t*-test, $p < 0.05$). Similarly, a two-way ANOVA for ethanol consumed per session yielded a significant main effect of session ($F_{(10,160)} = 3.18$, $p < 0.001$), but no main effect of exposure ($F_{(1,16)} = 0.04$, $p = 0.85$), and no significant session-by-exposure interaction ($F_{(10,160)} = 0.91$, $p = 0.53$). Specifically, rats self-administered an average of 0.6–1.2 g/kg ethanol in a session. We also compared body weight during these sessions (data not shown), and detected the expected effect of session (weight gain over days; ($F_{(10,160)} = 156.3$, $p < 0.001$), but no significant effect of exposure ($F_{(1,16)} = 0.25$, $p = 0.62$) or interaction ($F_{(10,160)} = 0.19$, $p = 0.99$). Thus, exposure to nicotine starting in adolescence but administered after the operant sessions had no impact on self-administration of sweetened ethanol.

TABLE 2 | Acquisition of operant self-administration, after water restriction.

Session	Saline	Nicotine
FR1, 10S/2.5E	131.9 ± 23.7	82.3 ± 16.7
FR1 10S/2.5E[a]	155.6 ± 14.2	135.3 ± 12.7
FR1, 10S/5E	151.1 ± 18.5	112.8 ± 13.0
FR1, 10S/5E	146.3 ± 20.8	135.4 ± 14.9
FR1, 10S/10E[b]	78.5 ± 11.1	85.2 ± 10.4
FR3, 10S/10E	115.4 ± 21.8	116.2 ± 12.5

Data shown as mean nosepokes ± SEM. [a]Collapsed across exposure group, higher than the previous session. [b]Collapsed across exposure group, lower than the previous three sessions.

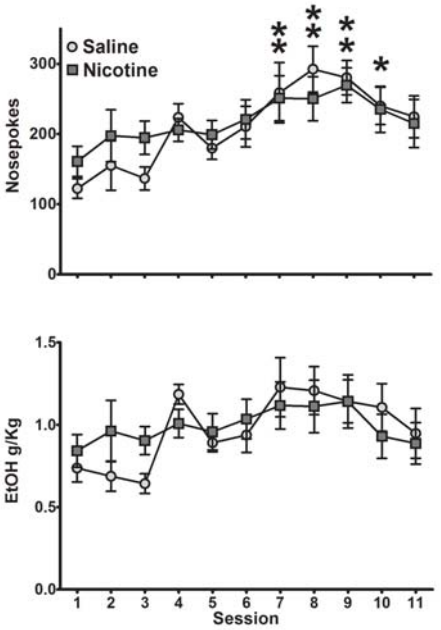


FIGURE 1 | Animals exposed to saline and nicotine self-administered sweetened ethanol similarly. Both nosepokes (Top) and consumption (Bottom) were not significantly different between exposure groups (Bonferroni *post hoc* *t*-test, collapsed across drug exposure groups: $^{*}p < 0.05$, $^{**}p < 0.01$ compared to first session; $N = 8$ saline, 10 nicotine).

Naltrexone Effects on Sweetened Ethanol Seeking

To determine if adolescent nicotine exposure affects the ability of NTX to alter ethanol seeking in animals, we administered a dose range of NTX that has been shown to differentially decrease ethanol vs. sucrose self-administration (Czachowski and Delory, 2009; Hay et al., 2013), using a within-subject design (**Figure 2**). A two-way ANOVA on nosepokes revealed a significant main effect of NTX ($F_{(3,48)} = 8.03$, $p < 0.001$), but no main effect

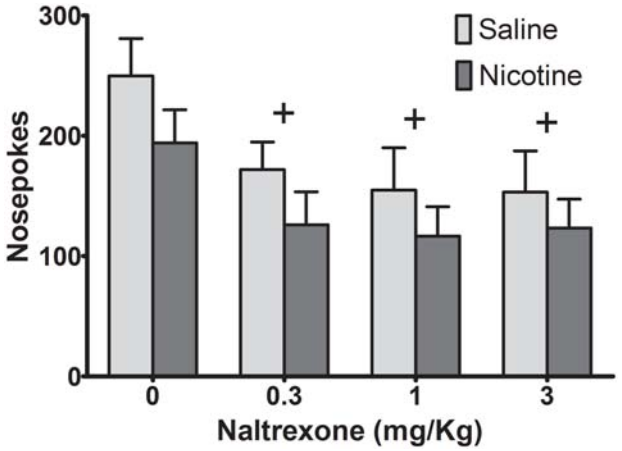


FIGURE 2 | Naltrexone (NTX) reduced operant behavior similarly in saline- and nicotine-exposed rats. While all doses of NTX decreased operant responding, no significant differences in responding were observed between groups (Bonferroni *post hoc t*-test, collapsed across drug exposure groups: $^{+}p < 0.01$ compared to vehicle; $N = 8$ saline, 10 nicotine).

of adolescent drug exposure history ($F_{(1,16)} = 1.81$, $p = 0.20$) and no significant dose-by-exposure interaction ($F_{(3,48)} = 0.15$, $p = 0.93$). *Post hoc* analysis collapsed across drug exposure history indicated significant decreases in sweetened ethanol seeking for all NTX doses compared to control ($p < 0.01$). Therefore, while NTX reduced sweetened ethanol self-administration, exposure to nicotine did not alter NTX's effects on ethanol seeking.

Satiety-Specific Reward Devaluation

Repeated exposure to nicotine can induce habitual nicotine seeking (Clemens et al., 2014). However, it is unknown if this inflexible behavior is generalizable to rewards other than nicotine. Thus, we used satiety-specific reward devaluation to determine if exposure to nicotine also renders an animal more susceptible to habitual behavior. Animals received *ad libitum* access to either 10S/10E or 2% maltodextrin control solution for 60 min prior to a 10 min extinction session (**Figure 3**). Saline-exposed animals did not alter reward seeking after 10S/10E devaluation compared to rats receiving the 2% maltodextrin control, indicating habitual reward seeking. In contrast, nicotine-exposed rats decreased operant responding following reward devaluation compared to rats under non-devalued conditions. A two-way ANOVA for nosepokes revealed no significant main effect of consumed solution ($F_{(1,14)} = 2.70$, $p = 0.12$), and no significant main effect of drug exposure ($F_{(1,14)} = 1.14$, $p = 0.30$), but a significant solution-by-exposure interaction ($F_{(1,14)} = 12.83$, $p < 0.01$). *Post hoc* analysis resulted in significant decrease in responding after 10S/10E devaluation compared to maltodextrin only in the nicotine-exposed rats ($p < 0.01$). This difference in reward seeking was not due to differences in liquid consumption during the 60-min access period, as there was no significant difference in volume of solution consumed prior to behavioral testing between exposure groups. A two-way ANOVA of liquid consumed

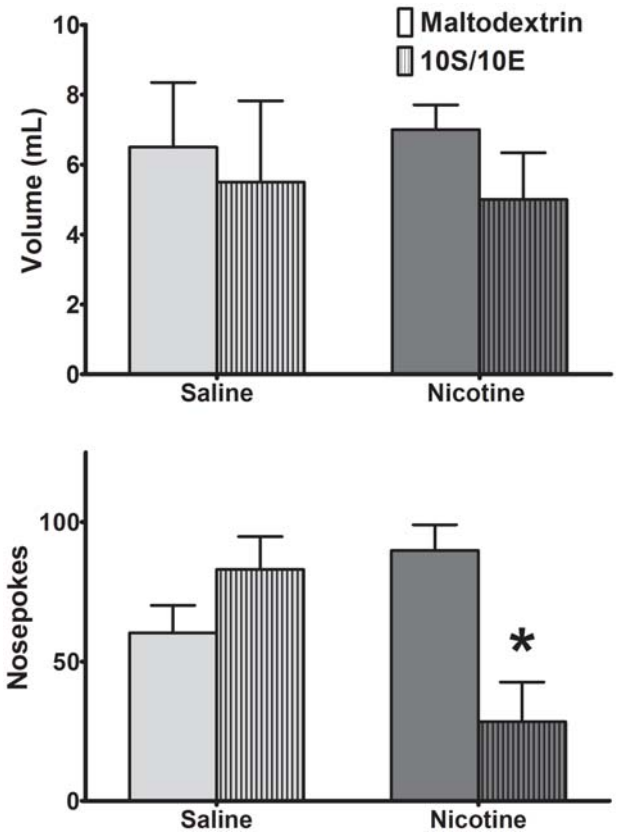


FIGURE 3 | Animals exhibited sustained habitual behavior following reward devaluation except when exposed to nicotine. (Top) Saline and nicotine animals consumed similar volumes of maltodextrin (solid bars) and sweetened ethanol (patterned bars) prior to behavioral measurement. (Bottom) Compared to maltodextrin, a control for consumed volume, pre-exposure to sweetened ethanol altered reward seeking only in nicotine exposed rats (Bonferroni *post hoc t*-test: $^{*}p < 0.01$ compared to maltodextrin access nicotine group; $N = 4$ Maltodextrin-Saline, 4 10S/10E-Saline, 5 Maltodextrin-Nicotine, 5 10S/10E-Nicotine).

prior to behavioral testing revealed no significant main effects of solution ($F_{(1,14)} = 0.92$, $p = 0.35$) and drug exposure ($F_{(1,14)} < 0.0001$, $p \approx 1.00$), nor a significant solution-by-exposure interaction ($F_{(1,14)} = 0.10$, $p = 0.75$). Though nicotine was expected to induce habitual behavior, nicotine exposed animals were more responsive to reward devaluation, and therefore resistant to habit formation towards sweetened ethanol in this experiment.

Progressive Ratio

Nicotine can enhance motivation for cue-reward pairing (Chaudhri et al., 2006, 2007). To test whether exposure to nicotine in adolescence alters motivation for sweetened ethanol, we recorded nosepokes and the breakpoint in a progressive ratio session (**Figures 4A,B**). Independent samples *t*-tests indicated that nicotine had no effect on nosepokes ($t_{(16)} = 0.49$, $p = 0.63$) or breakpoint ($t_{(16)} = 0.37$, $p = 0.72$). Thus, chronic nicotine exposure had no impact on motivation for sweetened ethanol.

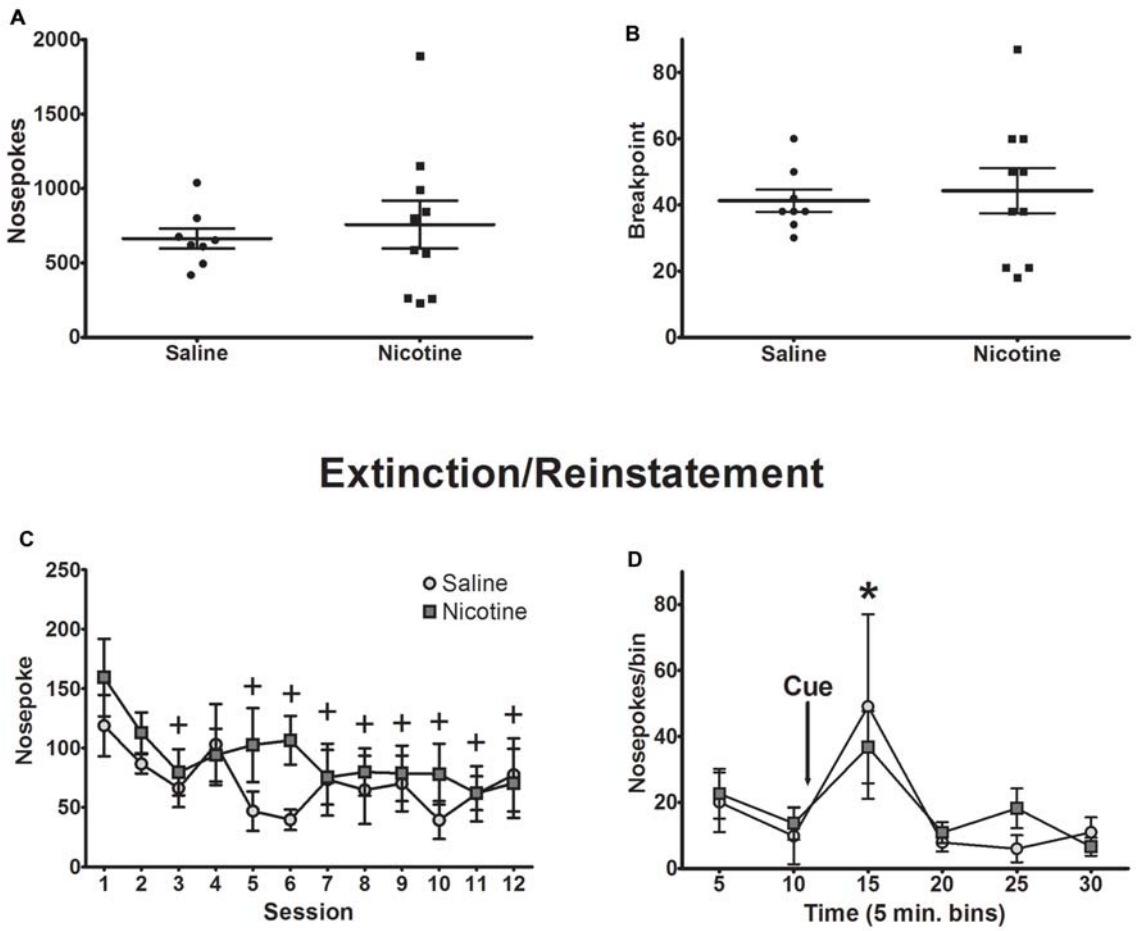


FIGURE 4 | Exposure to nicotine did not affect behavior during progressive ratio, extinction and reinstatement sessions. Motivation towards sweetened ethanol as measured by progressive ratio testing did not differ between exposure groups, indicated by **(A)** total nosepokes and **(B)** behavioral breakpoint. Individual rat data points are presented, with the mean ± SEM overlaid. **(C)** Behavioral responding decreased across 12 extinction sessions similarly between exposure groups. **(D)** Cue-induced reinstatement of operant responding was not different between groups (Bonferroni *post hoc t*-test, collapsed across drug exposure groups: $^{+}p < 0.01$ compared to session 1; $^{*}p < 0.05$ compared to 10, 20, 25 and 30 min bins; $N = 8$ saline, 10 nicotine).

Extinction and Reinstatement of Operant Behavior

Extinction learning was measured over 12 sessions. No significant difference in decreased reward seeking was observed between saline and nicotine exposed animals (**Figure 4C**). A two-way ANOVA of nosepokes resulted in a main effect of session ($F_{(11,176)} = 4.33$, $p < 0.0001$), but no main effect of drug exposure ($F_{(1,16)} = 0.59$, $p = 0.45$), and no significant session-by-exposure interaction ($F_{(11,176)} = 1.38$, $p = 0.18$). Nosepokes decreased significantly in the last eight sessions of extinction as indicated by *post hoc* analysis collapsed across drug exposure groups ($p < 0.01$).

As exposure to nicotine during adolescence has been shown to increase responding for conditioned reinforcers when compared to saline-exposed animals (Quick et al., 2014), we measured reinstatement of reward seeking after exposure to reward-associated cues. Reinstatement of operant responding for the cues previously associated with sweetened ethanol was measured in a single extinction-reinstatement session. Presentation of the cue (light and tone) 10 min into the session resulted in a significant increase in responding, but no significant difference was found between saline- and nicotine-exposed animals (**Figure 4D**). A two-way ANOVA of nosepokes across 5-min bins revealed a significant main effect of time bins ($F_{(5,80)} = 4.64$, $p < 0.001$), but no significant main effect of drug exposure ($F_{(1,16)} = 0.01$, $p = 0.92$) or significant time-by-exposure interaction ($F_{(5,80)} = 0.46$, $p = 0.80$). *Post hoc* analysis collapsed across drug exposure groups showed that cue presentation was sufficient to induce reinstatement of reward seeking, as nosepokes in the 15-min, post-cue time bin was

significantly higher than the 10-min, pre-cue time bin ($p < 0.05$). Collectively, these data show that nicotine exposure history had no impact on extinction of reward seeking or reinstatement of responding after re-exposure to reward-associated cues.

DISCUSSION

Epidemiological studies find that initiating nicotine consumption in adolescence correlates with progression to escalating use as well as to the propensity to pursue more illicit substances of abuse (Kandel and Faust, 1975; Kandel et al., 1992). The Gateway Hypothesis of addiction suggests that neurophysiological changes due to nicotine exposure would eventually lead to escalation of drug use due to increased reward valence to substances of abuse (Kandel and Kandel, 2014). Previous findings suggest exposure to nicotine only in adolescence is insufficient to alter adult cocaine self-administration (Pomfrey et al., 2015). Indeed, the escalation of use and progression to stronger drugs may rely on continuous drug exposure throughout adolescence as opposed to priming only (Kandel and Kandel, 2015). Given the propensity of nicotine and ethanol use in adolescence, it is also important to determine if nicotine can be a gateway drug to alcohol use. We hypothesized that animals exposed to daily injections of nicotine beginning in adolescence would be more sensitive to the rewarding and reinforcing properties of sweetened ethanol, and therefore would increase seeking and motivation for ethanol in a self-administration paradigm. However, as discussed below, our findings did not support this hypothesis.

Studies show that co-administration of nicotine increases alcohol seeking (Smith et al., 1999; Lê et al., 2014; Lárraga et al., 2017) and VTA dopamine neuron activity in rodents (Tolu et al., 2017). However, this may be due to nicotine's well-known ability to enhance the reinforcing properties of conditioned cues (Donny et al., 2003; Olausson et al., 2004a,b; Chaudhri et al., 2006; Palmatier et al., 2007, 2013; Caggiula et al., 2009; Guy and Fletcher, 2014a,b; Yager and Robinson, 2015). Indeed, several studies reported that nicotine administered prior to each behavioral session elevated conditioned responding to cues predicting either sucrose (Palmatier et al., 2013; Stringfield et al., 2017) or ethanol (Maddux and Chaudhri, 2017), supporting the contention that nicotine enhances approach behavior. Moreover, presentation of a nicotine-associated context, and not necessarily in the presence of nicotine, can increase ethanol self-administration (Zipori et al., 2017), suggesting the possibility that nicotine-enhancement of contextual cues is what actually drives enhanced ethanol seeking. Therefore, nicotine administered outside of the behavioral context may not alter self-administration, which is somewhat inconsistent with a molecular basis of the Gateway Hypothesis of nicotine (Kandel and Kandel, 2014). To test this, we gave adolescent rats daily injections of nicotine for 5 days prior to the start of behavioral training, and then, every day of behavioral training, animals received nicotine 2 h after the behavioral session. In short, rats received nicotine such that there was no association with the behavioral sessions and minimal pharmacological interaction with the ethanol consumed. We found that this nicotine regimen resulted in no significant impact on self-administration of sweetened ethanol; therefore, these results do not support a strictly "exposure" interpretation of the Gateway Hypothesis of nicotine. However, it should be noted that preclinical studies supporting the Gateway Hypothesis used a continuous exposure model of nicotine in drinking water (Levine et al., 2011), whereas we administered a single bolus of nicotine per day.

NTX is an opioid receptor antagonist that reduces ethanol seeking and the rewarding properties of ethanol (e.g., Kreek et al., 2002; Ripley and Stephens, 2011). We chose a dose range of NTX that has been shown to decrease ethanol seeking with nominal effect on sucrose seeking (Czachowski and Delory, 2009; Hay et al., 2013). NTX is more effective in reducing ethanol use in cigarette smokers compared to nonsmokers (Fucito et al., 2012) and in animals co-administering nicotine and ethanol during self-administration compared to ethanol alone (Lê et al., 2014). However, we found that NTX decreased sweetened ethanol seeking similarly in animals exposed to nicotine and saline. Our study was performed in a peri-adolescent period of development (∼P60), whereas most studies showing nicotine promotion of ethanol drinking have been done in adults. However, there is no evidence suggesting that NTX lacks efficacy in curbing ethanol seeking during adolescence. Indeed, NTX has been shown to decrease ethanol seeking in adolescent humans (Deas et al., 2005) and rodents (Sable et al., 2006). Thus, while previous findings show that NTX is a viable tool to decrease ethanol seeking in patients with a history of smoking, our results suggest this is likely due to nicotine-enhanced seeking behavior as opposed to nicotine exposure *per se*. As discussed above, our nicotine administration paradigm was such that animals were not exposed to nicotine in the context of the behavioral paradigm or in tandem with ethanol. As such, the cue reinforcing effects of nicotine (e.g., Caggiula et al., 2009) were likely not contributing factors to the results observed here.

Habit-directed behavior is a form of behavioral inflexibility, in that an animal is less likely to alter habitual behavior following a change in reward value. Ethanol consumption can shift operant behavior from goal- to habit-directed (Dickinson et al., 2002; Corbit et al., 2012; Mangieri et al., 2012). In turn, adolescent ethanol exposure can also lead to reduced behavioral flexibility in adulthood (Coleman et al., 2014; Gass et al., 2014; Madayag et al., 2017). Therefore, it is not surprising that the control animals in our study exhibited heightened habit-directed alcohol seeking in a reward devaluation paradigm, although extended operant training can also be sufficient to induce habit formation (e.g., Ostlund and Balleine, 2008). Repeated use of nicotine leads to habitual nicotine-seeking behavior (Clemens et al., 2014; Loughlin et al., 2017), though this can depend on the number of prior self-administration sessions, as 47 sessions, but not 10 sessions, produced insensitivity to nicotine devaluation (Clemens et al., 2014). Thus, one may expect that rats exposed to nicotine over 40 days would be predisposed to habitual reward seeking, but that is not what we observed in the present study. In fact, animals exposed to nicotine exhibited sensitivity to reward devaluation compared to animals that received saline control. This is not

likely due to acute effects of nicotine as the latest injection of nicotine was approximately 22 h prior to the behavioral session. Alternatively, it is possible that the ability of nicotine to maintain goal-directed behavior towards sweetened ethanol seeking was due to long-term exposure, as chronic exposure to nicotine can result in different nicotinic receptor subunit expression. For example, when administered in adolescence, repeated nicotine increases β2 subunit-containing nicotinic receptors (Counotte et al., 2012a). In turn, compounds that selectively target β2 subunit-containing receptors can enhance behavioral flexibility in humans and primates withdrawn from drugs of abuse (Gould et al., 2013; Terry et al., 2016; Lesage et al., 2017). Therefore, the administration of nicotine outside of the behavioral context may have enhanced behavioral flexibility and, therefore, rendered animals in our study resistant to habit-directed behavior.

Contingent nicotine co-administered with ethanol and non-contingent nicotine administered prior to ethanol self-administration sessions increase the motivation for ethanol seeking both in rodents (Bespalov et al., 1999) and humans (Barrett et al., 2006). When administered in conjunction with self-administered ethanol, nicotine enhances the motivation for ethanol in dependent rats (Leão et al., 2015). Consistent with this, administration of nicotine vs. non-nicotine cigarettes in human smokers increased the breakpoint for alcoholic beverages (Barrett et al., 2006). However, we observed no effect on motivation (nosepokes, breakpoint) for sweetened ethanol in animals administered nicotine when the nicotine was given outside of the behavioral context. This indicates that the motivationally enhancing effects of nicotine on ethanol seeking is likely to be highly dependent on the temporal aspect of administration.

Nicotinic receptor agonists including nicotine can be used to enhance cognition and behavioral flexibility (for review see Counotte et al., 2012b). For example, when given after induction of fear conditioning, nicotine has been shown to enhance extinction learning (Elias et al., 2010). Therefore, we expected animals exposed to nicotine to exhibit faster extinction from sweetened ethanol seeking compared to animals exposed to saline. Contrary to this prediction, we observed no significant difference in extinction behavior between the two groups, suggesting that the cognitive-enhancing effects of nicotine did not extend to extinction learning in the present study. Furthermore, when administered during ethanol withdrawal, nicotine can increase reinstatement of ethanol self-administration (López-Moreno et al., 2004). However, we observed no differences in reinstatement behavior after re-exposure to the associated cue. This likely occurred because we avoided acute nicotine effects on the reinstatement session, consistent with previous reports in which a temporally distant exposure had no effect on reinstatement behavior (Hauser et al., 2012).

Synergistic or additive effects on reinforcement circuitry due to co-administration or co-consumption of nicotine and ethanol (Leão et al., 2015; Tolu et al., 2017) are unlikely to contribute to the present results. The half-life of available nicotine in the brain is approximately 52 min (Ghosheh et al., 1999). Therefore, sufficient pharmacologically active nicotine should be available during the behavior session when administered up to 3–4 h prior (Hauser et al., 2012) to render behavioral effects from an acute injection, but not likely when administered approximately 22 h prior to the behavior session as in the present study. On the other hand, Doyon et al. (2013) reported long-term effects of nicotine on ethanol-evoked dopamine levels, lasting up to 40 h. Thus, the lack of effect of nicotine on behavior in the present study is consistent with expected pharmacokinetics, and while it is possible that this nicotine regimen produced persistent effects on the dopamine system, they were evidently insufficient to alter sweetened ethanol self-administration in the present study.

One caveat to the present study is that it used only female rats, as ethanol self-administration extended from mid-adolescence into adulthood, and female rodents are well-known to drink more ethanol than males in adulthood (Becker and Koob, 2016). However, in adolescence the sex difference is less clear, as some studies found that males drank more ethanol than females (e.g., Vetter-O'Hagen et al., 2009) and others found more drinking in females (e.g., Varlinskaya et al., 2015). When administered only during adolescence, nicotine enhanced conditioned reinforcement in both male and female adults, but increased Pavlovian conditioned approach only in adult males (Quick et al., 2014). Thus, it is possible that nicotine would have different effects in males under the current study design, which may be addressed by future studies.

A related issue is that behavioral neuroscience has historically largely used male rodents based on the assumption that females would introduce greater day-to-day variability due to hormonal effects across their estrous cycle (McCarthy, 2015; Guizzetti et al., 2016). Thus, it is possible that the present study "missed" effects of nicotine due to hormone-related variability in female self-administration. However, recent studies analyzing published data found this assumption to be erroneous: in general, female rodents exhibit no different variance in most metrics (physiological, behavioral, histological, etc.) compared to males (Prendergast et al., 2014; Becker et al., 2016). Even for those traits that showed sex differences, female data were not inherently more variable than male data (Becker et al., 2016). In humans, the primary discrepancy between sexes is that women metabolize nicotine faster than men (Benowitz et al., 2006), although menstrual cycle has no impact on the rate of metabolism in human subjects (Hukkanen et al., 2005). Collectively, it appears unlikely that using only females inserted more variability in behavior metrics. However, future studies that directly compare the effects of nicotine on ethanol self-administration in males and females are needed to determine sex differences in day-to-day variability of intake and the potential effects of nicotine.

According to the Gateway Hypothesis of addiction, prior exposure to nicotine leads to an increase in sensitivity to the rewarding properties of other substances of abuse (Kandel and Kandel, 2014). We observed that initiating nicotine administration during adolescence and continuing

into adulthood was insufficient to increase ethanol seeking. Therefore, it is likely that nicotine must be self-administered or associative cues must be present while experimenter-administered nicotine is physiologically active for it to produce its reward-amplifying properties. The Gateway Hypothesis should not be discounted, but further investigation should aim to determine in what capacity "gateway drugs" contribute to progression to and escalation of consumption of substances of abuse.

AUTHOR CONTRIBUTIONS

ACM designed the experimental methods and was the primary contributor to authoring the manuscript. KSC performed the experiments and was a secondary contributor to authoring the manuscript. LMW performed the experiments and reviewed the manuscript. DLR helped design the experimental method, reviewed the manuscript and is the principal investigator.

ACKNOWLEDGMENTS

The authors thank Sofia Neira, Hannah Jaggers, Madeline Watson and Brandi Lawrence for valuable technical assistance. Dr. Sierra Stringfield provided valuable discussion and insight. This research was funded by the National Institutes of Health (P60 AA011605). ACM was supported on T32 AA007573 and KSC was supported by the Science and Math Achievement and Resourcefulness Track (SMART) fellowship from the Office of Undergraduate Research at the University of North Carolina, Chapel Hill, NC, USA.

REFERENCES

Bachman, J. G. (2002). *The Decline of Substance Use in Young Adulthood: Changes in Social Activities, Roles, and Beliefs.* Mahwah, NJ: Lawrence Erlbaum Associates.

Barrett, S. P., Tichauer, M., Leyton, M., and Pihl, R. O. (2006). Nicotine increases alcohol self-administration in non-dependent male smokers. *Drug Alcohol Depend.* 81, 197–204. doi: 10.1016/j.drugalcdep.2005.06.009

Becker, J. B., and Koob, G. F. (2016). Sex differenecs in animal models: focus on addiction. *Pharmacol. Rev.* 68, 242–263. doi: 10.1124/pr.115.011163

Becker, J. B., Prendergast, B. J., and Liang, J. W. (2016). Female rats are not more variable than male rats: a meta-analysis of neuroscience studies. *Biol. Sex Differ.* 7:34. doi: 10.1186/s13293-016-0087-5

Benowitz, N. L., Lessov-Schlaggar, C. N., Swan, G. E., and Jacob, P. III (2006). Female sex and oral contraceptive use accelerate nicotine metabolism. *Clin. Pharmacol. Ther.* 79, 480–488. doi: 10.1016/j.clpt.2006.01.008

Bespalov, A., Lebedev, A., Panchenko, G., and Zvartau, E. (1999). Effects of abused drugs on thresholds and breaking points of intracranial self-stimulation in rats. *Eur. Neuropsychopharmacol.* 9, 377–383. doi: 10.1016/s0924-977x(99)00008-5

Bienkowski, P., Koros, E., Kostowski, W., and Bogucka-Bonikowska, A. (2000). Reinstatement of ethanol seeking in rats: behavioral analysis. *Pharmacol. Biochem. Behav.* 66, 123–128. doi: 10.1016/S0091-3057(00)00194-5

Brown, S. A., Tapert, S. F., Tate, S. R., and Abrantes, A. M. (2000). The role of alcohol in adolescent relapse and outcome. *J. Psychoactive Drugs* 32, 107–115. doi: 10.1080/02791072.2000.10400216

Caggiula, A. R., Donny, E. C., Palmatier, M. I., Liu, X., Chaudhri, N., and Sved, A. F. (2009). The role of nicotine in smoking: a dual-reinforcement model. *Nebr. Symp. Motiv.* 55, 91–109. doi: 10.1007/978-0-387-78748-0_6

Chaudhri, N., Caggiula, A. R., Donny, E. C., Booth, S., Gharib, M., Craven, L., et al. (2006). Operant responding for conditioned and unconditioned reinforcers in rats is differentially enhanced by the primary reinforcing and reinforcement-enhancing effects of nicotine. *Psychopharmacology (Berl)* 189, 27–36. doi: 10.1007/s00213-006-0522-0

Chaudhri, N., Caggiula, A. R., Donny, E. C., Booth, S., Gharib, M. A., Craven, L. A., et al. (2005). Sex differences in the contribution of nicotine and nonpharmacological stimuli to nicotine self-administration in rats. *Psychopharmacology (Berl)* 180, 258–266. doi: 10.1007/s00213-005-2152-3

Chaudhri, N., Caggiula, A. R., Donny, E. C., Booth, S., Gharib, M., Craven, L., et al. (2007). Self-administered and noncontingent nicotine enhance reinforced operant responding in rats: impact of nicotine dose and reinforcement schedule. *Psychopharmacology (Berl)* 190, 353–362. doi: 10.1007/s00213-006-0454-8

Clemens, K. J., Castino, M. R., Cornish, J. L., Goodchild, A. K., and Holmes, N. M. (2014). Behavioral and neural substrates of habit formation in rats intravenously self-administering nicotine. *Neuropsychopharmacology* 39, 2584–2593. doi: 10.1038/npp.2014.111

Coleman, L. G. Jr., Liu, W., Oguz, I., Styner, M., and Crews, F. T. (2014). Adolescent binge ethanol treatment alters adult brain regional volumes, cortical extracellular matrix protein and behavioral flexibility. *Pharmacol. Biochem. Behav.* 116, 142–151. doi: 10.1016/j.pbb.2013.11.021

Corbit, L. H., Nie, H., and Janak, P. H. (2012). Habitual alcohol seeking: time course and the contribution of subregions of the dorsal striatum. *Biol. Psychiatry* 72, 389–395. doi: 10.1016/j.biopsych.2012.02.024

Counotte, D. S., Goriounova, N. A., Moretti, M., Smoluch, M. T., Irth, H., Clementi, F., et al. (2012a). Adolescent nicotine exposure transiently increases high-affinity nicotinic receptors and modulates inhibitory synaptic transmission in rat medial prefrontal cortex. *FASEB J.* 26, 1810–1820. doi: 10.1096/fj.11-198994

Counotte, D. S., Smit, A. B., and Spijker, S. (2012b). The yin and yang of nicotine: harmful during development, beneficial in adult patient populations. *Front. Pharmacol.* 3:180. doi: 10.3389/fphar.2012.00180

Czachowski, C. L., and Delory, M. J. (2009). Acamprosate and naltrexone treatment effects on ethanol and sucrose seeking and intake in ethanol-dependent and nondependent rats. *Psychopharmacology (Berl)* 204, 335–348. doi: 10.1007/s00213-009-1465-z

Deas, D., May, M. P., Randall, C., Johnson, N., and Anton, R. (2005). Naltrexone treatment of adolescent alcoholics: an open-label pilot study. *J. Child Adolesc. Psychopharmacol.* 15, 723–728. doi: 10.1089/cap.2005.15.723

Degenhardt, L., Dierker, L., Chiu, W. T., Medina-Mora, M. E., Neumark, Y., Sampson, N., et al. (2010). Evaluating the drug use "gateway" theory using cross-national data: consistency and associations of the order of initiation of drug use among participants in the WHO World Mental Health Surveys. *Drug Alcohol Depend.* 108, 84–97. doi: 10.1016/j.drugalcdep.2009.12.001

Dickinson, A., Wood, N., and Smith, J. W. (2002). Alcohol seeking by rats: action or habit? *Q. J. Exp. Psychol. B* 55, 331–348. doi: 10.1080/02724990244000016

Doherty, J. M., and Gonzales, R. A. (2015). Operant self-administration of sweetened ethanol and time course of blood ethanol levels in adolescent and adult male Long-Evans rats. *Alcohol. Clin. Exp. Res.* 39, 485–495. doi: 10.1111/acer.12630

Donny, E. C., Chaudhri, N., Caggiula, A. R., Evans-Martin, F. F., Booth, S., Gharib, M. A., et al. (2003). Operant responding for a visual reinforcer in rats is enhanced by noncontingent nicotine: implications for nicotine self-administration and reinforcement. *Psychopharmacology (Berl)* 169, 68–76. doi: 10.1007/s00213-003-1473-3

Doyon, W. M., Dong, Y., Ostroumov, A., Thomas, A. M., Zhang, T. A., and Dani, J. A. (2013). Nicotine decreases ethanol-induced dopamine signaling and increases self-administration via stress hormones. *Neuron* 79, 530–540. doi: 10.1016/j.neuron.2013.06.006

Elias, G. A., Gulick, D., Wilkinson, D. S., and Gould, T. J. (2010). Nicotine and extinction of fear conditioning. *Neuroscience* 165, 1063–1073. doi: 10.1016/j.neuroscience.2009.11.022

Fucito, L. M., Park, A., Gulliver, S. B., Mattson, M. E., Gueorguieva, R. V., and O'Malley, S. S. (2012). Cigarette smoking predicts differential benefit from naltrexone for alcohol dependence. *Biol. Psychiatry* 72, 832–838. doi: 10.1016/j.biopsych.2012.03.023

Gass, J. T., Glen, W. B. Jr., McGonigal, J. T., Trantham-Davidson, H., Lopez, M. F., Randall, P. K., et al. (2014). Adolescent alcohol exposure reduces behavioral flexibility, promotes disinhibition and increases resistance to extinction of ethanol self-administration in adulthood. *Neuropsychopharmacology* 39, 2570–2583. doi: 10.1038/npp.2014.109

Ghosheh, O., Dwoskin, L. P., Li, W. K., and Crooks, P. A. (1999). Residence times and half-lives of nicotine metabolites in rat brain after acute peripheral administration of [2′-^{14}C]nicotine. *Drug Metab. Dispos.* 27, 1448–1455.

Gould, R. W., Garg, P. K., Garg, S., and Nader, M. A. (2013). Effects of nicotinic acetylcholine receptor agonists on cognition in rhesus monkeys with a chronic cocaine self-administration history. *Neuropharmacology* 64, 479–488. doi: 10.1016/j.neuropharm.2012.08.004

Guizzetti, M., Davies, D. L., Egli, M., Finn, D. A., Molina, P., Regunathan, S., et al. (2016). Sex and the lab: an alcohol-focused commentary on the NIH initiative to balance sex in cell and animal studies. *Alcohol. Clin. Exp. Res.* 40, 1182–1191. doi: 10.1111/acer.13072

Guy, E. G., and Fletcher, P. J. (2014a). The effects of nicotine exposure during Pavlovian conditioning in rats on several measures of incentive motivation for a conditioned stimulus paired with water. *Psychopharmacology (Berl)* 231, 2261–2271. doi: 10.1007/s00213-013-3375-3

Guy, E. G., and Fletcher, P. J. (2014b). Responding for a conditioned reinforcer, and its enhancement by nicotine, is blocked by dopamine receptor antagonists and a 5-HT_{2C} receptor agonist but not by a 5-HT_{2A} receptor antagonist. *Pharmacol. Biochem. Behav.* 125, 40–47. doi: 10.1016/j.pbb.2014.08.006

Hauser, S. R., Getachew, B., Oster, S. M., Dhaher, R., Ding, Z. M., Bell, R. L., et al. (2012). Nicotine modulates alcohol-seeking and relapse by alcohol-preferring (P) rats in a time-dependent manner. *Alcohol. Clin. Exp. Res.* 36, 43–54. doi: 10.1111/j.1530-0277.2011.01579.x

Hay, R. A., Jennings, J. H., Zitzman, D. L., Hodge, C. W., and Robinson, D. L. (2013). Specific and nonspecific effects of naltrexone on goal-directed and habitual models of alcohol seeking and drinking. *Alcohol. Clin. Exp. Res.* 37, 1100–1110. doi: 10.1111/acer.12081

Hukkanen, J., Gourlay, S. G., Kenkare, S., and Benowitz, N. L. (2005). Influence of menstrual cycle on cytochrome P450 2A6 activity and cardiovascular effects of nicotine. *Clin. Pharmacol. Ther.* 77, 159–169. doi: 10.1016/j.clpt.2004.10.012

Kandel, D., and Faust, R. (1975). Sequence and stages in patterns of adolescent drug use. *Arch. Gen. Psychiatry* 32, 923–932. doi: 10.1001/archpsyc.1975.01760250115013

Kandel, E. R., and Kandel, D. B. (2014). A molecular basis for nicotine as a gateway drug. *N. Eng. J. Med.* 371, 932–943. doi: 10.1056/nejmsa1405092

Kandel, D., and Kandel, E. (2015). The gateway hypothesis of substance abuse: developmental, biological and societal perspectives. *Acta Paediatr.* 104, 130–137. doi: 10.1111/apa.12851

Kandel, D. B., Yamaguchi, K., and Chen, K. (1992). Stages of progression in drug involvement from adolescence to adulthood: further evidence for the gateway theory. *J. Stud. Alcohol* 53, 447–457. doi: 10.15288/jsa.1992.53.447

Kreek, M. J., Laforge, K. S., and Butelman, E. (2002). Pharmacotherapy of addictions. *Nat. Rev. Drug Discov.* 1, 710–726. doi: 10.1038/nrd897

Lárraga, A., Belluzzi, J. D., and Leslie, F. M. (2017). Nicotine increases alcohol intake in adolescent male rats. *Front. Behav. Neurosci.* 11:25. doi: 10.3389/fnbeh.2017.00025

Lê, A. D., Funk, D., Lo, S., and Coen, K. (2014). Operant self-administration of alcohol and nicotine in a preclinical model of co-abuse. *Psychopharmacology (Berl)* 231, 4019–4029. doi: 10.1007/s00213-014-3541-2

Leão, R. M., Cruz, F. C., Vendruscolo, L. F., de Guglielmo, G., Logrip, M. L., Planeta, C. S., et al. (2015). Chronic nicotine activates stress/reward-related brain regions and facilitates the transition to compulsive alcohol drinking. *J. Neurosci.* 35, 6241–6253. doi: 10.1523/JNEUROSCI.3302-14.2015

Lesage, E., Aronson, S. E., Sutherland, M. T., Ross, T. J., Salmeron, B. J., and Stein, E. A. (2017). Neural signatures of cognitive flexibility and reward sensitivity following nicotinic receptor stimulation in dependent smokers: a randomized trial. *JAMA Psychiatry* 74, 632–640. doi: 10.1001/jamapsychiatry.2017.0400

Levine, A., Huang, Y., Drisaldi, B., Griffin, E. A. Jr., Pollak, D. D., Xu, S., et al. (2011). Molecular mechanism for a gateway drug: epigenetic changes initiated by nicotine prime gene expression by cocaine. *Sci. Transl. Med.* 3:107ra109. doi: 10.1126/scitranslmed.3003062

López-Moreno, J. A., Trigo-Díaz, J. M., Rodríguez de Fonseca, F., González Cuevas, G., Gómez de Heras, R., Crespo Galán, I., et al. (2004). Nicotine in alcohol deprivation increases alcohol operant self-administration during reinstatement. *Neuropharmacology* 47, 1036–1044. doi: 10.1016/j.neuropharm.2004.08.002

Loughlin, A., Funk, D., Coen, K., and Lê, A. D. (2017). Habitual nicotine-seeking in rats following limited training. *Psychopharmacology (Berl)* doi: 10.1007/s00213-017-4655-0 [Epub ahead of print].

Madayag, A. C., Stringfield, S. J., Reissner, K. J., Boettiger, C. A., and Robinson, D. L. (2017). Sex and adolescent ethanol exposure influence Pavlovian conditioned approach. *Alcohol. Clin. Exp. Res.* 41, 846–856. doi: 10.1111/acer.13354

Maddux, J. N., and Chaudhri, N. (2017). Nicotine-induced enhancement of Pavlovian alcohol-seeking behavior in rats. *Psychopharmacology (Berl)* 234, 727–738. doi: 10.1007/s00213-016-4508-2

Mangieri, R. A., Cofresi, R. U., and Gonzales, R. A. (2012). Ethanol seeking by Long Evans rats is not always a goal-directed behavior. *PLoS One* 7:e42886. doi: 10.1371/journal.pone.0042886

McCarthy, M. M. (2015). Incorporating sex as a variable in preclinical neuropsychiatric research. *Schizophr. Bull.* 41, 1016–1020. doi: 10.1093/schbul/sbv077

Melendez, R. I. (2011). Intermittent (every-other-day) drinking induces rapid escalation of ethanol intake and preference in adolescent and adult C57BL/6J mice. *Alcohol. Clin. Exp. Res.* 35, 652–658. doi: 10.1111/j.1530-0277.2010.01383.x

Nesil, T., Kanit, L., Collins, A. C., and Pogun, S. (2011). Individual differences in oral nicotine intake in rats. *Neuropharmacology* 61, 189–201. doi: 10.1016/j.neuropharm.2011.03.027

Olausson, P., Jentsch, J. D., and Taylor, J. R. (2004a). Nicotine enhances responding with conditioned reinforcement. *Psychopharmacology (Berl)* 171, 173–178. doi: 10.1007/s00213-003-1575-y

Olausson, P., Jentsch, J. D., and Taylor, J. R. (2004b). Repeated nicotine exposure enhances responding with conditioned reinforcement. *Psychopharmacology (Berl)* 173, 98–104. doi: 10.1007/s00213-003-1702-9

Ostlund, S. B., and Balleine, B. W. (2008). On habits and addiction: an associative analysis of compulsive drug seeking. *Drug Discov. Today Dis. Models* 5, 235–245. doi: 10.1016/j.ddmod.2009.07.004

Palmatier, M. I., Liu, X., Matteson, G. L., Donny, E. C., Caggiula, A. R., and Sved, A. F. (2007). Conditioned reinforcement in rats established with self-administered nicotine and enhanced by noncontingent nicotine. *Psychopharmacology (Berl)* 195, 235–243. doi: 10.1007/s00213-007-0897-6

Palmatier, M. I., Marks, K. R., Jones, S. A., Freeman, K. S., Wissman, K. M., and Sheppard, A. B. (2013). The effect of nicotine on sign-tracking and goal-tracking in a Pavlovian conditioned approach paradigm in rats. *Psychopharmacology (Berl)* 226, 247–259. doi: 10.1007/s00213-012-2892-9

Palmer, R. H., Button, T. M., Rhee, S. H., Corley, R. P., Young, S. E., Stallings, M. C., et al. (2012). Genetic etiology of the common liability to drug dependence: evidence of common and specific mechanisms for DSM-IV dependence symptoms. *Drug Alcohol Depend.* 123, S24–S32. doi: 10.1016/j.drugalcdep.2011.12.015

Pomfrey, R. L., Bostwick, T. A., Wetzell, B. B., and Riley, A. L. (2015). Adolescent nicotine exposure fails to impact cocaine reward, aversion and self-administration in adult male rats. *Pharmacol. Biochem. Behav.* 137, 30–37. doi: 10.1016/j.pbb.2015.08.004

Prendergast, B. J., Onishi, K. G., and Zucker, I. (2014). Female mice liberated for inclusion in neuroscience and biomedical research. *Neurosci. Biobehav. Rev.* 40, 1–5. doi: 10.1016/j.neubiorev.2014.01.001

Quick, S. L., Olausson, P., Addy, N. A., and Taylor, J. R. (2014). Repeated nicotine exposure during adolescence alters reward-related learning in male and female rats. *Behav. Brain Res.* 261, 171–176. doi: 10.1016/j.bbr.2013.12.001

Ripley, T. L., and Stephens, D. N. (2011). Critical thoughts on current rodent models for evaluating potential treatments of alcohol addiction and withdrawal. *Br. J. Pharmacol.* 164, 1335–1356. doi: 10.1111/j.1476-5381.2011.01406.x

Sable, H. J., Bell, R. L., Rodd, Z. A., and Mcbride, W. J. (2006). Effects of naltrexone on the acquisition of alcohol intake in male and female periadolescent and adult alcohol-preferring (P) rats. *Int. J. Adolesc. Med. Health* 18, 139–149. doi: 10.1515/ijamh.2006.18.1.139

Schassburger, R. L., Pitzer, E. M., Smith, T. T., Rupprecht, L. E., Thiels, E., Donny, E. C., et al. (2016). Adolescent rats self-administer less nicotine than adults at low doses. *Nicotine Tob. Res.* 18, 1861–1868. doi: 10.1093/ntr/ntw006

Serlin, H., and Torregrossa, M. M. (2015). Adolescent rats are resistant to forming ethanol seeking habits. *Dev. Cogn. Neurosci.* 16, 183–190. doi: 10.1016/j.dcn.2014.12.002

Shillinglaw, J. E., Everitt, I. K., and Robinson, D. L. (2014). Assessing behavioral control across reinforcer solutions on a fixed-ratio schedule of reinforcement in rats. *Alcohol* 48, 337–344. doi: 10.1016/j.alcohol.2013.12.006

Shnitko, T. A., and Robinson, D. L. (2015). Regional variation in phasic dopamine encoding of alcohol self-administration in rats. *ACS Chem. Neurosci.* 6, 147–154. doi: 10.1021/cn500251j

Shram, M. J., Funk, D., Li, Z., and Lê, A. D. (2008). Nicotine self-administration, extinction responding and reinstatement in adolescent and adult male rats: evidence against a biological vulnerability to nicotine addiction during adolescence. *Neuropsychopharmacology* 33, 739–748. doi: 10.1038/sj.npp.1301454

Shram, M. J., and Lê, A. D. (2010). Adolescent male Wistar rats are more responsive than adult rats to the conditioned rewarding effects of intravenously administered nicotine in the place conditioning procedure. *Behav. Brain Res.* 206, 240–244. doi: 10.1016/j.bbr.2009.09.018

Smith, B. R., Horan, J. T., Gaskin, S., and Amit, Z. (1999). Exposure to nicotine enhances acquisition of ethanol drinking by laboratory rats in a limited access paradigm. *Psychopharmacology (Berl)* 142, 408–412. doi: 10.1007/s002130050906

Stringfield, S. J., Palmatier, M. I., Boettiger, C. A., and Robinson, D. L. (2017). Orbitofrontal participation in sign- and goal-tracking conditioned responses: effects of nicotine. *Neuropharmacology* 116, 208–223. doi: 10.1016/j.neuropharm.2016.12.020

Tambour, S., Brown, L. L., and Crabbe, J. C. (2008). Gender and age at drinking onset affect voluntary alcohol consumption but neither the alcohol deprivation effect nor the response to stress in mice. *Alcohol. Clin. Exp. Res.* 32, 2100–2106. doi: 10.1111/j.1530-0277.2008.00798.x

Terry, A. V. Jr., Plagenhoef, M., and Callahan, P. M. (2016). Effects of the nicotinic agonist varenicline on the performance of tasks of cognition in aged and middle-aged rhesus and pigtail monkeys. *Psychopharmacology (Berl)* 233, 761–771. doi: 10.1007/s00213-015-4154-0

Tolu, S., Marti, F., Morel, C., Perrier, C., Torquet, N., Pons, S., et al. (2017). Nicotine enhances alcohol intake and dopaminergic responses through β2* and β4* nicotinic acetylcholine receptors. *Sci. Rep.* 7:45116. doi: 10.1038/srep45116

Torres, O. V., Natividad, L. A., Tejeda, H. A., Van Weelden, S. A., and O'Dell, L. E. (2009). Female rats display dose-dependent differences to the rewarding and aversive effects of nicotine in an age-, hormone-, and sex-dependent manner. *Psychopharmacology (Berl)* 206, 303–312. doi: 10.1007/s00213-009-1607-3

Truxell, E. M., Molina, J. C., and Spear, N. E. (2007). Ethanol intake in the juvenile, adolescent, and adult rat: effects of age and prior exposure to ethanol. *Alcohol. Clin. Exp. Res.* 31, 755–765. doi: 10.1111/j.1530-0277.2007.00358.x

Varlinskaya, E. I., Truxell, E. M., and Spear, L. P. (2015). Ethanol intake under social circumstances or alone in Sprague-Dawley rats: impact of age, sex, social activity, and social anxiety-like behavior. *Alcohol. Clin. Exp. Res.* 39, 117–125. doi: 10.1111/acer.12604

Vetter-O'Hagen, C., Varlinskaya, E., and Spear, L. (2009). Sex differences in ethanol intake and sensitivity to aversive effects during adolescence and adulthood. *Alcohol Alcohol.* 44, 547–554. doi: 10.1093/alcalc/agp048

Walker, B. M., and Ehlers, C. L. (2009). Age-related differences in the blood alcohol levels of Wistar rats. *Pharmacol. Biochem. Behav.* 91, 560–565. doi: 10.1016/j.pbb.2008.09.017

Walker, B. M., and Koob, G. F. (2007). The γ-aminobutyric acid-B receptor agonist baclofen attenuates responding for ethanol in ethanol-dependent rats. *Alcohol. Clin. Exp. Res.* 31, 11–18. doi: 10.1111/j.1530-0277.2006.00259.x

Yager, L. M., and Robinson, T. E. (2015). Individual variation in the motivational properties of a nicotine cue: sign-trackers vs. goal-trackers. *Psychopharmacology (Berl)* 232, 3149–3160. doi: 10.1007/s00213-015-3962-6

Zipori, D., Sadot-Sogrin, Y., Goltseker, K., Even-Chen, O., Rahamim, N., Shaham, O., et al. (2017). Re-exposure to nicotine-associated context from adolescence enhances alcohol intake in adulthood. *Sci. Rep.* 7:2479. doi: 10.1038/s41598-017-02177-2

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: 07 September 2017
doi: 10.3389/fnbeh.2017.00167

Nucleus Accumbens MC4-R Stimulation Reduces Food and Ethanol Intake in Adult Rats Regardless of Binge-Like Ethanol Exposure during Adolescence

Francisca Carvajal[1†], José M. Lerma-Cabrera[1,2†], Manuel Alcaraz-Iborra[3], Montserrat Navarro[4], Todd E. Thiele[4] and Inmaculada Cubero[1,3]*

[1]*Institute of Biomedical Sciences, Universidad Autonoma de Chile, Santiago, Chile,* [2]*Department of Psychology, University of Oviedo, Oviedo, Spain,* [3]*Department of Psychology, University of Almeria, Almeria, Spain,* [4]*Department of Psychology and Neuroscience, University of North Carolina, Chapel Hill, NC, United States*

The melanocortin (MC) system regulates feeding and ethanol consumption. Recent evidence shows that melanocortin 4 receptor (MC4-R) stimulation within the nucleus accumbens (NAc) elicits anorectic responses and reduces ethanol consumption and ethanol palatability in adult rats. Ethanol exposure during adolescence causes long-lasting changes in neural pathways critically involved in neurobehavioral responses to ethanol. In this regard, binge-like ethanol exposure during adolescence reduces basal alpha-melanocyte-stimulating hormone (α-MSH) and alters the levels of agouti-related peptide (AgRP) in hypothalamic and limbic areas. Given the protective role of MC against excessive ethanol consumption, disturbances in the MC system induced by binge-like ethanol exposure during adolescence might contribute to excessive ethanol consumption during adulthood. In the present study, we evaluated whether binge-like ethanol exposure during adolescence leads to elevated ethanol intake and/or eating disturbance during adulthood. Toward that aim, Sprague-Dawley rats were treated with ethanol (3 g/kg i.p.; BEP group) or saline (SP group) for 14 days (PND 25 to PND 38). On PND73, all the groups were given access to 20% ethanol on an intermittent schedule. Our results showed that adult rats given intermittent access (IAE) to 20% ethanol achieved high spontaneous ethanol intake that was not significantly enhanced by binge-like ethanol pretreatment during adolescence. However, BEP group exhibited an increase in food intake without a parallel increase in body weight (BW) relative to SP group suggesting caloric efficiency disturbance. Additionally, we evaluated whether binge-like ethanol exposure during adolescence alters the expected reduction in feeding and ethanol consumption following NAc shell administration of a selective MC4-R agonist in adult rats showing high rates of ethanol consumption. For that, animals in each pretreatment condition (SP and BEP) were divided into three subgroups and given bilateral NAc infusions of the selective MC4-R agonist cyclo(NH-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Glu)-NH_2 (0, 0.75 or 1.5 μg). Results revealed that MC4-R stimulation within the NAc reduced feeding and ethanol intake in high ethanol-drinking adult rats, regardless

OPEN ACCESS

Edited by:
Jacqueline M. Barker, Medical University of South Carolina, United States

Reviewed by:
Devon L. Graham, Florida State University College of Medicine, United States
Shauna L. Parkes, Centre National de la Recherche Scientifique (CNRS), France

****Correspondence:***
Inmaculada Cubero
icubero@ual.es

[†]These authors have contributed equally to this work.

Received: *15 March 2017*
Accepted: *22 August 2017*
Published: *07 September 2017*

Citation:
Carvajal F, Lerma-Cabrera JM, Alcaraz-Iborra M, Navarro M, Thiele TE and Cubero I (2017) Nucleus Accumbens MC4-R Stimulation Reduces Food and Ethanol Intake in Adult Rats Regardless of Binge-Like Ethanol Exposure during Adolescence. Front. Behav. Neurosci. 11:167. doi: 10.3389/fnbeh.2017.00167

of previous binge-like ethanol exposure during adolescence, which adds new evidence regarding the dual ability of MC compounds to control excessive ethanol and food intake.

Keywords: melanocortin, MC4-R, binge-like ethanol exposure, adolescence, intermittent-access ethanol paradigm, nucleus accumbens

INTRODUCTION

The melanocortin (MC) system is an important neural system involved in the regulation of energy balance and feeding behavior (for review see Gantz and Fong, 2003). Alpha-melanocyte-stimulating hormone (α-MSH) is an endogenous agonist of this system, and agouti-related peptide (AgRP) an inverse agonist that exerts the opposite effect on the same MC receptors (Cone, 2005; De Jonghe et al., 2011). The role of MC signaling within the hypothalamus in modulating feeding behavior, body weight (BW) regulation (Adan et al., 2006; Krashes et al., 2016; You et al., 2016) and food selection (Hillebrand et al., 2006) by homeostatic mechanisms (Pandit et al., 2011; Lerma-Cabrera et al., 2012) has been well established. Moreover, the MC system, especially the melanocortin 4 receptor (MC4-R), are currently a promising target system for the development of drugs intended to treat obesity and eating disorders in humans (Hillebrand et al., 2006; Moriya et al., 2006; Laviano et al., 2008; Steinman and DeBoer, 2013; Girardet and Butler, 2014).

Pharmacological and genetic studies have provided additional evidence that MC plays an important role in modulating neurobiological responses to ethanol, indicating that MC compounds may hold potential for treating alcohol abuse disorders. For example, intracerebroventricular (i.c.v.) infusion of the nonselective MC3/4-R agonist, melanotan-II (MTII) significantly reduces ethanol consumption in alcohol-preferring AA rats (Ploj et al., 2002) and C57BL/6J mice (Navarro et al., 2003). Conversely, i.c.v. infusion of AgRP-(83–132), a nonselective MC3/4-R inverse agonist, increases voluntary ethanol consumption in C57BL/6J mice (Navarro et al., 2005). Consistent with pharmacological studies, genetic deletion of AgRP blunts ethanol self-administration and binge-like ethanol drinking (Navarro et al., 2009). Furthermore, it was found that i.c.v. infusion of MTII reduces ethanol intake in mutant mice lacking MC3-R (MC3R−/−; Navarro et al., 2005) but failed to alter ethanol drinking in MC4R−/− mice (Navarro et al., 2011), suggesting that the protective effects of MC are modulated by MC4-R signaling. We have recently provided additional evidence that MC4-R stimulation within the nucleus accumbens (NAc) elicits anorectic responses and reduces ethanol consumption in rats that drink ethanol at moderate rates (Lerma-Cabrera et al., 2012), and the action of MC on MC4-R in the NAc helps modulate non-homeostatic aspects (palatability) of ethanol consumption (Lerma-Cabrera et al., 2013b). However, whether MC4-R signaling within the NAc plays a role in ethanol intake and/or feeding in rats showing high rates of ethanol intake remains unexplored.

Binge-drinking, defined as drinking excessive amounts of alcohol in a short period of time, is a popular pattern of consumption within the adolescent population (Pedersen and von Soest, 2015; Tanumihardjo et al., 2015). Adolescence is an important period of brain development during which heavy drinking triggers long-lasting adaptive changes in neural pathways critically involved in neurobehavioral responses to ethanol (Pascual et al., 2007, 2009, 2012; Maldonado-Devincci et al., 2010b). Several studies have shown that early alcohol use correlates with an increased likelihood of alcohol abuse (Pascual et al., 2009; Maldonado-Devincci et al., 2010a) and eating disorders in adulthood (Stickley et al., 2015). However, the underlying mechanisms leading to enhanced vulnerability to alcohol abuse and eating disorders after ethanol exposure during adolescence are still unclear.

Importantly, we have demonstrated that binge-like ethanol exposure during adolescence reduces basal α-MSH activity in hypothalamic and limbic areas and alters AgRP responses to acute ethanol administration (Lerma-Cabrera et al., 2013a). These data, together with previous evidence showing a key role of MC in feeding and ethanol drinking, suggest that changes in the MC system induced by binge-like ethanol exposure during adolescence may contribute to excessive ethanol consumption and/or eating disturbances during adulthood.

First, the present study assessed whether binge-like ethanol exposure during adolescence leads to elevated ethanol intake in adult rats with high rates of ethanol consumption as a consequence of exposure to an intermittent-access (IAE) 20% ethanol-drinking paradigm. Additionally, we evaluated whether binge-like ethanol exposure during adolescence alters the expected reduction in feeding and ethanol consumption following NAc shell administration of a selective MC4-R agonist in adult rats showing high rates of ethanol consumption.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rat pups on postnatal day (PND 21; Charles River Laboratories, Spain) arrived at the laboratory and were housed in groups of four rats per cage in an environmentally controlled room (22°C temperature on a 12:12 h light-dark cycle with lights off at 8 am). After an acclimatization period of 4 days (until PND 25) during which the animals were handled briefly every day, the paradigm of binge-like ethanol exposure began. At adulthood, rats were cannulated bilaterally in the NAc and thereafter were housed individually. Standard rodent chow and water were provided *ad libitum* throughout the experiments, and the manipulations were conducted at the onset of the dark phase. All the procedures used were in accordance with the animal care guidelines established by Spanish Royal Decree 53/2013 and were

approved by the University of Almeria Bioethical Animal Care and Use Committee.

Ethanol Exposure

Morning doses of either 25% (w/v) ethanol (3.0 g/kg) in isotonic saline (binge ethanol pretreatment group, BEP) or saline (saline pretreatment group, SP) were administered intraperitoneally (i.p.) to 25-day-old pups on a 4-day cycle, consisting of two injection days followed by 2 days without injections, for 2 weeks. Specifically, pups were injected at PND 25, 26, 29, 30, 33, 34, 37 and 38 (Pascual et al., 2009; Lerma-Cabrera et al., 2013a). Consistent with previous studies, this paradigm of binge-like ethanol exposure produced a blood ethanol concentration (BEC) of 210 ± 11 mg/dl 30 min after each single dose of ethanol (Pascual et al., 2007; **Figure 1A**).

Surgery

Twenty-five days after the last ethanol injection administered during adolescence (adult stage, PND 63), rats were anesthetized with Equithesin (0.3 ml/100 g) for cannula placement. Bilateral 26-G cannulae (Plastics One, Inc., Germany) were aimed at the NAc shell, using the following stereotaxic coordinates (Paxinos and Watson, 1998): AP: −1.7 mm, ML: ±0.8 mm and DV: −5.4 mm. The rats were allowed to recover for 10 days before any experimental manipulation.

During the recovery phase, animals were maintained warm until righting reflex has returned and also, sterile isotonic fluid was administered subcutaneously to facilitate proper hydration. Finally, to minimize wound contamination, a new cage with clean bedding is provided. During the first week after surgery, the general condition of the animal (BW, food/water intake, sign of pain or distress. . .) was monitored according to a monitoring protocol adapted from Morton and Griffiths (1985). Also, sign of infection at the incision were checked daily and treated when it needed.

When all procedures were complete, cannula placement was histologically verified (**Figure 1B**). Animals with injector tips outside the target regions (1–2 per treatment in each group) were discarded from the analysis, yielding final group sizes of n = 20 for the BEP group and n = 24 for the SP group.

Behavioral Procedures

Intermittent-Access 20% Ethanol 2-Bottle-Choice Drinking Paradigm

After 10 days of postsurgical recovery and before NAc infusion of the selective MC4-R agonist, the rats received 24 h of free access to two bottles (one containing water and the other containing 20% (v/v) ethanol) three times per week across 45 days as described previously by Simms et al. (2008). Given that ethanol concentration (25%) used in the protocol of binge-like ethanol exposure is probably to lead to aversive reactions, we decided to use the same ethanol concentration proposed by Simms et al. (2008). On Monday, Wednesday and Friday, each rat was given access to one bottle of 20% (v/v) ethanol and one bottle of water. After 24 h, the ethanol bottle was replaced with a second water bottle that was available for the next 24 h. Thus, the animals were exposed to a total of 19 sessions in which the ethanol bottle was available. The positions of the bottles were changed every ethanol drinking session to control for position preferences. To obtain measures of consumption that corrected for individual differences in rat size, we calculated intake in g of ethanol consumed per kg of BW for each 24-h measurement of ethanol intake (g/kg/24 h). Throughout the experiments, intake measures (ethanol, food and water) and BW were assessed.

Procedure for NAc Administration of the Selective MC4-R Agonist

Following 45 days during which baseline intake measures were collected (**Figure 1A**), both the BEP and SP groups were distributed into three subgroups matched for ethanol

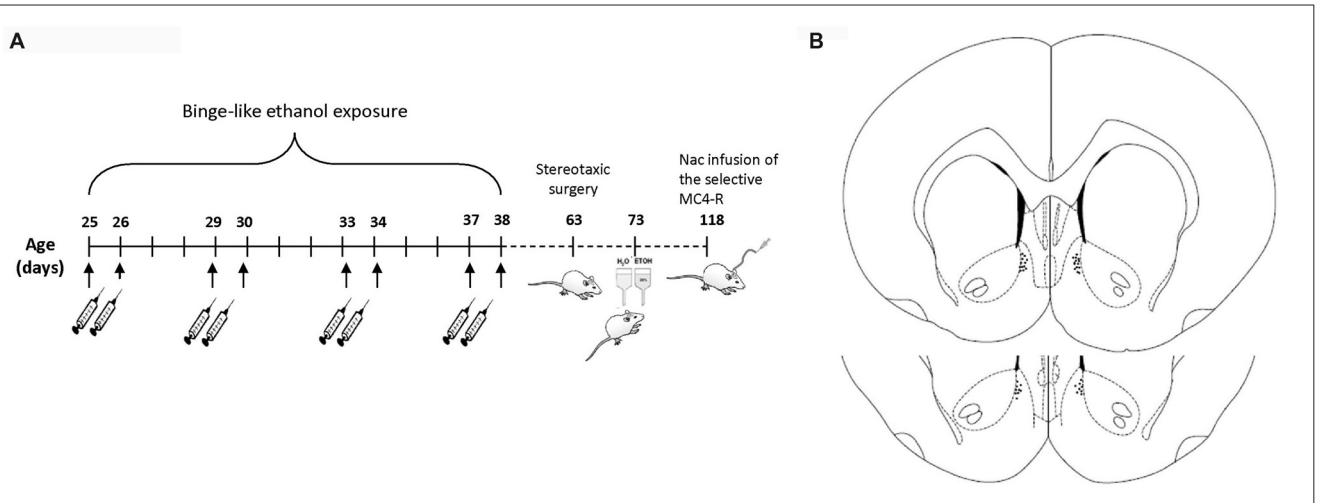


FIGURE 1 | (A) Illustration of the timeline of binge-like ethanol exposure, behavioral measures and pharmacological treatment. **(B)** Histological reconstruction of location of injection sites in the Nucleus accumbens (NAc) adapted from Paxinos and Watson (1998).

consumption (g/kg/24 h) over the final 3 days of IAE. In this way, we ensured that the baseline level of ethanol consumption was equal between the groups. One hour before the beginning of the dark phase of the light:dark cycle, the rats were weighed and the ethanol, water and food were removed from the cages. Then, isotonic saline or the selective MC4-R agonist cyclo(NH-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Glu)-NH_2 (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) at one of two possible doses (1.5 or 0.75 μg) was bilaterally injected at a volume of 0.5/site into the NAc (n = 10 per treatment in each group). The MC4-R agonist used is 90-fold and 3400-fold selective for MC4-R over MC3-R and MC5-R, respectively (Bednarek et al., 2001). We have previously found that the selected doses of that MC4-R agonist were effective in reducing feeding and ethanol consumption in Sprague-Dawley rats (Lerma-Cabrera et al., 2012). Site-directed infusions were given manually over a 1-min period using a 1.0 μl Hamilton syringe connected to a 33-G injector cannula (Plastics One, Inc., Germany) that extended 1 mm beyond the guide cannula. The injector was left in place for an additional 60 s to prevent reflux. Then, rats were immediately returned to their home cage. Within a period of 15 min after drug treatment, ethanol, water and food were returned to the cages. Previous evidence showed that the highest level of food intake in rats occurs during the first 4-h of dark cycle (Tabarin et al., 2007). Moreover, given that differences in food and ethanol intake could vary over this time as described in Lerma-Cabrera et al. (2012), non-cumulative intake measures were collected in two 2-h intervals following drug administration.

Blood Ethanol Concentration (BEC)

One week after NAc administration of the selective MC4-R agonist, when consumption measures had stabilized and returned to baseline levels, approximately 10 μl of blood was collected from the tail vein of each rat 4 h after the beginning of the ethanol exposure session. The samples were centrifuged, and 5 μl of plasma from each sample was analyzed for BEC measured in mg/dl (Analox Instruments, Lunenburg, MA, USA). Additionally, 4-h ethanol consumption was recorded (g/kg/4 h) to study the correlation between the level of ethanol intake and BEC for SP and BEP animals.

Data Analyses

Data from the intermittent-access ethanol drinking paradigm were analyzed using a repeated measures analysis of variance (ANOVA) with pretreatment (BEP and SP group) as the between-subject variable. Non-cumulative ethanol and food intake data were compared using a two-way (drug × pretreatment) repeated measures ANOVA that evaluated the short-term effects of NAc-administration of two doses of the selective MC4-R agonist or saline, at 2 and 4 h after infusion in binge ethanol- or saline-pretreated rats during adolescence. Additionally, total calories (cal/kg/4 h; calories from chow + ethanol solution) consumed in the first 4 h before treatment were analyzed with 3 × 2 (drug × pretreatment) ANOVAs. Finally, one-way ANOVAs were used to analyze BECs (mg/dl) and ethanol consumption (g/kg/4 h). When significant differences were found ($p < 0.05$), pairwise comparisons were conducted with a *post hoc* Fisher's LSD test. All data are presented as the means ± SEM.

RESULTS

Intermittent-Access 20% Ethanol Drinking Paradigm in Saline (SP) and Binge-Ethanol (BEP) Pretreated Groups: Measures of Ethanol and Food Intake

In agreement with previous reports (Simms et al., 2008; Hwa et al., 2011), the intermittent-access 20% ethanol 2-bottle-choice drinking procedure induced high levels of ethanol intake in both the SP and BEP groups (average ethanol consumption during the last week: SP = 4.36 ± 0.31 g/kg/24 h and BEP = 4.73 ± 0.4 g/kg/24-h). Data showing 24-h voluntary ethanol consumption in the SP and BEP groups using the intermittent-access 20% ethanol drinking paradigm are presented in **Figure 2A**. A repeated-measures ANOVA performed on ethanol consumption data over 6 weeks revealed a significant main effect of week ($F_{(5,215)} = 6.00$; $p < 0.01$) and a pretreatment × week interaction ($F_{(5,215)} = 3.48$; $p < 0.01$). With respect to the main effect of time, the data revealed that ethanol intake was higher during the first, third and fourth weeks ($p < 0.01$ for all comparisons). Further pairwise comparison using Fisher's LSD test revealed that the SP group drank significantly more ethanol than the BEP group during the first week of ethanol consumption ($p < 0.05$); however, these differences disappeared over time. At the end of the experimental procedure, the two groups showed similar, high rates of voluntary ethanol consumption.

Data showing 24-h food intake in the SP and BEP groups are represented in **Figure 2B**. A repeated-measures ANOVA performed on food intake data over 6 weeks revealed significant main effects of pretreatment ($F_{(1,44)} = 19.57$; $p < 0.01$) and week ($F_{(5,220)} = 193.72$; $p < 0.01$) but not pretreatment × week interaction. These data showed that the BEP group ate significantly more than the SP group and both groups decreased their food intake over time ($p < 0.01$ for all comparisons).

Because MC4-Rs are implicated in BW regulation (Adan et al., 2006; Krashes et al., 2016; You et al., 2016), we evaluated whether group differences in food intake were primarily associated with altered BW induced by previous binge-like ethanol exposure during adolescence. Data showing the BW of the SP and BEP groups during the intermittent-access 20% ethanol drinking paradigm are represented in **Figure 2C**. The repeated-measures ANOVA exhibited a significant main effect of week ($F_{(5,220)} = 625.24$; $p < 0.01$) showing that BW increased across the weeks. The pretreatment × week interaction attained statistical significance ($F_{(5,220)} = 9.59$, $p < 0.01$). The analysis of the interaction revealed that the BEP group showed reduced BW compared with the SP group during the first ($p < 0.001$) and second weeks ($p < 0.01$). No statistical significance was reported for the main factor of pretreatment. Consistent with previous reports (Maldonado-Devincci et al., 2010a), these data suggest that binge-like ethanol exposure during adolescence did not

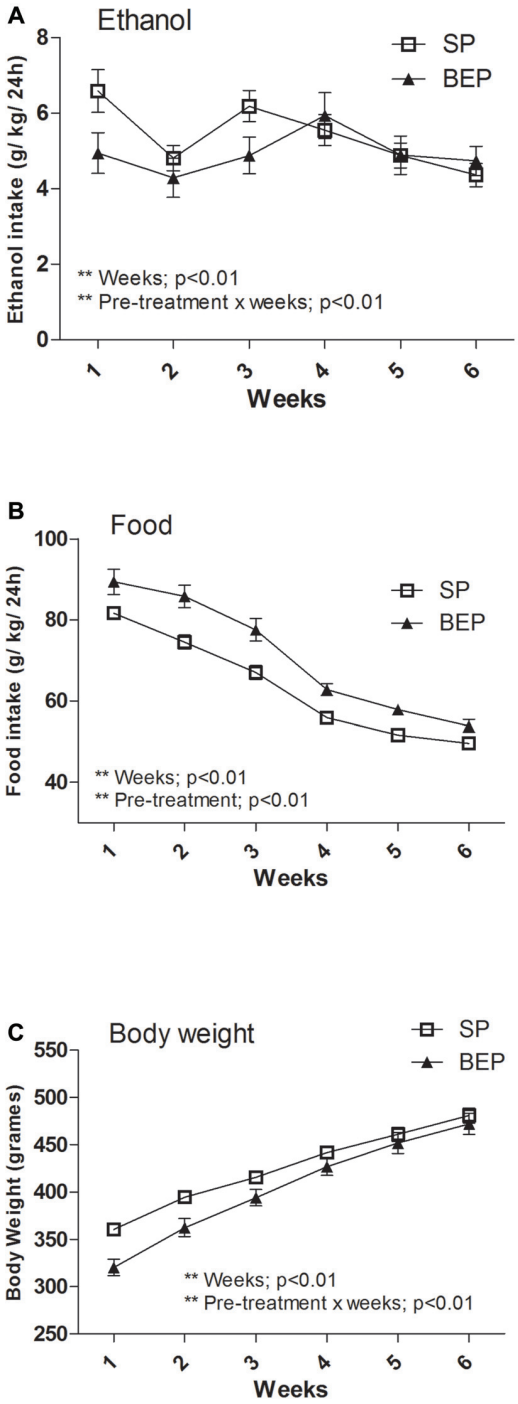


FIGURE 2 | Weekly average 24-h ethanol consumption **(A)**, food intake **(B)** and body weight (BW) **(C)**, collected for the first six complete weeks in an intermittent-access (IAE) 20% ethanol 2-bottle-choice drinking paradigm in both the binge-like ethanol pre-treatment (BEP) and saline pretreatment (SP) groups. Panel **(A)** shows baseline ethanol consumption of 4.36 ± 0.31 and 4.74 ± 0.39 g/kg/24 h for the SP and BEP groups, respectively. All values are means ± SEM.

cause relevant and/or permanent disturbances in BW regulation during adulthood.

Effects of the Selective MC4-R Agonist on Food, Ethanol and Total Calories Consumed by the Saline (SP) and Binge Ethanol-Pretreated (BEP) Groups

Figure 3A represents non-cumulative food consumption over two 2-h intervals following NAc infusion of the selective MC4-R agonist in the SP and BEP groups. A repeated-measures ANOVA performed on non-cumulative food consumption data revealed statistically significant main effects of drug ($F_{(2,37)}$ = 48.14; $p < 0.01$) and time ($F_{(1,37)}$ = 17.12, $p < 0.01$; food consumption at 2–4 h < consumption at 0–2 h). Specifically, *post hoc* Fisher's LSD test revealed that administration of 0.75 μg and 1.5 μg significantly decreased food consumption relative to saline ($p < 0.01$) in a manner unrelated to binge-like ethanol pre-exposure.

The ANOVA performed on non-cumulative ethanol consumption data following NAc infusion of the selective MC4-R agonist (**Figure 3B**) showed a statistically significant drug × time interaction ($F_{(2,37)}$ = 3.55; $p < 0.05$). *Post hoc* analysis revealed that both doses of the selective MC4-R agonist (0.75 μg and 1.5 μg) significantly reduced ethanol consumption relative to saline at the second 2-h interval

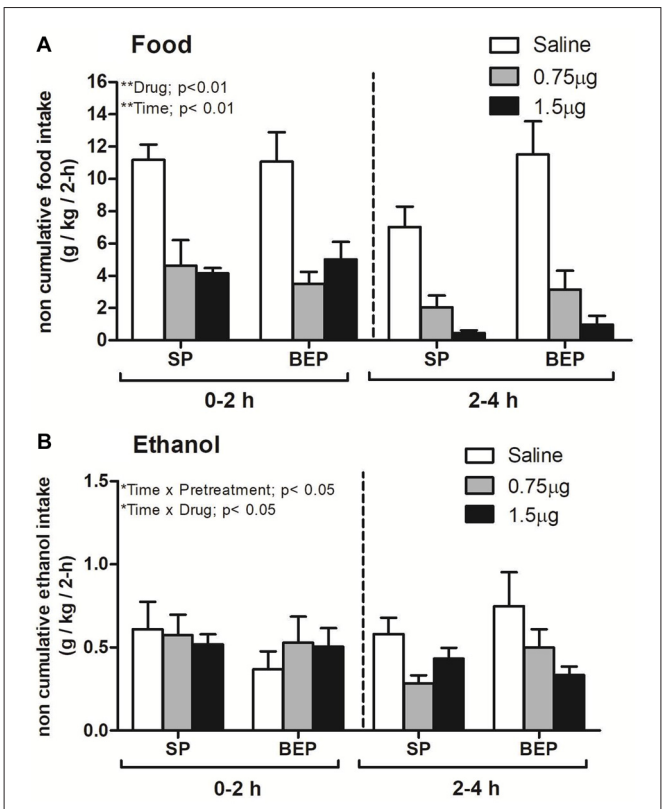


FIGURE 3 | Non-cumulative food **(A)** and ethanol consumption **(B)** in the SP and BEP groups over two 2-h intervals after NAc administration of the selective melanocortin 4 receptor (MC4-R) agonist cyclo(NH-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Glu)-NH_2 (0, 0.75 μg or 1.5 μg). Administration of the selective agonist significantly decreased food and ethanol consumption relative to saline administration in both the SP and BEP groups. All values are means ± SEM.

TABLE 1 | Total calories consumed in 4 h (cal/kg/4 h) by the SP and BEP groups after infusion of a selective MC4-R agonist (0.75 μg or 1.5 μg) or isotonic saline into the NAc. Mean ± SEM.

	BEP group	SP group
MC4-R agonist		
Saline	73.31 ± 7.99	60.76 ± 6.47
0.75 μg	26.47 ± 4.27[a]	25.32 ± 6.44[a]
1.5 μg	23.24 ± 4.39[a]	19.67 ± 1.04[a]

[a]$p < 0.05$ with respect to saline control.

($p < 0.05$). A pretreatment × time interaction was also observed ($F_{(1,37)} = 4.67$; $p < 0.05$) and Fisher's LSD test revealed that SP group decrease ethanol intake between the first and the second interval ($p < 0.05$); however, the natural tendency to decrease ethanol intake over time showed in SP group was not present in BEP group. Thus, the BEP group continues to consume large amount of ethanol both at first and second 2-h interval ($p < 0.05$).

Finally, the administration of the MC4-R agonist into the NAc was able to decrease total calories (cal/kg/4 h) consumed within 4 h after treatment (main effect of drug [$F_{(2,37)} = 40.29$; $p < 0.01$]). Concretely, both doses of the selective MC4-R agonist significantly decreased caloric intake compared with saline administration ($p < 0.01$; **Table 1**). Neither the main effect of pretreatment nor the pretreatment × drug interaction attained statistical significance.

Blood Ethanol Concentration Following 4 h of Voluntary Ethanol Consumption in SP and BEP Animals Exposed to an Intermittent-Access 20% Ethanol Drinking Paradigm

Average ethanol consumed (g/kg/4 h) and BECs (mg/dl/4 h) for SP and BEP rats following 4 h of free access to ethanol using the intermittent-access 20% ethanol drinking paradigm 1 week after agonist administration are presented in **Figure 4** [Ethanol intake: SP rats 1.50 ± 0.17 g/kg/4-h and BEP rats 1.52 ± 0.22 g/kg/4-h; BECs: SP rats 43.5 ± 3.66 (mg/dl/4-h) and BEP rats 41.5 ± 3.23, (mg/dl/4-h)]. Independent one-way ANOVAs performed on ethanol consumption and BEC data revealed no significant main effect of pretreatment ($F_{(1,14)} = 0.00$; $p > 0.05$ and $F_{(1,14)} = 0.15$; $p > 0.50$, respectively).

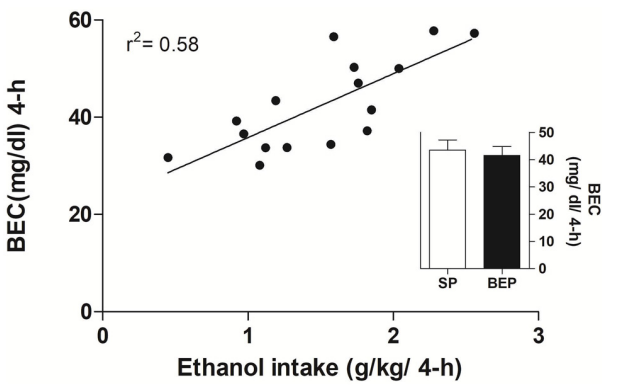


FIGURE 4 | Blood ethanol concentration (BEC; mg/dl) and ethanol intake (g/kg/4 h) were evaluated across 4 h in the SP and BEP groups 1 week before NAc administration of the selective MC4-R agonist. There was no difference in BEC or ethanol consumption between groups. The amount of ethanol consumed in a 4-h drinking session significantly correlated with the measured BEC (linear regression).

DISCUSSION

The most important observations in the study are as follows: (1) intermittent 20% ethanol drinking triggered high spontaneous ethanol intake in adult animals of both the SP and BEP groups; suggesting that under the experimental conditions we employed, binge-like ethanol pre-exposure during adolescence did not enhance vulnerability to ethanol consumption in adulthood; (2) a combination of binge-like ethanol pretreatment during adolescence (BEP) and high levels of ethanol intake due to intermittent ethanol exposure during adulthood triggered increased food intake without a parallel increase in BW in BEP animals; and (3) NAc infusion of a selective MC4-R agonist significantly reduced feeding, ethanol intake and total calories in adult animals with high rates of ethanol consumption, independent of pretreatment.

Regarding the first observation, several studies have previously demonstrated that standard adult laboratory rats, despite having no genetic vulnerability to high rates of spontaneous ethanol consumption, would consume high and pharmacologically relevant levels of ethanol when given intermittent access to 20% ethanol in a 2-bottle-choice drinking paradigm (Simms et al., 2008; Hwa et al., 2011; Li et al., 2011). Consistent with this, we report here that intermittent exposure to 20% ethanol induced high rates of ethanol intake in adult Sprague-Dawley rats, correlated with pharmacologically relevant BEC, as measured during the first 4 h of ethanol access.

More importantly, this behavioral profile was unrelated to any previous history of binge-like ethanol exposure during adolescence. This fact contrasts with previous research showing that binge-like ethanol exposure during adolescence increases voluntary ethanol consumption in adulthood (Pascual et al., 2009; Maldonado-Devincci et al., 2010a). It has been demonstrated that ethanol exposure during adolescence elicits neurobiological changes, such as up-regulation of neuroinflammatory mediators (Pascual et al., 2007), down-regulation of dopamine and glutamate receptors (Pascual et al., 2009) or increased expression of corticotrophin-releasing hormone (Przybycien-Szymanska et al., 2011). Moreover, we previously showed that binge-like ethanol exposure during adolescence reduces basal α-MSH and alters AgRP expression in hypothalamic and limbic areas in adult rats (Lerma-Cabrera et al., 2013a). Additionally, pharmacological studies showed that administration of a selective MC4-R antagonist into the NAc increases ethanol drinking in Sprague-Dawley rats (Lerma-Cabrera et al., 2012), suggesting that endogenous α-MSH might have a protective role against excessive ethanol intake by negatively modulating the rewarding properties of ethanol via MC4-R signaling in the

NAc. When the evidence is taken together, it is tempting to propose that a long-lasting reduction of α-MSH in hypothalamic and limbic areas, triggered by binge-like ethanol pretreatment during adolescence, might enhance vulnerability to ethanol consumption during adulthood. Surprisingly, in our study, binge-ethanol administration during adolescence did not enhance ethanol consumption as tested through an intermittent 20% ethanol drinking paradigm during adulthood. Because this paradigm triggers high rates of voluntary ethanol consumption in all groups (Simms et al., 2008), the possibility that a ceiling effect obscured detection of changes in ethanol consumption between BEP and SP group exists. On the other hand, there is alternative experimental evidence that exposure to ethanol during adolescence does not necessarily increase the risk of ethanol abuse and dependence during adulthood (Siegmund et al., 2005; Clark et al., 2008; Gurkovskaya et al., 2009) and that differences do not emerge until subjects experience a stressor such as exposure to foot shock or forced swim (Siegmund et al., 2005). Furthermore Gilpin et al. (2012) reported that forced exposure to binge-like alcohol during adolescence causes long-lasting reduction, rather than increase, in voluntary ethanol consumption in adulthood (Gilpin et al., 2012). The contrasting results in these studies may be related to the use of different protocol of ethanol exposure during adolescence as well as the paradigm used to evaluate ethanol intake in adulthood. Therefore, the present data cannot rule out that our protocol of binge-like ethanol exposure during adolescence might have elicited unobserved or silent adaptations in neural mechanisms involved in ethanol consumption. But these neurobiological alterations could be only evident under certain condition such as stress or ethanol withdrawal as proposed elsewhere (Siegmund et al., 2005; Gilpin et al., 2012). Our results emphasize the need to further characterize the conditions under which binge-like ethanol exposure during adolescence might affect ethanol consumption during adulthood.

Regarding the second observation, we show here that a combination of binge-like ethanol pretreatment during adolescence (BEP) and high levels of ethanol intake due to ethanol intermittent exposure during adulthood triggered increased food intake without a parallel increase in BW in BEP animals. The present data are in accordance with clinical observations showing a high rate of co-morbidity between alcohol abuse and eating disorders (Gregorowski et al., 2013; Fouladi et al., 2015; Rush et al., 2016). A recent study determined that the rate of co-occurrence between eating disorders and binge drinking in young people between 18 and 22 years old is as high as 18% (Rush et al., 2016). Additionally, it has been demonstrated that severity and frequency of alcohol consumption in adolescents are positively associated with number of eating disorder symptoms (Arias et al., 2009). Although these epidemiological studies show the relationship between alcohol intake during adolescence and eating disorders, studies evaluating the impact of alcohol consumption during adolescence on eating disorders in adulthood are scarce. Previous studies carried out in animal models had shown the existence of a set threshold for the stimulation of appetite and food intake by alcohol (Caton et al., 2004). This could explain why adult rats that consume high levels of ethanol and were pre-exposed to binge ethanol during adolescence are more sensitive to the stimulatory effect of alcohol on food intake than those not pre-exposed to ethanol during adolescence.

Further experiments are needed to evaluate the potential implication of ethanol exposure during adolescence regarding energy balance in adulthood. However, given that enhanced feeding in the BEP group was not parallel to increased BW, there is the possibility that binge-like ethanol exposure during adolescence might have elicited brain alterations, such as changes in hypothalamic neuropeptides function, triggering caloric efficiency disturbances in adult rats exhibiting high ethanol consumption. One possible explanation for why BEP group exhibited increased food intake but no difference in BW with respect to SP would be group differences in spontaneous locomotor activity. However, ethanol exposure during adolescence reduces, rather than increases, spontaneous locomotor activity (Pascual et al., 2009; Teixeira et al., 2014), suggesting that other factors may be responsible for our data. Another hypothesis that could explain the present data relies on the effect of binge-like ethanol exposure during adolescence on the MC system, a key neural pathway involved in the regulation of food intake and energy balance (De Jonghe et al., 2011; Krashes et al., 2016). First, binge-like ethanol exposure during adolescence significantly reduces basal α-MSH in hypothalamic and limbic areas and increases AgRP immunoreactivity in response to a high dose of ethanol during adulthood (Lerma-Cabrera et al., 2013a). Second, i.c.v. administration of α-MSH in rats (Abbott et al., 2000) or of MTII, a nonselective MCR agonist, in mice reduces food intake (Navarro et al., 2003). Moreover, MC4-R knockout mice do not reduce their food intake following i.c.v. administration of MTII (Marsh et al., 1999; Navarro et al., 2011). Conversely, increased food intake has been reported after administration of a selective antagonist of MC4-R into the lateral hypothalamus, NAc and VTA (Lerma-Cabrera et al., 2012). Finally, it has recently been demonstrated that AgRP cell activity is essential for ethanol-induced overeating (Cains et al., 2017). Together, these data suggest the critical role of MC4-R signaling in energy balance. Given this fact and our present data, there is a possibility that binge-like ethanol exposure during adolescence combined with high rates of ethanol intake during adulthood alters MC function, which might contribute to energy metabolism disturbances during adulthood.

Finally, we report here that NAc infusion of a selective MC4-R agonist triggered a strong anorectic response and reduced total calories consumed over a 4-h testing period in animals showing high rates of ethanol consumption (more than 4 g/kg/24 h), unrelated to previous history of binge-like ethanol exposure during adolescence. It has been reported that administration of an MC4-R agonist does not cause significant changes in locomotor activity (Klenerová et al., 2008; Bertolini et al., 2009); therefore, it is unlikely that reduced food and ethanol intake following MC4-R activation were the result of generalized behavioral inhibition. Our data, showing reduced

ethanol and food intake in high ethanol drinkers as a result of the administration of a selective MC4-R agonist, extend our previous observation reported in low ethanol drinkers (Lerma-Cabrera et al., 2012). Additionally, they are consistent with our previous studies demonstrating that an MC4-R agonist blunts ethanol drinking in high-ethanol-consuming C57BL/6J mice (Navarro et al., 2005) and that MTII, a nonselective MC4/3-R agonist, fails to reduce ethanol drinking in MC4-R knockout mice (Navarro et al., 2011). The present findings add relevant preclinical information regarding the relevance of MC4-R signaling into the NAc as a potential target for therapeutic interventions for eating disorders (Adan et al., 2006; Girardet and Butler, 2014) and alcohol use disorders (AUDs) in humans (Olney et al., 2014a).

Importantly, we have previously demonstrated that binge-like ethanol exposure during adolescence reduces basal α-MSH and alters AgRP expression in hypothalamic and limbic areas in adult rats (Lerma-Cabrera et al., 2013a). However, pre-exposure to ethanol during adolescence did not alter the effect of administration of selective MC4-R agonist into NAc on feeding and ethanol consumption. It is possible that reduction in basal α-MSH-induced by binge-like ethanol pre-exposure during adolescence was not sufficient to blunt the effect of selective agonist of MC4-R into NAc. Additional studies aimed to quantify protein level of α-MSH and MC4-R after NAc administration of selective agonist of MC4-R in adult rats pre-exposed to ethanol during adolescence may help to test this hypothesis. Another possible explanation for the present data relies on the fact that α-MSH exerts its effects by binding to both MC3-R and MC4-R. However, we used a selective agonist of MC4-R leaving MC3-R functioning unimpeded. It is known that MC3-R regulate the endogenous α-MSH signaling onto MC4R, probably acting as an inhibitory autoreceptor on POMC neurons (Renquist et al., 2011). Moreover, a recent study demonstrated that MC3R−/− mice were more sensitive to the protective effects of MTII than MC3R+/+ mice (Olney et al., 2014b) suggesting that MC3-R may contribute to binge-like ethanol drinking. Given that in this study we used an intermittent ethanol access schedule, a model of binge-like alcohol drinking in rats (Simms et al., 2014), the contribution of MC3-R to ethanol consumption in adults rats pre-exposed to binge-like ethanol paradigm during adolescence should be evaluated in future studies.

Because ethanol has both caloric and reward properties, the mechanisms underlying the effect of NAc administration of a selective MC4-R agonist on ethanol intake remain unclear. Taking into account that direct ingestive responses are not valid direct measures of the hedonic value of ethanol/food (Salamone et al., 2016), we cannot conclude whether intake disturbances observed after NAc infusion of the selective MC4-R agonist were calorically driven or whether they were the result of hedonically driven non-homeostatic processes. Furthermore, given our previous data (Lerma-Cabrera et al., 2013b), it is tempting to speculate that MC4-R signaling within the NAc shell has a key role in non-homeostatic aspects (palatability) of ethanol as well as food intake also in high ethanol drinkers. Although the precise nature of the neurochemical mechanisms involved remains uncertain, MC/opioid interactions within the NAc have been proposed and discussed elsewhere (Lerma-Cabrera et al., 2012). Anatomical (Grossman et al., 2003; Bertolini et al., 2009) and pharmacological studies (Ploj et al., 2002; Starowicz et al., 2003; Kalange et al., 2007) have suggested the existence of an important neurobiological interaction between MC and opioids. Recently, it has been shown that administration of MTII synergistically augments the ability of naltrexone to blunt binge-like ethanol intake in mice (Navarro et al., 2015). Given the role of opioids in ethanol palatability (Peciña, 2008; Katsuura and Taha, 2014; Ikeda et al., 2015; Uhari-Väänänen et al., 2016), one interesting avenue for future research will be to explore the contribution of MC-opioid interactions within the NAc to the hedonic aspects of ethanol consumption in high ethanol drinkers.

We report here that a combined history of binge-like ethanol pre-exposure during adolescence and high ethanol consumption during adulthood elicits an increase in food intake without BW changes in adulthood, which suggests caloric efficiency disturbances. Additionally, this study reports that MC4-R stimulation within the NAc reduces feeding and ethanol intake in high ethanol-drinking adult rats, unrelated to previous episodes of binge-like ethanol exposure during adolescence, which adds new evidence regarding the dual ability of MC compounds to control excessive ethanol and food intake.

AUTHOR CONTRIBUTIONS

IC, MN and TET made substantial contribution to the study concept and design. FC, JML-C and MA-I conducted the experiments. FC, JML-C, MA-I and IC analyzed the data. All the authors critically reviewed content and approved the final version for publication.

ACKNOWLEDGMENTS

This work was supported by MEC (Spain; SEJ2006-03629, PSI2009-07677 and CTS1350), FEDER UALM05-23-006, the NIH (AA013573, AA015148, AA022048), the Department of Defense (W81XWH-09-1-0293) and CONICYT (Chile; Fondecyt 1140284 and 1150308 and Programa de Investigacion Asociativa ACT1411.

REFERENCES

Abbott, C. R., Rossi, M., Kim, M. S., AlAhmed, H., Taylor, G. M., Ghatei, M. A., et al. (2000). Investigation of the melanocyte stimulating hormones on food intake. Lack of evidence to support a role for the melanocortin-3-receptor. *Brain Res.* 869, 203–210. doi: 10.1016/S0006-8993(00)02386-6

Adan, R. A., Tiesjema, B., Hillebrand, J. J., la Fleur, S. E., Kas, M. J., and de Krom, M. (2006). The MC4 receptor and control of appetite. *Br. J. Pharmacol.* 149, 815–827. doi: 10.1038/sj.bjp.0706929

Arias, J. E., Hawke, J. M., Arias, A. J., and Kaminer, Y. (2009). Eating disorder symptoms and alcohol use among adolescents in substance abuse treatment. *Subst. Abuse* 3, 81–91.

Bednarek, M. A., MacNeil, T., Tang, R., Kalyani, R. N., Van der Ploeg, L. H., and Weinberg, D. H. (2001). Potent and selective peptide agonists of alpha-melanotropin action at human melanocortin receptor 4: their synthesis and biological evaluation *in vitro*. *Biochem. Biophys. Res. Commun.* 286, 641–645. doi: 10.1006/bbrc.2001.5444

Bertolini, A., Tacchi, R., and Vergoni, A. V. (2009). Brain effects of melanocortins. *Pharmacol. Res.* 1, 13–47. doi: 10.1016/j.phrs.2008.10.005

Cains, S., Blomeley, C., Kollo, M., Rácz, R., and Burdakov, D. (2017). Agrp neuron activity is required for alcohol-induced overeating. *Nat. Commun.* 8:14014. doi: 10.1038/ncomms14014

Caton, S. J., Ball, M., Ahern, M. M., and Hetherington, M. M. (2004). Dose-dependent effects of alcohol on appetite and food intake. *Physiol. Behav.* 81, 51–58. doi: 10.1016/j.physbeh.2003.12.017

Clark, D. B., Thatcher, D. L., and Tapert, S. F. (2008). Alcohol, psychological dysregulation, and adolescent brain development. *Alcohol. Clin. Exp. Res.* 32, 375–385. doi: 10.1111/j.1530-0277.2007.00601.x

Cone, R. D. (2005). Anatomy and regulation of the central melanocortin system. *Nat. Neurosci.* 8, 571–578. doi: 10.1038/nn1455

De Jonghe, B. C., Hayes, M. R., and Bence, K. K. (2011). Melanocortin control of energy balance: evidence from rodent models. *Cell. Mol. Life Sci.* 68, 2569–2588. doi: 10.1007/s00018-011-0707-5

Fouladi, F., Mitchell, J. E., Crosby, R. D., Engel, S. G., Crow, S., Hill, L., et al. (2015). Prevalence of alcohol and other substance use in patients with eating disorders. *Eur. Eat. Disord. Rev.* 23, 531–536. doi: 10.1002/erv.2410

Gantz, I., and Fong, T. M. (2003). The melanocortin system. *Am. J. Physiol. Endocrinol. Metab.* 284, E468–E474. doi: 10.1152/ajpendo.00434.2002

Gilpin, N. W., Karanikas, C. A., and Richardson, H. N. (2012). Adolescent binge drinking leads to changes in alcohol drinking, anxiety, and amygdalar corticotropin releasing factor cells in adulthood in male rats. *PLoS One* 7:e31466. doi: 10.1371/journal.pone.0031466

Girardet, C., and Butler, A. A. (2014). Neural melanocortin receptors in obesity and related metabolic disorders. *Biochim. Biophys. Acta* 1842, 482–494. doi: 10.1016/j.bbadis.2013.05.004

Gregorowski, C., Seedat, S., and Jordaan, G. P. (2013). A clinical approach to the assessment and management of co-morbid eating disorders and substance use disorders. *BMC Psychiatry* 13:289. doi: 10.1186/1471-244x-13-289

Grossman, H. C., Hadjimarkou, M. M., Silva, R. M., Giraudo, S. Q., and Bodnar, R. J. (2003). Interrelationships between μ opioid and melanocortin receptors in mediating food intake in rats. *Brain Res.* 991, 240–244. doi: 10.1016/s0006-8993(03)03442-5

Gurkovskaya, O. V., Leonard, S. T., Lewis, P. B., and Winsauer, P. J. (2009). Effects of pregnanolone and dehydroepiandrosterone on ethanol intake in rats administered ethanol or saline during adolescence. *Alcohol. Clin. Exp. Res.* 33, 1252–1264. doi: 10.1111/j.1530-0277.2009.00951.x

Hillebrand, J. J., Kas, M. J., and Adan, R. A. (2006). To eat or not to eat; regulation by the melanocortin system. *Physiol. Behav.* 89, 97–102. doi: 10.1016/j.physbeh.2006.01.034

Hwa, L. S., Chu, A., Levinson, S. A., Kayyali, T. M., DeBold, J. F., and Miczek, K. A. (2011). Persistent escalation of alcohol drinking in C57BL/6J mice with intermittent access to 20% ethanol. *Alcohol. Clin. Exp. Res.* 35, 1938–1947. doi: 10.1111/j.1530-0277.2011.01545.x

Ikeda, H., Ardianto, C., Yonemochi, N., Yang, L., Ohashi, T., Ikegami, M., et al. (2015). Inhibition of opioid systems in the hypothalamus as well as the mesolimbic area suppresses feeding behavior of mice. *Neuroscience* 311, 9–21. doi: 10.1016/j.neuroscience.2015.10.002

Kalange, A. S., Kokare, D. M., Singru, P. S., Upadhya, M. A., Chopde, C. T., and Subhedar, N. K. (2007). Central administration of selective melanocortin 4 receptor antagonist HS014 prevents morphine tolerance and withdrawal hyperalgesia. *Brain Res.* 1181, 10–20. doi: 10.1016/j.brainres.2007.08.054

Katsuura, Y., and Taha, S. A. (2014). Mu opioid receptor antagonism in the nucleus accumbens shell blocks consumption of a preferred sucrose solution in an anticipatory contrast paradigm. *Neuroscience* 261, 144–152. doi: 10.1016/j.neuroscience.2013.12.004

Klenerová, V., Krejcí, I., Sída, P., Hlinák, Z., and Hynie, S. (2008). Effects of melanotan II, a melanocortin agonist, on grooming and exploration in rats after repeated restraint/immobilization. *Neurosci. Lett.* 432, 202–205. doi: 10.1016/j.neulet.2007.12.020

Krashes, M. J., Lowell, B. B., and Garfield, A. S. (2016). Melanocortin-4 receptor-regulated energy homeostasis. *Nat. Neurosci.* 19, 206–219. doi: 10.1038/nn.4202

Laviano, A., Inui, A., Marks, D. L., Meguid, M. M., Pichard, C., Rossi Fanelli, F., et al. (2008). Neural control of the anorexia-cachexia syndrome. *Am. J. Physiol. Endocrinol. Metab.* 295, E1000–E1008. doi: 10.1152/ajpendo.90252.2008

Lerma-Cabrera, J. M., Carvajal, F., de la Torre, L., de la Fuente, L., Navarro, M., Thiele, T. E., et al. (2012). Control of food intake by MC4-R signaling in the lateral hypothalamus, nucleus accumbens shell and ventral tegmental area: interactions with ethanol. *Behav. Brain Res.* 234, 51–60. doi: 10.1016/j.bbr.2012.06.006

Lerma-Cabrera, J. M., Carvajal, F., Alcaraz-Iborra, M., de la Fuente, L., Navarro, M., Thiele, T. E., et al. (2013a). Adolescent binge-like ethanol exposure reduces basal α-MSH expression in the hypothalamus and the amygdala of adult rats. *Pharmacol. Biochem. Behav.* 110, 66–74. doi: 10.1016/j.pbb.2013.06.006

Lerma-Cabrera, J. M., Carvajal, F., Chotro, G., Gaztañaga, M., Navarro, M., Thiele, T. E., et al. (2013b). MC4-R signaling within the nucleus accumbens shell, but not the lateral hypothalamus, modulates ethanol palatability in rats. *Behav. Brain Res.* 239, 51–54. doi: 10.1016/j.bbr.2012.10.055

Li, J., Zou, Y., and Ye, J. H. (2011). Low frequency electroacupuncture selectively decreases voluntarily ethanol intake in rats. *Brain Res. Bull.* 86, 428–434. doi: 10.1016/j.brainresbull.2011.08.013

Maldonado-Devincci, A. M., Alipour, K. K., Michael, L. A., and Kirstein, C. L. (2010a). Repeated binge ethanol administration during adolescence enhances voluntary sweetened ethanol intake in young adulthood in male and female rats. *Pharmacol. Biochem. Behav.* 96, 476–487. doi: 10.1016/j.pbb.2010.07.008

Maldonado-Devincci, A. M., Badanich, K. A., and Kirstein, C. L. (2010b). Alcohol during adolescence selectively alters immediate and long-term behavior and neurochemistry. *Alcohol* 44, 57–66. doi: 10.1016/j.alcohol.2009.09.035

Marsh, D. J., Hollopeter, G., Huszar, D., Laufer, R., Yagaloff, K. A., Fisher, S. L., et al. (1999). Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides. *Nat. Genet.* 21, 119–122. doi: 10.1038/5070

Moriya, J., Takimoto, Y., Yoshiuchi, K., Shimosawa, T., and Akabayashi, A. (2006). Plasma agouti-related protein levels in women with anorexia nervosa. *Psychoneuroendocrinology* 31, 1057–1061. doi: 10.1016/j.psyneuen.2006.06.006

Morton, D. B., and Griffiths, P. H. (1985). Guidelines of the recognition on pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Vet. Rec.* 116, 431–436.doi: 10.1136/vr.116.16.431

Navarro, M., Carvajal, F., Lerma-Cabrera, J. M., Cubero, I., Picker, M. J., and Thiele, T. E. (2015). Evidence that melanocortin receptor agonist melanotan-ii synergistically augments the ability of naltrexone to blunt binge-like ethanol intake in male C57BL/6J mice. *Alcohol. Clin. Exp. Res.* 39, 1425–1433. doi: 10.1111/acer.12774

Navarro, M., Cubero, I., Chen, A. S., Chen, H. Y., Knapp, D. J., Breese, G. R., et al. (2005). Effects of melanocortin receptor activation and blockade on ethanol intake: a possible role for the melanocortin-4 receptor. *Alcohol. Clin. Exp. Res.* 29, 949–957. doi: 10.1097/01.alc.0000167740.19702.8c

Navarro, M., Cubero, I., Knapp, D. J., and Thiele, T. E. (2003). MTII-induced reduction of voluntary ethanol drinking is blocked by pretreatment with AgRP-(83–132). *Neuropeptides* 37, 338–344. doi: 10.1016/j.npep.2003.10.003

Navarro, M., Cubero, I., Ko, L., and Thiele, T. E. (2009). Deletion of agouti-related protein blunts ethanol self-administration and binge-like drinking in mice. *Genes Brain Behav.* 8, 450–458. doi: 10.1111/j.1601-183x.2009.00493.x

Navarro, M., Lerma-Cabrera, J. M., Carvajal, F., Lowery, E. G., Cubero, I., and Thiele, T. E. (2011). Assessment of voluntary ethanol consumption and the effects of a melanocortin (MC) receptor agonist on ethanol intake in mutant C57BL/6J mice lacking the MC-4 receptor. *Alcohol. Clin. Exp. Res.* 35, 1058–1066. doi: 10.1111/j.1530-0277.2011.01438.x

Olney, J. J., Navarro, M., and Thiele, T. E. (2014a). Targeting central melanocortin receptors: a promising novel approach for treating alcohol abuse disorders. *Front. Neurosci.* 8:128. doi: 10.3389/fnins.2014.00128

Olney, J. J., Sprow, G. M., Navarro, M., and Thiele, T. E. (2014b). The protective effects of the melanocortin receptor (MCR) agonist, melanotan-II (MTII), against binge-like ethanol drinking are facilitated by deletion of the MC3 receptor in mice. *Neuropeptides* 48, 47–51. doi: 10.1016/j.npep.2013.11.001

Pandit, R., de Jong, J. W., Vanderschuren, L. J., and Adan, R. A. (2011). Neurobiology of overeating and obesity: the role of melanocortins and beyond. *Eur. J. Pharmacol.* 660, 28–42. doi: 10.1016/j.ejphar.2011.01.034

Pascual, M., Blanco, A. M., Cauli, O., Miñarro, J., and Guerri, C. (2007). Intermittent ethanol exposure induces inflammatory brain damage and causes long-term behavioural alterations in adolescent rats. *Eur. J. Neurosci.* 25, 541–550. doi: 10.1111/j.1460-9568.2006.05298.x

Pascual, M., Boix, J., Felipo, V., and Guerri, C. (2009). Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. *J. Neurochem.* 108, 920–931. doi: 10.1111/j.1471-4159.2008.05835.x

Pascual, M., Do Cout, B. R., Alfonso-Loeches, S., Aguilar, M. A., Rodriguez-Arias, M., and Guerri, C. (2012). Changes in histone acetylation in the prefrontal cortex of ethanol-exposed adolescent rats are associated with ethanol-induced place conditioning. *Neuropharmacology*. 62, 2309–2319. doi: 10.1016/j.neuropharm.2012.01.011

Paxinos, G., and Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates*. 4th Edn. San Diego, CA: Academic Press.

Peciña, S. (2008). Opioid reward 'liking' and 'wanting' in the nucleus accumbens. *Physiol. Behav.* 94, 675–680. doi: 10.1016/j.physbeh.2008.04.006

Pedersen, W., and von Soest, T. (2015). Adolescent alcohol use and binge drinking: an 18-year trend study of prevalence and correlates. *Alcohol Alcohol.* 50, 219–225. doi: 10.1093/alcalc/agu091

Ploj, K., Roman, E., Kask, A., Hyytiä, P., Schiöth, H. B., Wikberg, J., et al. (2002). Effects of melanocortin receptor ligands on ethanol intake and opioid levels in alcohol-preferring AA rats. *Brain Res. Bull.* 59, 97–104. doi: 10.1016/s0361-9230(02)00844-4

Przybycien-Szymanska, M. M., Mott, N. N., Paul, C. R., Gillespie, R. A., and Pak, T. R. (2011). Binge-pattern alcohol exposure during puberty induces long-term changes in HPA axis reactivity. *PLoS One* 6:e18350. doi: 10.1371/journal.pone.0018350

Renquist, B. J., Lippert, R. N., Sebag, J. A., Ellacott, K. L. J., and Cone, R. D. (2011). Physiological roles of the melanocortin MC_3 receptor. *Eur. J. Pharmacol.* 660, 13–20. doi: 10.1016/j.ejphar.2010.12.025

Rush, C. C., Curry, J. F., and Looney, J. G. (2016). Alcohol expectancies and drinking behaviors among college students with disordered eating. *J. Am. Coll. Health.* 64, 195–204. doi: 10.1080/07448481.2015.1107837

Salamone, J. D., Pardo, M., Yohn, S. E., López-Cruz, L., San Miguel, N., and Correa, M. (2016). Mesolimbic dopamine and the regulation of motivated behavior. *Curr. Top. Behav. Neurosci.* 27, 231–257. doi: 10.1007/7854_2015_383

Siegmund, S., Vengeliene, V., Singer, M. V., and Spanagel, R. (2005). Influence of age at drinking onset on long-term ethanol self-administration with deprivation and stress phases. *Alcohol. Clin. Exp. Res.* 29, 1139–1145. doi: 10.1097/01.alc.0000171928.40418.46

Simms, J. A., Nielsen, C. K., Li, R., and Bartlett, S. E. (2014). Intermittent access ethanol consumption dysregulates CRF function in the hypothalamus and is attenuated by the CRF-R1 antagonist, CP-376395. *Addict. Biol.* 19, 606–611. doi: 10.1111/adb.12024

Simms, J. A., Steensland, P., Medina, B., Abernathy, K. E., Chandler, L. J., Wise, R., et al. (2008). Intermittent access to 20% ethanol induces high ethanol consumption in long-evans and wistar rats. *Alcohol. Clin. Exp. Res.* 32, 1816–1823. doi: 10.1111/j.1530-0277.2008.00753.x

Starowicz, K., Sieja, A., Bilecki, W., Obara, I., and Przewlocka, B. (2003). The effect of morphine on MC4 and CRF receptor mRNAs in the rat amygdala and attenuation of tolerance after their blockade. *Brain Res.* 990, 113–119. doi: 10.1016/s0006-8993(03)03444-9

Steinman, J., and DeBoer, M. D. (2013). Treatment of cachexia: melanocortin and ghrelin interventions. *Vitam. Horm.* 92, 197–242. doi: 10.1016/B978-0-12-410473-0.00008-8

Stickley, A., Koyanagi, A., Koposov, R., McKee, M., Murphy, A., and Ruchkin, V. (2015). Binge drinking and eating problems in Russian adolescents. *Alcohol. Clin. Exp. Res.* 39, 540–547. doi: 10.1111/acer.12644

Tabarin, A., Diz-Chaves, Y., Consoli, D., Monsaingeon, M., Bale, T. L., Culler, M. D., et al. (2007). Role of the corticotropin-releasing factor receptor type 2 in the control of food intake in mice: a meal pattern analysis. *Eur. J. Neurosci.* 26, 2303–2314. doi: 10.1111/j.1460-9568.2007.05856.x

Tanumihardjo, J., Shoff, S. M., Koenings, M., Zhang, Z., and Lai, H. J. (2015). Association between alcohol use among college students and alcohol outlet proximity and densities. *WMJ* 114, 143–147.

Teixeira, F. B., Santana, L. N., Bezerra, F. R., De Carvalho, S., Fontes-Júnior, E. A., Prediger, R. D., et al. (2014). Chronic ethanol exposure during adolescence in rats induces motor impairments and cerebral cortex damage associated with oxidative stress. *PLoS One* 9:e101074. doi: 10.1371/journal.pone.0101074

Uhari-Väänänen, J., Raasmaja, A., Bäckström, P., Oinio, V., Airavaara, M., Piepponen, P., et al. (2016). Accumbal μ-Opioid receptors modulate ethanol intake in alcohol-preferring alko alcohol rats. *Alcohol. Clin. Exp. Res.* 40, 2114–2123. doi: 10.1111/acer.13176

You, P., Hu, H., Chen, Y., Zhao, Y., Yang, Y., Wang, T., et al. (2016). Effects of melanocortin 3 and 4 receptor deficiency on energy homeostasis in rats. *Sci. Rep.* 6:34938. doi: 10.1038/srep34938

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 Psychiatry

ORIGINAL RESEARCH
published: 20 April 2017
doi: 10.3389/fpsyt.2017.00061

Investigating the Role of Serotonin in Methamphetamine Psychosis: Unaltered Behavioral Effects of Chronic Methamphetamine in 5-HT$_{1A}$ Knockout Mice

*Emily J. Jaehne[1], Dzeneta Ameti[1], Tehani Paiva[1] and Maarten van den Buuse[1,2,3]**

[1] Department Psychology and Counselling, School of Psychology and Public Health, La Trobe University, Melbourne, VIC, Australia, [2] Department of Pharmacology, University of Melbourne, Melbourne, VIC, Australia, [3] The College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, QLD, Australia

OPEN ACCESS

Edited by:
Jacqueline M. Barker, Medical University of South Carolina, USA

Reviewed by:
Eugene A. Kiyatkin, National Institute on Drug Abuse, USA
Jean-Philippe Guilloux, Université Paris Sud UMRS-1178, France

***Correspondence:**
Maarten van den Buuse
m.vandenbuuse@latrobe.edu.au

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Psychiatry

Received: *15 February 2017*
Accepted: *04 April 2017*
Published: *20 April 2017*

Citation:
Jaehne EJ, Ameti D, Paiva T and van den Buuse M (2017) Investigating the Role of Serotonin in Methamphetamine Psychosis: Unaltered Behavioral Effects of Chronic Methamphetamine in 5-HT$_{1A}$ Knockout Mice. Front. Psychiatry 8:61. doi: 10.3389/fpsyt.2017.00061

Methamphetamine (Meth) is a widely abused stimulant drug, but this abuse is associated with an increased risk of developing psychosis. In addition to its well-known action on brain dopamine, Meth also affects serotonergic (5-HT) neurons. The aim of this study was to investigate this role in mice, which lack one of the main serotonin receptors, the 5-HT$_{1A}$ receptor, which has been implicated in both schizophrenia and Meth-induced psychosis. Male and female wild-type or 5-HT$_{1A}$ knockout (KO) mice received daily treatment with increasing doses of methamphetamine from 6 to 9 weeks of age (1–4 mg/kg/day twice a day). At least 2 weeks after the last injection, the mice underwent a battery of behavioral tests focusing on psychosis-related behaviors, including Meth-induced hyperactivity, prepulse inhibition (PPI), social interaction, elevated plus maze (EPM), and Y-maze. Meth pretreatment resulted in significantly increased hyperlocomotion in response to an acute Meth challenge, but this effect was independent of genotype. Chronic Meth treatment resulted in decreased levels of anxiety in the EPM in both sexes, as well as increased startle responses in female mice only, again independent of genotype. 5-HT$_{1A}$ KO mice showed an increased locomotor response to acute Meth in both sexes, as well as increased PPI and decreased startle responses in female mice only, independent of Meth pretreatment. In conclusion, the effects of chronic Meth appear unaffected by the absence of the 5-HT$_{1A}$ receptor. These results do not support a role of the 5-HT$_{1A}$ receptor in Meth-induced psychosis.

Keywords: methamphetamine, psychosis, serotonin, 5-HT$_{1A}$ receptors, neuroplasticity

INTRODUCTION

Methamphetamine (Meth) use and availability has been increasing worldwide and is placing a huge burden on users, their relatives and friends, and society at large (1). One of the most severe consequences of Meth abuse in some users is the development of psychosis (2, 3), either acutely when the drug is active or as long-term symptoms, which may be similar to schizophrenia (2–4). Users may also develop other persistent psychiatric symptoms or impaired cognitive abilities following

prolonged use of the drug (4, 5). However, the mechanisms involved in Meth psychosis, and its overlap with schizophrenia symptoms, remain unclear.

We have previously investigated the effects of chronic Meth on psychosis-like behavior in adulthood using a dosing schedule of escalating exposure during late adolescence/early adulthood. At least 2 weeks after the chronic treatment ended, the long-lasting effects of chronic Meth on a locomotor hyperactivity model of psychosis, as well as on cognition and other behaviors with relevance to psychiatric symptoms, were studied (6–8). Acute amphetamine- or Meth-induced locomotor hyperactivity is a widely used behavioral test in preclinical schizophrenia research, as it models the increase in dopamine signaling thought to contribute to psychosis (9). Sensitization of this dopaminergic signaling has been postulated to mimic developmental mechanisms in psychosis (10, 11). In our experiments, pretreatment with Meth led to sensitization to the acute effects of amphetamine in the hyperactivity model compared to control pretreatment (6), while Meth pretreatment reduced the effect of an acute injection of amphetamine on prepulse inhibition (PPI) of acoustic startle (PPI) (7). Further studies showed that the chronic dosing protocol altered social novelty behavior but not short-term memory in the Y-maze (8).

There is increasing evidence for a role of serotonin, as well as dopaminergic mechanisms, in schizophrenia. Postmortem studies have shown increased expression of 5-HT$_{1A}$ receptors in the frontal cortex (12) but decreased binding in the amygdala in patients with schizophrenia (13). A polymorphism in the 5-HT$_{1A}$ receptor was associated with schizophrenia psychopathology (14) and several atypical antipsychotic drugs have high affinity for serotonergic receptors, including the 5-HT$_{1A}$ receptor (15–19). Extensive evidence suggests that 5-HT$_{1A}$ receptor activation modulates dopamine activity and may enhance cognition in schizophrenia (15, 17, 20, 21). While much of the animal literature has focused on the role of 5-HT$_{1A}$ in anxiety, where studies have shown 5-HT$_{1A}$ knockout (KO) mice to have a robust anxiety phenotype behavior in the elevated plus maze (EPM) (22–25) and certain fear conditioning paradigms (26), more recent work has investigated its role in schizophrenia-related behaviors. For example, 5-HT$_{1A}$ KO mice have an enhanced locomotor response to D-amphetamine compared to wild-type (WT) mice (27), an altered PPI response to methylenedioxymethamphetamine (28), and impaired cognition in the Morris Water maze (25, 29). The 5-HT$_{1A}$ antagonist WAY100635 has also been shown to increase PPI in C57BL/6 mice (30).

These previous studies suggest a role for 5-HT$_{1A}$ receptors in schizophrenia although it is less clear if this extends to Meth psychosis. While several studies have suggested a role for 5-HT$_{1A}$ receptors in addiction to this drug (31), a role for this receptor in sensitization of Meth-induced hyperlocomotion is less clear (32). For example, the 5-HT$_{1A}$ receptor antagonist, WAY100635, had no effect on amphetamine or Meth-induced hyperlocomotor activity (33, 34), while in contrast, the agonist, 8-OH-DPAT, was able to inhibit hyperlocomotion (33). 8-OH-DPAT and the 5-HT$_{1A}$ receptor agonist, osemozotan, also prevented development and expression of amphetamine or Meth-induced behavioral sensitization (35, 36). However, the long-lasting effects of chronic Meth to induce psychosis-like behavior have not been studied in animals with genetically modified 5-HT$_{1A}$ receptor levels. The current study was, therefore, designed to investigate the effect of 5-HT$_{1A}$ receptor KO on the action of Meth to induce psychosis- and schizophrenia-related behaviors. We used the acute Meth-induced hyperactivity model of psychosis as well as a range of other relevant behavioral tests, including PPI for sensorimotor gating, social interaction as a model of some of the negative symptoms of schizophrenia, Y-maze and fear conditioning to assess cognitive changes, and EPM for anxiety. 5-HT$_{1A}$ KO mice or WT controls were tested during adulthood after a 3-week binge protocol of Meth administration during late adolescence. As previous studies have shown sex differences in animal models of psychosis (37) as well as the effect of this Meth dosing protocol on some behaviors (7, 8), both male and female mice were included in this study.

MATERIALS AND METHODS

Animals

5-HT$_{1A}$ receptor KO mice and their C57BL/6 WT control littermates (WT) (27) were derived from a breeding colony at the La Trobe Animal Research and Teaching Facility. Heterozygous mice were used as breeders to obtain WT and KO littermates for the current studies, while heterozygous offspring was not used. Genotypes were confirmed at weaning by Transnetyx Inc. (Cordova, TN, USA).

A total of 104 male and female mice were used for experiments ($n = 10$–16/group; **Table 1**). All mice were housed in groups of two to five during the experimental period in individually ventilated cages (Tecniplast, Buguggiate, Italy) with standard pellet food and water available *ad libitum*. Ambient temperature of housing and testing rooms was 21 ± 2°C and mice were housed under a 12-h light–dark cycle, lights on at 0700 hours, with all behavioral testing conducted between 0800 and 1600 hours.

Chronic Methamphetamine Treatment and Experimental Procedure

Mice were given either Meth or saline as a vehicle control for five consecutive days a week for a period of 3 weeks during

TABLE 1 | Number and mean weight of experimental groups.

Group	*n*	Body weight (g) at end of testing
Males		
WT-Saline	12	28.0 ± 0.7
WT-Meth	12	30.4 ± 0.6
5-HT$_{1A}$ KO-Saline	12	28.8 ± 0.7
5-HT$_{1A}$ KO-Meth	16	29.6 ± 0.5
Females		
WT-Saline	12	22.4 ± 0.3
WT-Meth	13	22.3 ± 0.3
5-HT$_{1A}$ KO-Saline	16	22.5 ± 0.4
5-HT$_{1A}$ KO-Meth	11	23.4 ± 0.4

Genotype: 5-HT$_{1A}$ knockout (KO) or wild-type (WT).
Pretreatment: saline or methamphetamine (Meth).
Body weight: mean (±SEM).

adolescence, from the age of 6 to 9 weeks. Mice received 1 injection/day of 1 mg/kg during the first week, 2 injections/day of 2 mg/kg the second week, and 2 injections/day of 4 mg/kg the third week (6–8). This protocol was based on a binge-type Meth intake pattern seen in many abusers of the drug, including occasional interruptions of administration and a gradual increase of doses (38). The 2-week washout is required to be able to see the long-term effects of these chronic doses, which reflect increased effects in relapsed chronic users (38) as well as sensitization mechanisms similar to those postulated in psychosis development (10, 11). Meth and saline solutions were given intraperitoneally at a volume of 5 ml/kg. Meth was purchased from the National Measurement Institute (Pymble, NSW, Australia) and dissolved in 0.9% sterile saline.

Starting 2 weeks following the final injection, at 11 weeks of age, mice underwent a battery of behavioral tests over a period of 4 weeks, with less stressful tests performed first and more stressful tests, or tests involving acute drug challenge, performed at the end. Mice were given at least 2–3 days between behavioral tests. Tests were performed in the following order: Y-maze, social interaction, EPM, fear conditioning, PPI, and Meth-induced locomotor hyperactivity.

Behavioral Testing

Methamphetamine-Induced Locomotor Hyperactivity

Mice were placed into automated photocell arenas (Med Associates, Fairfax, VT, USA), 27 cm × 27 cm with walls 40 cm high, with a 16 × 16 array of photobeam sensors for detecting movement. Each time, they were first habituated in the arenas for 1 h, then were removed briefly and injected with the challenge drug, then placed back into the arena for a further 2 h (6, 27). During the first session, all mice received saline, followed by 1 mg/kg Meth in the second and 3 mg/kg Meth in the final session, with 3–4 days washout in between sessions. Distance traveled was automatically calculated in 5-min time bins.

PPI of Acoustic Startle

Prepulse inhibition was assessed as a measure of sensorimotor gating using automated SR-Lab startle chambers (San Diego Instruments, San Diego, CA, USA). Mice were placed in individual plexiglass cylinders (5 cm diameter) and the test session consisted of 104 stimulus trials as previously described (7, 27). PPI was quantified as the difference between stimulus responses during prepulse-pulse and pulse-alone trials and expressed as a percentage of pulse-alone responses. At 30 ms ISI, mice showed no effect of genotype or pretreatment, therefore, results analysis will focus on the 100 ms ISI.

Social Interaction

The test apparatus consists of a rectangular three chambered enclosure, 43 cm × 64 cm, with transparent walls 23 cm high. Two "stranger" enclosures, diameter 9 cm, height 10 cm, were placed in the two outer chambers. The test consisted of three phases, each 10 min in duration, which were conducted immediately after one another (8, 39). Stranger mice were adolescent 5-HT$_{1A}$ heterozygous mice of the same sex as the test mouse. Interaction time was measured using Ethovision video tracking (Noldus, Wageningen, The Netherlands), with time spent in a "sniffing zone," 2.5 cm immediately surrounding stranger cages, defined as interaction.

Elevated Plus Maze

The EPM consisted of an elevated plus-shaped platform with two open and two closed arms, with a length of 40 cm and width of 5 cm, 50 cm above the ground, with a central square section between arms (40, 41). During the 5-min trial, time spent in open and closed arms and number of entries into arms were measured using Ethovision video tracking (Noldus). Mice that spend more time in the open arms are considered to have a lesser anxiety phenotype. The total number of arm entries was used as a control measure of locomotor activity.

Y-Maze

The Y-maze was a Y-shaped apparatus with three arms (start arm and two test arms), each 32 cm long and 10 cm wide with walls 15 cm high. The arms were at a 120° angle from each other. The test arms had different black and white symbols on either end wall. Behavior was tracked using Ethovision (Noldus), which measured time spent in, and number of entries to, each arm (42, 43). Sessions included a 10-min trial with access to only two arms followed 1 h later but a 5 min re-trial with access to all three arms. Time spent in the novel test arm compared to the other arms (familiar and start) during the retention phase was used as a measure of short-term spatial recognition memory.

Fear Conditioning

Fear memory was assessed using a 3-day fear conditioning protocol as previously used in mice (43, 44) using chambers equipped with footshock grid floors (Med Associates). Two different conditioning contexts were used, which differed in lux, scent, bedding, and structure due to a concave wall insert, and mice were pseudorandomly assigned to one context or the other. Freezing was defined as complete lack of any movement besides breathing and was measured in response to (1) the context where mice had previously been exposed to an unconditioned stimulus (scrambled foot-shocks of 1 s duration, 0.7 mA) or (2) a conditioned stimulus (30 s duration, 7,500 Hz, 70 dB) previously presented prior to the US, but in a new context.

Statistical Analysis

Data were expressed as the mean ± SEM and differences between groups were analyzed with analysis of variance (ANOVA), with repeated measures where appropriate, using IBM SPSS Statistics 23 (Armonk, New York, NY, USA). All data were first analyzed with genotype, Meth pretreatment and sex as between-group statistical factors. If significant interactions were seen, data were split and further analyzed by genotype, pretreatment, or sex as stated in the results. For locomotor activity analysis, repeated-measures factors were time and acute Meth treatment, while for social interaction and Y-maze analysis, repeated measures factors were stranger mouse interaction time and time spent in arms, respectively. Differences between groups were considered significant when $p < 0.05$.

RESULTS

Locomotor Activity

Analysis of locomotor activity following 1 and 3 mg/kg Meth compared to saline control showed a significant main effect of an acute Meth challenge [$F_{(2, 188)} = 273$, $p < 0.001$] and a treatment × pretreatment interaction [$F_{(2, 188)} = 73.3$, $p < 0.001$]. Data were, therefore, split and further ANOVAs were used to compare the effect of each dose of acute Meth with saline to further explore this relationship. Although females showed higher activity than males [$F_{(1, 94)} = 16.4$, $p < 0.001$], there were no significant interactions between sex and genotype, acute treatment or pretreatment, therefore, locomotor activity results for males and females were combined (**Figure 1**).

Following acute injection with 3 mg/kg Meth, all groups showed a significant main effect of treatment compared to saline injection [$F_{(1, 94)} = 307$, $p < 0.001$]. This effect was much greater in Meth-pretreated mice [treatment × pretreatment $F_{(1, 94)} = 87.3$, $p < 0.001$], indicating sensitization to a challenge dose of Meth following the binge dosing protocol used, and was also greater in 5-HT$_{1A}$ KO mice than in WT controls [treatment × genotype $F_{(1, 94)} = 5.10$, $p = 0.026$]. There was, however, no treatment × pretreatment × genotype interaction, suggesting the genotype differences and Meth sensitization were independent of each other.

Acute injection with 1 mg/kg Meth showed similar results [treatment $F_{(1, 94)} = 24.9$, $p < 0.001$; treatment × pretreatment $F_{(1, 94)} = 18.0$, $p < 0.001$]; however, the effect of genotype in response to acute Meth failed to reach significance at this dose.

Prepulse Inhibition

Analysis of PPI at 100 ms ISI showed a significant genotype × sex interaction [$F_{(1, 104)} = 5.77$, $p = 0.018$]; therefore, data were further analyzed separately for male and female mice (**Figure 2A**). Female, but not male, 5-HT$_{1A}$ KO mice showed significantly higher PPI than WT mice [main effect of genotype $F_{(1, 54)} = 9.08$, $p = 0.004$]. However, there was no effect of Meth pretreatment on PPI in any of the groups.

Analysis of startle response showed that male mice had significantly higher startle compared to females [$F_{(1, 104)} = 15.1$, $p < 0.001$] while there was also a significant main effect of genotype [$F_{(1, 104)} = 4.43$, $p = 0.038$] and a genotype × sex interaction [$F_{(1, 104)} = 4.52$, $p = 0.036$] suggesting a genotype effect, which was dependent on sex (**Figure 2B**). We, therefore, again further analyzed results separately for male and female mice. As with PPI, male mice showed no difference between groups, while in females, 5-HT$_{1A}$ KO mice showed significantly lower startle than WT mice [main effect of genotype $F_{(1, 54)} = 17.6$, $p < 0.001$]. It was also shown that female Meth pretreated mice had significantly higher startle compared to controls [main effect of pretreatment $F_{(1, 54)} = 4.08$, $p = 0.048$]; however, there was no genotype × pretreatment effect, suggesting that these results were independent of each other.

Social Interaction

Analysis of total time spent interacting with the stranger mouse and empty cage showed a significant preference for the stranger mouse [$F_{(1, 78)} = 150$, $p < 0.001$], but there was no statistical interaction with genotype, pretreatment, or sex, indicating all groups showed similar sociability (**Figure 3A**).

In contrast, while analysis of the total time spent interacting with the familiar and novel stranger mice again showed a significant preference for the novel stranger [$F_{(1, 78)} = 51.6$, $p < 0.001$] and no statistical interaction with genotype or pretreatment, there was a significant interaction between stranger time and sex [$F_{(1, 78)} = 5.6$, $p = 0.021$; **Figure 3B**]. This suggests that there were no effects of genotype or Meth pretreatment on social novelty behavior, but that female mice show a decreased preference for the novel over the familiar stranger mouse.

Elevated Plus Maze

Analysis of time spent in the open arms of the EPM showed a significant main effect of Meth pretreatment [$F_{(1, 94)} = 8.17$, $p = 0.005$] reflecting that this pretreatment leads to increased time spent on the open arms of the EPM, suggesting decreased anxiety in these mice (**Figure 4A**). There was no significant main effect of genotype or sex; therefore, results have been presented for both sexes combined.

Total number of arm entries was analyzed as an indicator of activity on the plus maze (WT-Saline 42.2 ± 4.2, KO-Saline

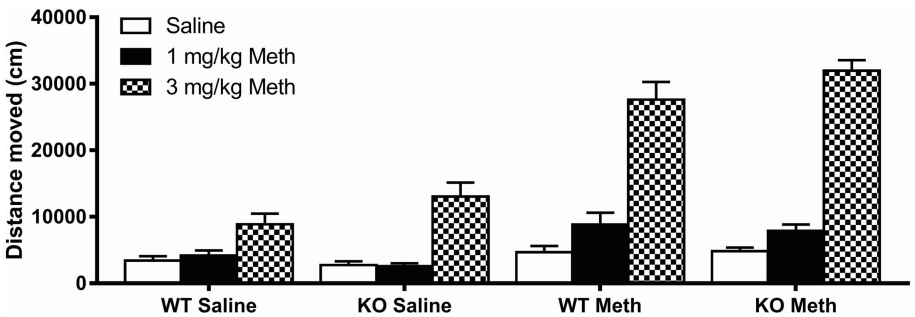


FIGURE 1 | Acute methamphetamine (Meth) induced hyperactivity following chronic Meth exposure. A low acute challenge dose of Meth (1 mg/kg) induced hyperactivity in mice previously pretreated with Meth. There was no genotype effect at this dose. A high acute challenge dose of Meth (3 mg/kg) induced hyperactivity to a significantly greater extent in Meth pretreated compared to saline pretreated mice. This dose also induced hyperactivity to a significantly greater extent in 5-HT$_{1A}$ knockout (KO) mice compared to wild-type (WT) mice. Results are shown for males and females combined.

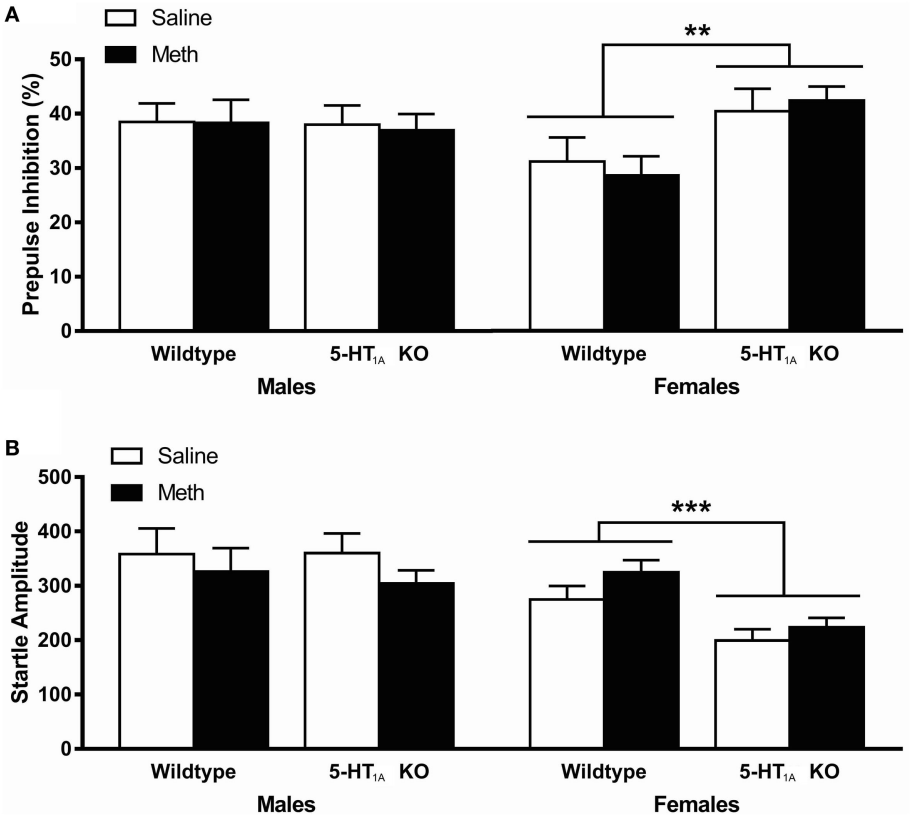


FIGURE 2 | **(A)** Average prepulse inhibition (PPI) across all four PP intensities was significantly higher in 5-HT$_{1A}$ knockout (KO) females compared to wild-type (WT) females. There was no difference in PPI between any groups in male mice. **(B)** Average startle responses across all four startle blocks were significantly lower in female 5-HT$_{1A}$ KO mice compared to WT, and higher in female Meth pretreated compared to saline pretreated mice. There was no difference in startle between any groups in male mice. ** signifies genotype effect $p < 0.01$, *** signifies $p < 0.001$.

45.1 ± 3.6, WT-Meth 46.5 ± 4.3, KO-Meth 47.0 ± 3.8). The results showed that there were no differences in activity between groups; therefore, overall activity did not have any effect on the different anxiety levels seen.

Y-Maze

Analysis of total time spent in each arm of the Y-maze showed a significant main effect of arms [$F_{(2, 184)} = 91.6$, $p < 0.001$], but no significant interaction of time in arms with genotype, pretreatment, or sex indicating all groups showed a similar preference for the novel arm and have intact short-term spatial memory (**Figure 4B**). There was no significant main effect of sex; therefore, results have been presented for both sexes combined.

Fear Conditioning

Analysis of context freezing (WT-Saline 42.3 ± 4.3, KO-Saline 45.6 ± 3.4, WT-Meth 44.7 ± 3.6, KO-Meth 40.9 ± 3.5%) and tone freezing (WT-Saline 43.5 ± 4.9, KO-Saline 55.5 ± 4.3, WT-Meth 49.0 ± 4.9, KO-Meth 48.1 ± 4.1) showed that there were no differences between the groups, suggesting that neither Meth pretreatment nor 5-HT$_{1A}$ genotype have any effect on fear memory.

DISCUSSION

This study showed that, while both the Meth binge dosing and 5-HT$_{1A}$ receptor KO genotype alter behavior in mice, 5-HT$_{1A}$ KO mice did not respond differently to chronic Meth pretreatment in any of the behaviors tested. Meth pretreatment resulted in a heightened response to acute Meth and decreased levels of anxiety in both sexes, as well as increased startle responses in female mice only, independent of genotype. However, 5-HT$_{1A}$ KO mice also showed an increased response to acute Meth in both sexes, as well as increased PPI and decreased startle response in female mice only, independent of Meth pretreatment. There were no effects on either short-term spatial memory in the Y-maze or conditioned fear memory induced by either Meth or 5-HT$_{1A}$ receptor KO genotype.

These studies were first able to confirm that chronic escalating Meth exposure during adolescence/young adulthood leads to sensitization to the effects of an acute challenge dose of Meth in adulthood, consistent with previous studies (6). We were also able to confirm that 5-HT$_{1A}$ KO mice have a greater response to acute Meth compared to WT consistent with their enhanced sensitivity seen following acute D-amphetamine previously (27). However, there was no statistical interaction between the

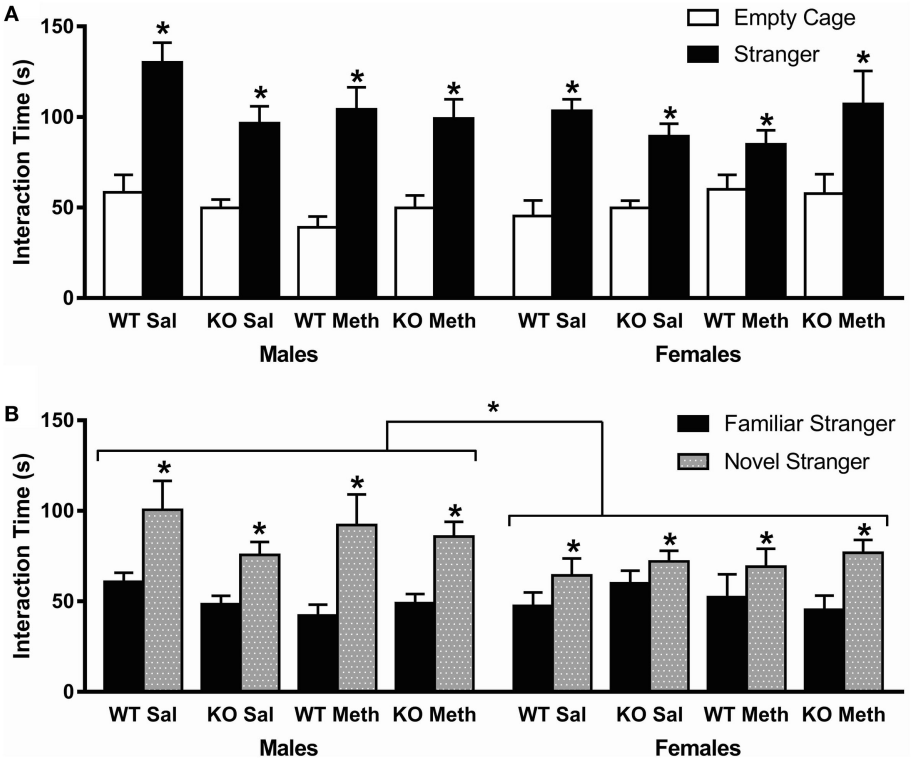


FIGURE 3 | **Social behavior in the three-chamber task**. **(A)** Sociability, as measured by preference to interact with a stranger mouse compared to an empty cage, was normal in all groups (indicated by * above stranger). **(B)** Social novelty preference, as measured by preference to interact with novel stranger mouse, was also normal in all groups (indicated by * above stranger), although female mice showed a lower preference compared to males. *$p < 0.05$.

two effects, as reflected by the observation that KO mice did not show an altered sensitivity to acute re-exposure to a single challenge dose of Meth compared to WT mice. These results suggest that the 5-HT$_{1A}$ receptor is likely important in the acute action of Meth to induce hyperactivity but is not required for the development of sensitization induced by prior binge dosing of Meth. A previous study in the laboratory showed that the binding density of the dopamine transporter (DAT) and dopamine receptors D_1 and D_2 is only changed subtly in 5-HT$_{1A}$ KO mice (27) and suggested that the interaction of 5-HT$_{1A}$ receptors with dopamine release is at a more immediate functional level (45) rather that *via* more long-term changes in the expression of DAT or dopamine receptors.

Male mice showed no effect of genotype or Meth pretreatment on either PPI or startle response. Female 5-HT$_{1A}$ KO mice, however, showed increased PPI and decreased startle responses compared to WT. The results are partly consistent with a previous study, which showed that administration of the 5-HT$_{1A}$ antagonist, WAY100635, led to an increase in PPI in C57BL/6 mice (30); however, this was only done in male mice, which showed no changes in PPI between genotypes in the current study. While Meth pretreatment did not alter PPI in either sex, there was also a small but significant effect of Meth pretreatment to increase startle response in female mice; however, the effects of genotype and pretreatment were again independent of each other. Therefore, as with locomotor hyperactivity, Meth pretreatment had no specific effect in 5-HT$_{1A}$ KO mice only. This is similar to previous studies using Meth pretreatment where no differences were seen in baseline PPI in either sex following binge Meth dosing (7).

There were no effects of Meth pretreatment or 5-HT$_{1A}$ genotype on social behavior using the three-chamber sociability and social novelty preference tests. A previous study in the lab showed the same results for sociability, although suggested that Meth pretreatment decreased preference for social novelty to a lower level than saline pretreated mice in males only (8), while other studies have also shown impaired social behavior in animals previously exposed to Meth (46, 47).

5-HT$_{1A}$ KO mice showed no signs of increased or decreased anxiety in the EPM, which is in contrast to other studies conducted in these mice, which have shown an anxious phenotype on the EPM and other tests of anxiety (22–26). Differences in experimental conditions such as conducting other behavioral tests prior to the elevated zero maze could contribute to the differences seen here compared to previous studies. Future studies should test for an anxiety phenotype in these mice prior to further behavioral testing to confirm presence of the phenotype. Importantly, however, Meth pretreatment led to increased time spent in the open arms of the plus maze, suggesting that these mice were less anxious than controls, but this effect was again

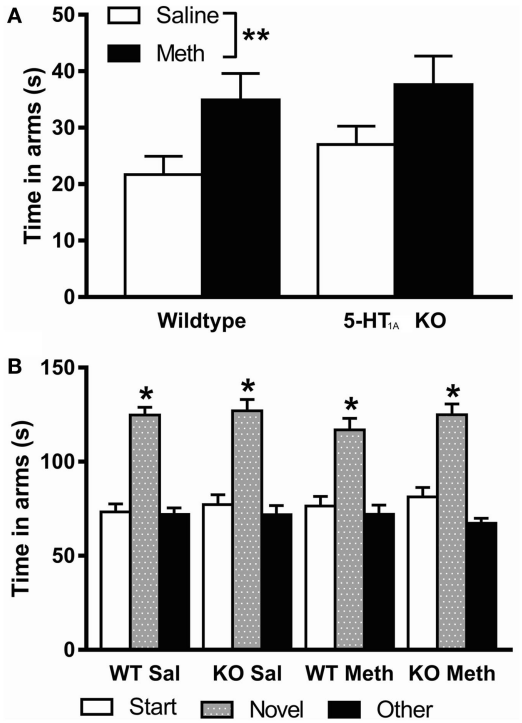


FIGURE 4 | **(A)** Time spent in the open arms of the elevated plus maze was higher in Meth pretreated mice compared to saline pretreated. **(B)** Short-term spatial memory, measured by preference to spend time in the novel arm of the Y-maze, was normal in all groups (indicated by * above novel arm). *$p < 0.05$, **$p < 0.01$. Results are shown for males and females combined.

independent of genotype. Previously, effects of chronic Meth pretreatment on anxiety have been variable. For example, a 10-day escalating dose protocol in mice induced no changes in anxiety measures in the EPM or light/dark box (48) while studies in rats showed increased anxiety or no change in the EPM depending on the age or extent of Meth exposure or time following exposure (49–51). These results suggest that dosing and testing protocol are very important for long-term effects of Meth on behavior.

A limitation of using 5-HT$_{1A}$ KO mice is that the absence of these receptors during embryogenesis and development may influence the role of these receptors on behavior and the development of Meth sensitization. For example, changes in receptor density or function may change over time, and this could be altered by absence of the receptors during early development. Future studies could use conditional KO mice in which the receptor is deleted only in adulthood. Any differential role of presynaptic and postsynaptic 5-HT$_{1A}$ receptors, which would both be absent in our KO mice, is also not addressed in this study. Previous research using 5-HT$_{1A}$ agonists suggest that, while antagonists have no effect on behavioral sensitization (33, 34), the effects of the agonists may have been due to the activation of the presynaptic receptors preferentially over postsynaptic receptors (52). Studies show that both 8-OH-DPAT and osemozotan are able to prevent the development and expression of amphetamine or Meth-induced behavioral sensitization (35, 36), these effects were also reversed by the antagonist WAY100635, which has a greater affinity for presynaptic 5-HT$_{1A}$ autoreceptors (53). Other limitations of this study are that the estrus cycle of female mice was not monitored, which could strengthen the results of any study using female animals, and the fact that we did not see an anxiety phenotype in the 5-HT$_{1A}$ KO mice as discussed above.

While this study showed that there was no interaction between Meth pretreatment and the 5-HT$_{1A}$ receptor in the protocols used, there is evidence that other 5-HT receptors may be involved in the long-term effects of Meth and Meth-induced sensitization. Repeated Meth administration failed to induce behavioral sensitization in 5-HT reuptake KO mice, but this was rescued by administration of a 5-HT$_{1B}$ antagonist (54). Many different 5-HT receptor agonists and antagonists have been investigated for their role in behavioral sensitization induced by Meth. For example, the 5-HT$_{1B}$ receptor antagonist, SB 216641, inhibited development but not expression of amphetamine-induced sensitization (55) while the 5-HT$_2$ receptor antagonist, ritanserin, inhibited the development, expression, and maintenance of Meth-induced behavioral sensitization (56, 57). Taken together, the current findings in 5-HT$_{1A}$ receptor KO mice do not support a role of these receptors in Meth-induced psychosis, although this does not rule out a role of other serotonin receptors, which can be addressed in future studies with selected KO lines such as 5-HT$_{1B}$ and 5-HT$_2$ receptor KO mice.

ETHICS STATEMENT

All experimentation was approved by the La Trobe University Animal Ethics Committee and was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes set out by the National Health and Medical Research Council of Australia.

AUTHOR CONTRIBUTIONS

MB designed the study. EJ, DA, and TP performed all behavioral testing and collected data. EJ performed analysis and wrote the manuscript with the help of MB who edited the final version. All authors contributed to and approved the final version of this manuscript.

ACKNOWLEDGMENTS

This study was supported by a Senior Research Fellowship (grant number 1041895) from the National Health and Medical Research Council of Australia. The authors would like to acknowledge and thank Camilla Hume for assistance with implementation and supervision of behavioral testing.

REFERENCES

1. United Nations. In: United Nations Office on Drugs and Crime, editor. *World Drug Report 2016*. Vienna (2016). Available from: http://www.unodc.org/wdr2016/
2. Callaghan RC, Cunningham JK, Allebeck P, Arenovich T, Sajeev G, Remington G, et al. Methamphetamine use and schizophrenia: a population-based cohort study in California. *Am J Psychiatry* (2012) 169(4):389–96. doi:10.1176/appi.ajp.2011.10070937
3. McKetin R, Hickey K, Devlin K, Lawrence K. The risk of psychotic symptoms associated with recreational methamphetamine use. *Drug Alcohol Rev* (2010) 29(4):358–63. doi:10.1111/j.1465-3362.2009.00160.x
4. Zweben JE, Cohen JB, Christian D, Galloway GP, Salinardi M, Parent D, et al. Psychiatric symptoms in methamphetamine users. *Am J Addict* (2004) 13(2):181–90. doi:10.1080/10550490490436055
5. Courtney KE, Ray LA. Methamphetamine: an update on epidemiology, pharmacology, clinical phenomenology, and treatment literature. *Drug Alcohol Depend* (2014) 143:11–21. doi:10.1016/j.drugalcdep.2014.08.003
6. Manning EE, Halberstadt AL, van den Buuse M. BDNF-deficient mice show reduced psychosis-related behaviors following chronic methamphetamine. *Int J Neuropsychopharmacol* (2016) 19(4):yv116. doi:10.1093/ijnp/pyv116
7. Manning EE, van den Buuse M. BDNF deficiency and young-adult methamphetamine induce sex-specific effects on prepulse inhibition regulation. *Front Cell Neurosci* (2013) 7:92. doi:10.3389/fncel.2013.00092
8. Manning EE, van den Buuse M. Altered social cognition in male BDNF heterozygous mice and following chronic methamphetamine exposure. *Behav Brain Res* (2016) 305:181–5. doi:10.1016/j.bbr.2016.03.014
9. van den Buuse M. Modeling the positive symptoms of schizophrenia in genetically modified mice: pharmacology and methodology aspects. *Schizophr Bull* (2010) 36(2):246–70. doi:10.1093/schbul/sbp132
10. Laruelle M. The role of endogenous sensitization in the pathophysiology of schizophrenia: implications from recent brain imaging studies. *Brain Res Brain Res Rev* (2000) 31(2–3):371–84. doi:10.1016/S0165-0173(99)00054-5
11. Weidenauer A, Bauer M, Sauerzopf U, Bartova L, Praschak-Rieder N, Sitte HH, et al. Making sense of: sensitization in schizophrenia. *Int J Neuropsychopharmacol* (2017) 20(1):1–10. doi:10.1093/ijnp/pyw081
12. Burnet PW, Eastwood SL, Harrison PJ. [^{3}H]WAY-100635 for 5-HT1A receptor autoradiography in human brain: a comparison with [^{3}H]8-OH-DPAT and demonstration of increased binding in the frontal cortex in schizophrenia. *Neurochem Int* (1997) 30(6):565–74. doi:10.1016/S0197-0186(96)00124-6
13. Yasuno F, Suhara T, Ichimiya T, Takano A, Ando T, Okubo Y. Decreased 5-HT1A receptor binding in amygdala of schizophrenia. *Biol Psychiatry* (2004) 55(5):439–44. doi:10.1016/j.biopsych.2003.11.016
14. Huang YY, Battistuzzi C, Oquendo MA, Harkavy-Friedman J, Greenhill L, Zalsman G, et al. Human 5-HT1A receptor C(-1019)G polymorphism and psychopathology. *Int J Neuropsychopharmacol* (2004) 7(4):441–51. doi:10.1017/s1461145704004663
15. De Deurwaerdere P, Di Giovanni G. Serotonergic modulation of the activity of mesencephalic dopaminergic systems: therapeutic implications. *Prog Neurobiol* (2017) 151:175–236. doi:10.1016/j.pneurobio.2016.03.004
16. Meltzer HY, Huang M. In vivo actions of atypical antipsychotic drug on serotonergic and dopaminergic systems. *Prog Brain Res* (2008) 172:177–97. doi:10.1016/s0079-6123(08)00909-6
17. Millan MJ. Improving the treatment of schizophrenia: focus on serotonin (5-HT)(1A) receptors. *J Pharmacol Exp Ther* (2000) 295(3):853–61.
18. Wood MD, Wren PB. Serotonin-dopamine interactions: implications for the design of novel therapeutic agents for psychiatric disorders. *Prog Brain Res* (2008) 172:213–30. doi:10.1016/s0079-6123(08)00911-4
19. Yadav PN, Abbas AI, Farrell MS, Setola V, Sciaky N, Huang XP, et al. The presynaptic component of the serotonergic system is required for clozapine's efficacy. *Neuropsychopharmacology* (2011) 36(3):638–51. doi:10.1038/npp.2010.195
20. Meltzer HY, Sumiyoshi T. Does stimulation of 5-HT(1A) receptors improve cognition in schizophrenia? *Behav Brain Res* (2008) 195(1):98–102. doi:10.1016/j.bbr.2008.05.016
21. Sumiyoshi T, Bubenikova-Valesova V, Horacek J, Bert B. Serotonin1A receptors in the pathophysiology of schizophrenia: development of novel cognition-enhancing therapeutics. *Adv Ther* (2008) 25(10):1037–56. doi:10.1007/s12325-008-0102-2
22. Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, et al. Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. *Proc Natl Acad Sci U S A* (1998) 95(25):15049–54. doi:10.1073/pnas.95.25.15049
23. Parks CL, Robinson PS, Sibille E, Shenk T, Toth M. Increased anxiety of mice lacking the serotonin1A receptor. *Proc Natl Acad Sci U S A* (1998) 95(18):10734–9. doi:10.1073/pnas.95.18.10734
24. Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, et al. Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. *Proc Natl Acad Sci U S A* (1998) 95(24):14476–81. doi:10.1073/pnas.95.24.14476
25. Rogers J, Vo U, Buret LS, Pang TY, Meiklejohn H, Zeleznikow-Johnston A, et al. Dissociating the therapeutic effects of environmental enrichment and exercise in a mouse model of anxiety with cognitive impairment. *Transl Psychiatry* (2016) 6:e794. doi:10.1038/tp.2016.52
26. Klemenhagen KC, Gordon JA, David DJ, Hen R, Gross CT. Increased fear response to contextual cues in mice lacking the 5-HT1A receptor. *Neuropsychopharmacology* (2006) 31(1):101–11. doi:10.1038/sj.npp.1300774
27. van den Buuse M, Ruimschotel E, Martin S, Risbrough VB, Halberstadt AL. Enhanced effects of amphetamine but reduced effects of the hallucinogen, 5-MeO-DMT, on locomotor activity in 5-HT(1A) receptor knockout mice: implications for schizophrenia. *Neuropharmacology* (2011) 61(1–2):209–16. doi:10.1016/j.neuropharm.2011.04.001
28. van den Buuse M, Becker T, Kwek P, Martin S, Ruimschotel E, Risbrough V. Disruption of prepulse inhibition by 3,4-methylenedioxymethamphetamine (MDMA): comparison between male and female wild-type and 5-HT(1A) receptor knockout mice. *Int J Neuropsychopharmacol* (2011) 14(6):856–61. doi:10.1017/s1461145711000101
29. Wolff M, Costet P, Gross C, Hen R, Segu L, Buhot MC. Age-dependent effects of serotonin-1A receptor gene deletion in spatial learning abilities in mice. *Brain Res Mol Brain Res* (2004) 130(1–2):39–48. doi:10.1016/j.molbrainres.2004.07.012
30. van den Buuse M. Exploring the role of 5-HT1A receptors in the regulation of prepulse inhibition in mice: implications for cross-species comparisons. *ACS Chem Neurosci* (2013) 4(1):149–60. doi:10.1021/cn300118t
31. Müller CP, Carey RJ, Huston JP, De Souza Silva MA. Serotonin and psychostimulant addiction: focus on 5-HT1A-receptors. *Prog Neurobiol* (2007) 81(3):133–78. doi:10.1016/j.pneurobio.2007.01.001
32. Muller CP, Homberg JR. The role of serotonin in drug use and addiction. *Behav Brain Res* (2015) 277:146–92. doi:10.1016/j.bbr.2014.04.007
33. Przegalinski E, Filip M. Stimulation of serotonin (5-HT)1A receptors attenuates the locomotor, but not the discriminative, effects of amphetamine and cocaine in rats. *Behav Pharmacol* (1997) 8(8):699–706. doi:10.1097/00008877-199712000-00004
34. Steed E, Jones CA, McCreary AC. Serotonergic involvement in methamphetamine-induced locomotor activity: a detailed pharmacological study. *Behav Brain Res* (2011) 220(1):9–19. doi:10.1016/j.bbr.2011.01.026
35. Ago Y, Nakamura S, Uda M, Kajii Y, Abe M, Baba A, et al. Attenuation by the 5-HT1A receptor agonist osemozotan of the behavioral effects of single and repeated methamphetamine in mice. *Neuropharmacology* (2006) 51(4):914–22. doi:10.1016/j.neuropharm.2006.06.001
36. Przegaliński E, Siwanowicz J, Baran L, Filip M. Activation of serotonin (5-HT)1A receptors inhibits amphetamine sensitization in mice. *Life Sci* (2000) 66(11):1011–9. doi:10.1016/S0024-3205(99)00666-9
37. Hill RA. Sex differences in animal models of schizophrenia shed light on the underlying pathophysiology. *Neurosci Biobehav Rev* (2016) 67:41–56. doi:10.1016/j.neubiorev.2015.10.014
38. Segal DS, Kuczenski R, O'Neil ML, Melega WP, Cho AK. Escalating dose methamphetamine pretreatment alters the behavioral and neurochemical profiles associated with exposure to a high-dose methamphetamine binge. *Neuropsychopharmacology* (2003) 28(10):1730–40. doi:10.1038/sj.npp.1300247
39. Jaehne EJ, Klarić TS, Koblar SA, Baune BT, Lewis MD. Effects of Npas4 deficiency on anxiety, depression-like, cognition and sociability behaviour. *Behav Brain Res* (2015) 281:276–82. doi:10.1016/j.bbr.2014.12.044
40. Dawson GR, Tricklebank MD. Use of the elevated plus maze in the search for novel anxiolytic agents. *Trends Pharmacol Sci* (1995) 16(2):33–6. doi:10.1016/S0165-6147(00)88973-7

41. Wall PM, Messier C. Methodological and conceptual issues in the use of the elevated plus-maze as a psychological measurement instrument of animal anxiety-like behavior. *Neurosci Biobehav Rev* (2001) 25(3):275–86. doi:10.1016/S0149-7634(01)00013-6
42. Jaehne EJ, Baune BT. Effects of chemokine receptor signalling on cognition-like, emotion-like and sociability behaviours of CCR6 and CCR7 knockout mice. *Behav Brain Res* (2014) 261:31–9. doi:10.1016/j.bbr.2013.12.006
43. Notaras M, Hill R, Gogos JA, van den Buuse M. BDNF Val66Met genotype determines hippocampus-dependent behavior via sensitivity to glucocorticoid signaling. *Mol Psychiatry* (2016) 21(6):730–2. doi:10.1038/mp.2015.152
44. Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, et al. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science* (2006) 314(5796):140–3. doi:10.1126/science.1129663
45. Fink KB, Gothert M. 5-HT receptor regulation of neurotransmitter release. *Pharmacol Rev* (2007) 59(4):360–417. doi:10.1124/pr.107.07103
46. Chiu HY, Chan MH, Lee MY, Chen ST, Zhan ZY, Chen HH. Long-lasting alterations in 5-HT2A receptor after a binge regimen of methamphetamine in mice. *Int J Neuropsychopharmacol* (2014) 17(10):1647–58. doi:10.1017/s1461145714000455
47. Strauss L, Brink CB, Möller M, Stein DJ, Harvey BH. Late-life effects of chronic methamphetamine exposure during puberty on behaviour and corticostriatal mono-amines in social isolation-reared rats. *Dev Neurosci* (2014) 36(1):18–28. doi:10.1159/000357495
48. Haidar M, Lam M, Chua BE, Smith CM, Gundlach AL. Sensitivity to chronic methamphetamine administration and withdrawal in mice with relaxin-3/RXFP3 deficiency. *Neurochem Res* (2016) 41(3):481–91. doi:10.1007/s11064-015-1621-2
49. Hruba L, Schutova B, Slamberova R. Sex differences in anxiety-like behavior and locomotor activity following prenatal and postnatal methamphetamine exposure in adult rats. *Physiol Behav* (2012) 105(2):364–70. doi:10.1016/j.physbeh.2011.08.016
50. Kitanaka N, Kitanaka J, Tatsuta T, Tanaka K, Watabe K, Nishiyama N, et al. Withdrawal from fixed-dose injection of methamphetamine decreases cerebral levels of 3-methoxy-4-hydroxyphenylglycol and induces the expression of anxiety-related behavior in mice. *Neurochem Res* (2010) 35(5):749–60. doi:10.1007/s11064-010-0132-4
51. Loxton D, Canales JJ. Long-term cognitive, emotional and neurogenic alterations induced by alcohol and methamphetamine exposure in adolescent rats. *Prog Neuropsychopharmacol Biol Psychiatry* (2017) 74:1–8. doi:10.1016/j.pnpbp.2016.11.003
52. Ago Y, Nakamura S, Baba A, Matsuda T. Neuropsychotoxicity of abused drugs: effects of serotonin receptor ligands on methamphetamine- and cocaine-induced behavioral sensitization in mice. *J Pharmacol Sci* (2008) 106(1):15–21. doi:10.1254/jphs.FM0070121
53. Ago Y, Koyama Y, Baba A, Matsuda T. Regulation by 5-HT1A receptors of the in vivo release of 5-HT and DA in mouse frontal cortex. *Neuropharmacology* (2003) 45(8):1050–6. doi:10.1016/S0028-3908(03)00304-6
54. Igari M, Shen HW, Hagino Y, Fukushima S, Kasahara Y, Lesch KP, et al. Attenuated methamphetamine-induced locomotor sensitization in serotonin transporter knockout mice is restored by serotonin 1B receptor antagonist treatment. *Behav Pharmacol* (2015) 26(1–2):167–79. doi:10.1097/fbp.0000000000000120
55. Przegalinski E, Siwanowicz J, Nowak E, Papla I, Filip M. Role of 5-HT(1B) receptors in the sensitization to amphetamine in mice. *Eur J Pharmacol* (2001) 422(1–3):91–9. doi:10.1016/S0014-2999(01)01079-2
56. Ago Y, Nakamura S, Kajita N, Uda M, Hashimoto H, Baba A, et al. Ritanserin reverses repeated methamphetamine-induced behavioral and neurochemical sensitization in mice. *Synapse* (2007) 61(9):757–63. doi:10.1002/syn.20421
57. Tanaka T, Ishigooka J, Watanabe S, Nagata E, Miura S. Partial inhibition of reverse tolerance by a high dose of ritanserin or low dose of haloperidol in methamphetamine-sensitized rat. *Nihon Shinkei Seishin Yakurigaku Zasshi* (1998) 18(1):1–7.

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 Behavioral Neuroscience

ORIGINAL RESEARCH
published: 21 July 2017
doi: 10.3389/fnbeh.2017.00137

Consequences of Adolescent Exposure to the Cannabinoid Receptor Agonist WIN55,212-2 on Working Memory in Female Rats

Erin K. Kirschmann[1†], *Daniel M. McCalley*[1†], *Caitlyn M. Edwards*[1,2] *and Mary M. Torregrossa*[1,2*]

[1]*Department of Psychiatry, Translational Neuroscience Program, University of Pittsburgh, Pittsburgh, PA, United States,*
[2]*Center for Neuroscience, University of Pittsburgh, Pittsburgh, PA, United States*

Marijuana is a prevalent illicit substance used by adolescents, and several studies have indicated that adolescent use can lead to long-term cognitive deficits including problems with attention and memory. However, preclinical animal studies that observe cognitive deficits after cannabinoid exposure during adolescence utilize experimenter administration of doses of cannabinoids that may exceed what an organism would choose to take, suggesting that contingency and dose are critical factors that need to be addressed in translational models of consequences of cannabinoid exposure. Indeed, we recently developed an adolescent cannabinoid self-administration paradigm in male rats, and found that prior adolescent self-administration of the cannabinoid receptor agonist WIN55,212-2 (WIN) resulted in improved working memory performance in adulthood. In addition, the doses self-administered were not as high as those that are found to produce memory deficits. However, given known sex differences in both drug self-administration and learning and memory processes, it is possible that cannabinoid self-administration could have different cognitive consequences in females. Therefore, we aimed to explore the effects of self-administered vs. experimenter-administered WIN in adolescent female rats on adult cognitive function. Female rats were trained to self-administer WIN daily throughout adolescence (postnatal day 34–59). A control group self-administered vehicle solution. The acute effects of adolescent WIN self-administration on memory were determined using a short-term spatial memory test 24 h after final SA session; and the long-term effects on cognitive performance were assessed during protracted abstinence in adulthood using a delayed-match-to-sample working memory task. In a separate experiment, females were given daily intraperitoneal (IP) injections of a low or high dose of WIN, corresponding to self-administered and typical experimenter-administered doses, respectively, or its vehicle during adolescence and working memory was assessed under drug-free conditions in adulthood. While self-administration of WIN in adolescence had no significant effects on short-term spatial memory or adult working memory, experimenter administration of WIN resulted in improved adult working memory performance that was more pronounced in the low dose group. Thus, low-dose adolescent WIN exposure, whether self-administered or

OPEN ACCESS

Edited by:
Valérie Doyère, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
James David Jentsch, Binghamton University, United States
Arnau Busquets-Garcia, Neurocentre Magendie, France

***Correspondence:**
Mary M. Torregrossa
torregrossam@upmc.edu

[†]*Co-first authors.*

Received: *24 April 2017*
Accepted: *07 July 2017*
Published: *21 July 2017*

Citation:
Kirschmann EK, McCalley DM, Edwards CM and Torregrossa MM (2017) Consequences of Adolescent Exposure to the Cannabinoid Receptor Agonist WIN55,212-2 on Working Memory in Female Rats. Front. Behav. Neurosci. 11:137. doi: 10.3389/fnbeh.2017.00137

experimenter-administered, results in either improvements or no change in adult working memory performance in female rats, similar to previous results found in males.

Keywords: cannabis, WIN55,212-2, adolescence, female, working memory, self-administration

INTRODUCTION

Legal policy in the U.S. regarding both medicinal and recreational use of marijuana has become increasingly relaxed over the last decade. Considering that the prevalence of marijuana use among adolescents is between 35 and 40 percent (Moss et al., 2014; Salas-Wright et al., 2016), it is crucial to understand the long-term effects of marijuana exposure during adolescence in order to properly inform any potential policy changes. Adolescence is a period of substantial neuronal development. Of particular importance, the development of both the prefrontal cortex, a brain region required for higher-order cognitive processing, and the endocannabinoid system, responsible for modulating various physiological functions as well as the binding of exogenous cannabinoids, occur simultaneously (Spear, 2000; Schneider, 2008). The primary psychoactive components of marijuana are cannabinoids, particularly Δ^9-tetrahydrocannabinol (THC), that activate cannabinoid receptors CB1 and CB2. Therefore, considering the concurrent changes in neuronal and cannabinoid system development, there is potential for marijuana use to cause cannabinoid-induced interference in the carefully orchestrated development of the adolescent brain, possibly resulting in long-term effects on cognition.

Several studies have been conducted in order to assess the long-term effects of cannabinoid use during adolescence; however, the findings have been mixed. Some clinical work has linked adolescent THC use to reduced IQ, increased risk for psychosis, and impaired working memory (Meier et al., 2012; Becker et al., 2014; Gage et al., 2016; Marconi et al., 2016). In contrast, other clinical studies have failed to find differences in performance between cannabis users and controls on cognitive tasks (Jager et al., 2006; Buchy et al., 2015; Mokrysz et al., 2016). In preclinical animal studies, the most common application of cannabinoids has been via experimenter administration, which does not model the volitional control over intake and choice over dose of intake observed in human populations, bringing to question the translational value of these studies. Nevertheless, these studies have generally found a relationship between chronic cannabinoid exposure and cognitive deficits, especially in working memory, object recognition and short-term spatial memory capacity (Hampson and Deadwyler, 2000; O'Shea et al., 2004, 2006; Schneider and Koch, 2007; Abush and Akirav, 2012; Renard et al., 2016).

We recently developed a model of adolescent intravenous (IV) cannabinoid self-administration, which more closely models the voluntary nature of drug use in humans (Kirschmann et al., 2017). We trained rats to self-administer the synthetic cannabinoid receptor agonist WIN55,212-2 (WIN), a full agonist of CB1 and CB2 cannabinoid receptors that is more potent than THC, because previous studies have found that rats will readily self-administer this compound, whereas rats did not readily self-administer THC, which is a partial agonist (Takahashi and Singer, 1979; Fattore et al., 2001; Deiana et al., 2007; Pertwee, 2010; Lefever et al., 2014). Although WIN's effects may be more directly comparable to the frequently abused synthetic cannabinoids such as K2 and spice, it provides us with the opportunity to investigate the effects of self-directed cannabinoid receptor activation on behavior. Interestingly, adolescent self-administration of WIN did not lead to acute memory deficits or long-term effects on working memory performance using a delayed-match-to-sample task. In fact, we found that rats that self-administered WIN had better working memory performance in adulthood (after several weeks of abstinence) than control animals that responded for sucrose pellets. However, the doses of WIN that rats were willing to self-administer were much lower than doses used in previous studies identifying memory impairments after experimenter-administration. Thus, it is possible that cannabinoid-induced memory deficits are likely to only be found in very heavy users, and/or are not likely to be observed after long periods of abstinence. Another potential difference between findings in rodents and humans is that almost all previous preclinical work, including our own study, was conducted in male subjects. Given that there are known sex differences in both drug self-administration and learning and memory processes, it is possible that adolescent cannabinoid exposure could produce different results in males and females. Therefore, the current study aimed to explore the long-term cognitive effects of self-administered versus experimenter-administered cannabinoids in adolescent female rats.

MATERIALS AND METHODS

Animals

A total of 42 female, Sprague–Dawley (Harlan, Frederick, MD, USA) rats were delivered on postnatal day (PND) 22 and were housed in a climate-controlled room on a 12-h dark/light cycle (lights on at 4:30 am) throughout the duration of the experiment. All behavioral experiments were conducted during the light phase. Rats were pair-housed unless otherwise indicated, and were food restricted to about 85%–90% of normal, free-feeding weight for the duration of all behavioral experiments. Food portions were adjusted daily throughout adolescence with females receiving a range of 13–15 g/day. Food restriction was necessary to maintain responding in behavioral tasks. All procedures were performed in accordance with the recommendations of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The protocol was approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

Drugs

The synthetic cannabinoid receptor agonist WIN55,212-2 mesylate (WIN; NIMH Chemical Synthesis and Drug Supply Program; Cayman Chemical Company, Ann Arbor, MI, USA), was dissolved in sterile 0.9% saline with a drop of Tween 80. Fresh stock solutions were made every 2–3 days, and were diluted with saline daily to a working concentration for IV administration of 0.0125 mg/kg/infusion. A vehicle solution of 0.9% saline and a drop of Tween 80 was created daily and was diluted with saline for IV administration and intraperitoneal (IP) administration. Fresh working solutions of 1.2 mg/mL and 0.2 mg/mL were prepared daily for IP administration.

Surgical Procedures

In animals responding for IV WIN/vehicle, surgery was performed to implant indwelling jugular catheters on PND 27–28. Rats were anesthetized with ketamine (90 mg/kg) and xylazine (5 mg/kg) and given 5 mg/kg of the analgesic Rimadyl. Catheters were constructed with silastic tubing (11 cm; Braintree Scientific, Braintree, MA, USA) attached to a bent steel guide cannula (22 gauge; Plastics One Inc., Roanoke, VA, USA), surrounded by Loctite medical epoxy (Grainger, Pittsburgh, PA, USA) and attached to a 2 cm square piece of mesh (Bard Mesh; Davol Inc., Cranston, RI, USA). Catheters were implanted in the right jugular vein and fed subcutaneously to the back, where they exited through a small incision between the shoulder blades, and were flushed daily with 0.1 ml of Gentamicin in heparinized saline. A recovery period of 7 days was allowed before initiation of behavioral testing.

Self-Administration

Between PNDs 34–59, rats (n = 10) were trained to self-administer the synthetic cannabinoid receptor agonist WIN in standard operant conditioning chambers (Med Associates, St. Albans, VT, USA; see **Figure 1** for an experimental timeline). The chambers contained two retractable levers, a house light, two stimulus lights, a tone generator, a food magazine and a fan for background noise. A press on the active lever resulted in a 0.0125 mg/kg/infusion delivery of WIN at a rate of 0.03 ml/s paired with a 10 s tone and light stimulus in combination with a 10 s timeout, during which the house light was extinguished. Infusion times were adjusted daily based on individual rats' weights to maintain the 0.0125 mg/kg/infusion dose. A press on the inactive lever yielded no programmed consequences. Rats were trained on a 1-h fixed-ratio 1 (FR1) schedule of reinforcement during the first 4 days of self-administration, and were then switched to 2-h sessions (FR1) for the remaining 18 days of self-administration. Control rats (n = 8) responded for IV infusions of a vehicle solution using identical training procedures as for WIN.

Extinction and Reinstatement Testing

Following the last day of self-administration training, rats underwent instrumental extinction (1-h sessions), where lever presses produced no cue presentations or IV infusions. Rats underwent extinction for 8–9 days and until the rat had fewer than 20 lever presses. The day following the last day of extinction, and again 20 days later, rats were tested for cue-induced reinstatement of WIN seeking in 30 min sessions. During these sessions, lever presses resulted in a 10 s presentation of the audiovisual cue on an FR1 schedule. No infusions were given during reinstatement.

Experimenter-Administration

In a separate experiment, female rats (n = 24) received IP injections daily throughout adolescence (PND 34–53) between 2:00–3:00 pm during the light phase (**Figure 1**). Rats received either vehicle (n = 8), a low dose of WIN, 0.2 mg/kg, which corresponds to the average total daily dose that males received during self-administration (Kirschmann et al., 2017; n = 8), or

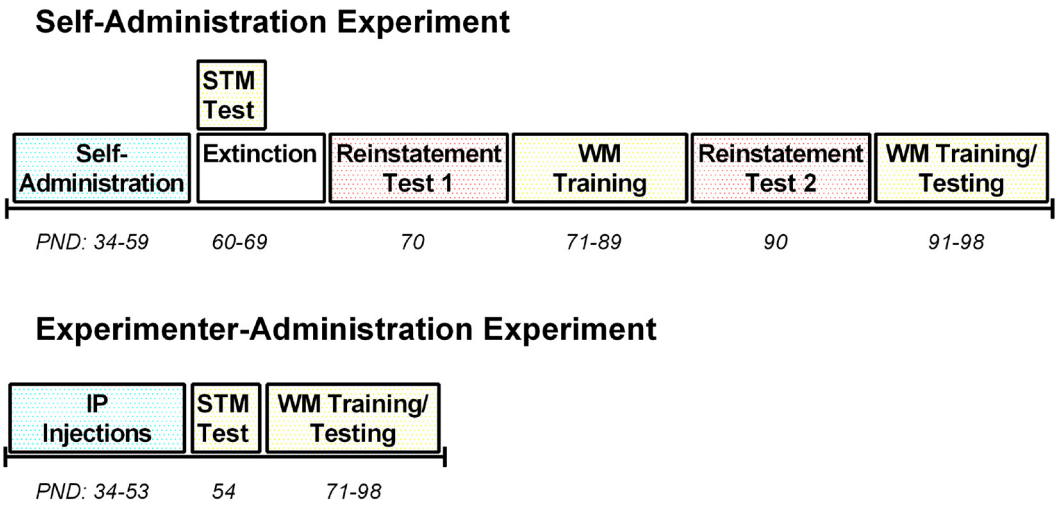


FIGURE 1 | Timeline of treatment and behavioral testing for both the self-administration and experimenter-administration experiments. PND, postnatal day; STM, short-term memory; WM, working memory; IP, intraperitoneal.

a high dose of WIN, 1.2 mg/kg, which corresponds to the dose commonly given in previous experiments that identified deficits in short-term memory (Schneider and Koch, 2003; O'Shea et al., 2006; $n = 8$). The total number of days of exposure to WIN was almost the same between the experimenter-administration and self-administration groups, though the age at which exposure ended varied by 6 days. The self-administration group did not have sessions on ∼3 days during the study due to experimental conflicts, and rats that self-administered tended to get fewer infusions, and thus a lower dose of WIN, on the initial day of self-administration. Thus, the low dose IP group and the self-administration group are not perfectly equated, but received similar amounts of WIN overall.

Short-Term Spatial Memory Task

All rats were allowed to habituate to an empty, open field chamber (43 cm × 43 cm; Med Associates) under dim light for a period of 5 min on three occasions. Within 24 h of the last day of WIN exposure (PND 60 for self-administration (SA) rats, PND 54 for IP rats), all rats underwent short-term spatial memory testing in the same open field chamber. Two objects were placed evenly from opposing corners in the chamber, and rats were allowed to explore the objects for a period of 5 min. Objects were similar in material (glass/ceramic) and dimension (11 cm × 11 cm). Rats were returned to their home cages, and one of the objects was moved to a new spatial location. Following a 35-min delay, rats were again allowed to explore the objects in the chamber. Typically, rats prefer novelty; hence a rat with intact spatial memory will spend more time examining the object in the novel location, compared to the time spent examining the object in the familiar location. All rats performed this task and the chamber was cleaned with 70% ethanol between trials. Data were collected and analyzed offline using AnyMaze software (Stoelting, Wood Dale, IL, USA).

Delayed-Match-to-Sample Working Memory Task

Following 10–11 days of abstinence (which allowed the rats to age into adulthood), all rats began training in a delayed-match-to-sample working memory task. Training was performed during 1-h sessions in chambers (Med Associates) equipped with five nose poke apertures and a food dispenser. Initially, all five apertures were illuminated and rats received a sucrose pellet (45 mg, Bioserv, Flemington, NJ, USA) reward upon response on any of these five holes on an FR1 schedule. Next, a single aperture was illuminated and only a response in that hole resulted in reward. On the following days of training, a response on the single, illuminated "sample" aperture resulted in the immediate illumination of the "sample" and two additional "choice" apertures. The sample aperture for each trial was selected at random using Med Associates random number generator software code. The additional two apertures illuminated during the choice phase were always the two closest apertures to the sample, resulting in the two flanking apertures for the middle three apertures, and the next two innermost apertures for outer apertures. Following illumination of the choice aperture, a second response on the "sample" resulted in sucrose reward (FR1). Responses in any of the other apertures resulted in a 2 s timeout where all lights were extinguished. Once animals performed this task reliably with accuracy greater than 75% correct, delays were introduced between the "sample" phase and the "choice" phase. Rats performed blocks of trials in which seven delays (0.5–6 s) were presented in random order; each of the seven delays occurred before a new block of trials began. Once rats reached training criterion (≥80% correct 0.5 s delay), the range was increased (0.5–12 s; 0.5–24 s; See **Tables 1**, **2** for summaries of SA and IP groups' training data). Working memory was assessed most critically on the first day in which the animals were presented with delays between 0.5 s and 24 s, ranging from age PND 93 to 98.

Estrous Cycle Monitoring

Vaginal lavage samples were taken by gently pipetting 150 μl of saline into the vagina to flush the vaginal canal. The saline

TABLE 1 | Training data for self-administration study.

		Days to meet criteria[a]	Reinforcers earned[b,c,d]	Accuracy across delays[e]
Phase 1	WIN	1.0 (±0)	60.1 (±4.3)	N/A
	VEH	1.0 (±0)	62.6 (±6.5)	
Phase 2	WIN	4.0 (±0.3)	59.1 (±6.0)	N/A
	VEH	4.1 (±0.1)	63.8 (±9.8)	
Phase 3	WIN	4.0 (±0.4)	58.2 (±2.3)	N/A
	VEH	3.9 (±0.3)	61.3 (±6.7)	
Phase 4	WIN	5.6 (±0.6)	49.9 (±2.3)	$F_{(1,16)} = 0.51$, $p = 0.49$
	VEH	5.6 (±0.5)	54.1 (±3.4)	
Phase 5	WIN	6.0 (±0.4)	47.2 (±2.4)	$F_{(1,16)} = 0.29$, $p = 0.60$
	VEH	6.0 (±0.2)	56.6 (±3.3)*	

*[a]Number of days WIN SA or VEH SA rats remained in current phase of training before advancing to next phase. Reinforcers earned: [b]Single day of phase 1; [c]Average over first 4 days in phases 2–3; [d]Average over first 6 days in phases 4–5. [e]Accuracy across delays on first day of training in phases 4–5. *p < 0.05, WIN vs. VEH.*

TABLE 2 | Training data for experimenter-administration study.

		Days to meet criteria[a]	Reinforcers earned[b,c,d]	Accuracy across delays[e]
Phase 1	Low	1.0 (±0)	23.9 (±2.8)	N/A
	High	1.0 (±0)	27.3 (±2.8)	
	Veh	1.0 (±0)	26.9 (±3.1)	
Phase 2	Low	7.0 (±0.6)	24.4 (±6.3)	N/A
	High	7.1 (±0.4)	18.4 (±4.4)*	
	Veh	6.0 (±0.5)	41.0 (±6.4)	
Phase 3	Low	3.9 (±0.7)	63.6 (±10.6)	N/A
	High	2.9 (±0.1)	84.0 (±7.6)	
	Veh	3.0 (±0.2)	95.3 (±23.7)	
Phase 4	Low	3.4 (±0.3)	57.5 (±5.3)	$F_{(2,21)} = 0.47$, $p = 0.63$
	High	4.0 (±0.3)	48.2 (±6.1)	
	Veh	4.0 (±0.3)	54.7 (±7.7)	
Phase 5	Low	5.6 (±0.3)	50.6 (±3.6)	$F_{(2,21)} = 0.78$, $p = 0.47$
	High	5.8 (±0.4)	45.8 (±5.8)	
	Veh	6.6 (±0.5)	52.0 (±6.0)	

*[a]Number of days Low WIN (0.2 mg/kg) IP, High WIN (1.2 mg/kg) IP or vehicle IP rats remained in current phase of training before advancing to next phase. Reinforcers earned: [b]Single day of phase 1; [c]Average over first 6 days in phases 2 and 5; [d]Average over first 3 days in phases 3–4. [e]Accuracy across delays on first day of training in phases 4–5. *p < 0.05, High WIN vs. VEH.*

was pipetted onto a clean microscope slide and covered with a coverslip. Cell morphology was examined under a compound microscope at a magnification of 100–200× to determine estrous cycle phase using standard procedures (Goldman et al., 2007). In order to allow for uninterrupted pubertal development, vaginal lavage sampling began on PND 46 (day 13 of the self-administration period) and was performed daily until the final day of self-administration. Samples were also taken on days of significant testing, specifically on the first day of each new delay range in working memory. Females were split into two groups of either high estradiol (proestrus or transitioning in or out of proestrus; PRO), or low estradiol (estrus, metestrus, or diestrus; EMD) and analyzed accordingly.

Statistical Analyses

Data were analyzed using Prism 6.0 Software (GraphPad Inc., LaJolla, CA, USA) and the SPSS software package version 21.0 (SPSS, Chicago, IL, USA). Self-administration and extinction data were analyzed using repeated measures (rm) ANOVA with a within-subjects factor of day of self-administration and between-subjects factor of group (WIN vs. VEH SA). Additionally, separate rmANOVA analyses of VEH and WIN SA groups were conducted with a within-subjects factor of day of self-administration and between-subjects factor of response type (active lever vs. infusions vs. inactive lever). Reinstatement data were analyzed by comparing within-subjects lever pressing on the last day of extinction to pressing across days of abstinence, comparing responses on the active and inactive levers by rmANOVA. Short-term spatial memory and working memory were also analyzed by rmANOVA, comparing test phase or delay length within-subjects and adolescent treatment type (WIN vs. VEH) between subjects. Fisher's least significant difference (LSD) test was used for *post hoc* analyses following identification of significant effects. Two-sided *t*-tests were used to determine if high or low estradiol phases of the estrous cycle [proestrus (PRO) vs. a combination of estrus, metestrus, and diestrus (EMD)] affected self-administration behaviors during the last 5 days of training.

RESULTS

Adolescent Self-Administration, Extinction and Cue-Induced Reinstatement

Analysis of infusions earned during self-administration by rmANOVA with group (WIN vs. VEH) as the between subjects factor showed that infusion number increased over the course of self-administration, regardless of whether responding was for WIN or for vehicle solution (VEH; main effect of SA day, $F_{(21,336)} = 5.41$, $p < 0.001$; no group × day interaction $F_{(21,336)} = 0.51$, $p = 0.97$). We also conducted separate rmANOVAs for WIN and VEH groups individually in order to compare the pattern of active vs. inactive lever pressing across days under each self-administration condition. Adolescent female rats that self-administered WIN reliably discriminated between the active and inactive levers (**Figure 2A**). Analysis by rmANOVA found a significant main effect of response type across days ($F_{(2,27)} = 14.83$, $p < 0.001$), and Fisher's LSD test indicated significant difference between inactive lever presses and both active lever presses ($p < 0.001$) and infusions earned ($p = 0.041$). Females responding for VEH did not significantly differ from WIN treated females in the number of active or inactive lever presses overall, and exhibited a significant main effect of response type ($F_{(2,21)} = 15.30$, $p < 0.001$). Further analysis indicated that only active and inactive lever presses significantly differed ($p < 0.001$), while there was no difference between infusions earned and inactive lever presses ($p = 0.65$; **Figure 2B**). Therefore, inactive pressing for VEH did not separate from infusions earned to the same degree as in the WIN group, though there were no overall between-drug differences. We hypothesize that the high degree of VEH responding was likely driven by presentation of the audiovisual cue. Importantly, the equatable levels of SA performance during adolescence in the VEH group serves as a better behavioral control for our later assessment of cognitive consequences, since both groups had similar experience with the cues. Females self-administered an average dose of 0.15–0.16 mg/kg WIN over the last 5 days of self-administration, similar to what we have previously observed in males, where the average daily dose was 0.22 mg/kg (Kirschmann et al., 2017).

Next, we examined extinction of the lever press response, where active lever presses produced neither the IV infusion, nor the audiovisual cue. Analysis of active lever responding by rmANOVA with day as within-subjects factor and group (WIN vs. VEH) as between subjects factor showed no effect of SA condition on extinction behavior (main effect of day ($F_{(7,112)} = 4.02$, $p = 0.001$), no group × day interaction ($F_{(7,112)} = 1.08$, $p = 0.384$)). We confirmed in separate rmANOVAs for WIN and VEH groups that both WIN and VEH self-administration groups exhibited a significant decrease in active lever responding over days (main effects of day ($F_{(7,126)} = 2.57$, $p = 0.017$) and ($F_{(7,98)} = 3.90$, $p < 0.001$), respectively), indicating acquisition of extinction (**Figures 2C,D**). However, the WIN group did not extinguish to as low of a degree of responding as the VEH group, exhibiting a significant difference between active and inactive lever presses across days of training ($F_{(1,18)} = 4.70$, $p = 0.044$), while the VEH group showed no significant differences between active and inactive lever responses over time ($F_{(1,14)} = 2.80$, $p = 0.12$).

Following extinction training, rats were tested for cue-induced reinstatement in two 30-min sessions where active lever presses resulted in presentation of the audiovisual cue. The first test occurred following the last day of extinction training, on day 9–10 of abstinence depending on when the rat met the extinction criterion. The second test occurred 20 days later, corresponding to day 29–30 of abstinence. Due to the difference in session length between extinction training and reinstatement tests, response rates were calculated (lever presses/minute) and compared between the last day of extinction (baseline) and the two reinstatement tests. Analysis by rmANOVA with group (WIN vs. VEH) as between-subjects factor identified a main effect of test phase ($F_{(2,32)} = 44.52$, $p < 0.001$), but no effect of group ($F_{(1,16)} = 0.616$, $p = 0.44$)

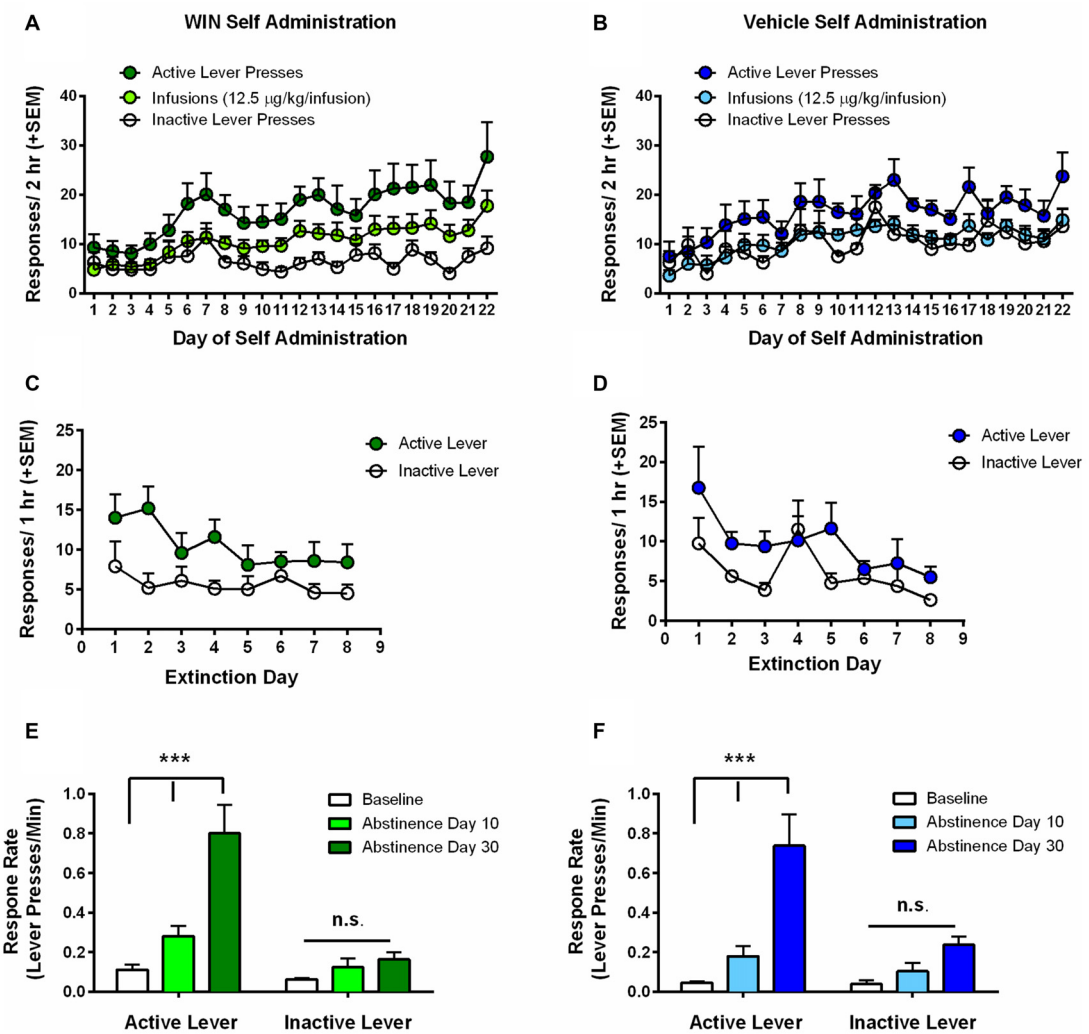


FIGURE 2 | Adolescent self-administration behaviors. **(A)** Rats self-administering WIN throughout adolescence showed clear discrimination between active and inactive lever responses over days, and rats earned significantly more infusions relative to inactive lever presses. **(B)** Rats self-administering vehicle showed a smaller, but significant differentiation between active and inactive lever responses, but no difference between inactive lever presses and infusions earned. **(C,D)** Significant extinction of the lever press response occurred in both the WIN **(C)** and VEH **(D)** groups. **(E)** Rats that self-administered WIN exhibited significant cue-induced reinstatement of lever pressing over response rates on the last day of extinction (i.e., baseline) on both 10 and 30 days of abstinence. Reinstatement on day 30 was also significantly greater than that observed on day 10. **(F)** Rats that self-administered VEH did not exhibit a significant cue-induced reinstatement of lever pressing on day 10, but responding on day 30 was significantly greater than baseline and day 10 response rates. ***$p < 0.001$.

or group × test phase interaction ($F_{(2,32)} = 0.04$, $p = 0.96$). Analysis of WIN and VEH SA groups separately by rmANOVA revealed that the WIN self-administration group exhibited significant differences based on test phase ($F_{(2,18)} = 26.09$, $p < 0.001$), and lever type ($F_{(1,9)} = 17.42$, $p = 0.002$), and a significant interaction between the two ($F_{(2,18)} = 23.60$, $p < 0.001$). *Post hoc* analysis indicated that there were no significant differences in inactive lever response rates across test days, but that there was a significant difference between baseline responding (i.e., on the last day of extinction) and response rates on day 10 ($p = 0.018$) and day 30 ($p < 0.001$) of abstinence, indicating significant cue-induced reinstatement of WIN seeking on both test days. Furthermore, there was also a significant increase in responding from day 10 to day 30 of abstinence ($p < 0.001$), indicating a significant incubation of craving effect (**Figure 2E**). Similar effects were observed in the VEH self-administration group, with significant effects of test day ($F_{(2,14)} = 28.02$, $p < 0.001$), lever type ($F_{(1,7)} = 9.48$, $p = 0.018$) and their interaction ($F_{(2,14)} = 7.28$, $p = 0.007$). However, on day 10 of "abstinence", there was no significant difference in response rate from the extinction baseline ($p = 0.20$). Nevertheless, on day 30 of "abstinence", response rates were significantly increased over both baseline ($p < 0.001$) and day 10 levels ($p < 0.001$). Therefore, while there was not a significant cue-induced reinstatement effect on day 10, there was still an increase in cue-motivated responding on day 30, despite the

cue never being paired with a drug (**Figure 2F**). These data suggest that cues can be strong motivators of behavior (at least in adolescent females), independent of conditioning to a reinforcer.

Estrous Cycle Phase Does Not Impact Self-Administration

The present study was conducted in freely cycling females, thus making it difficult to explicitly assess the relationship between circulating hormones and motivation to self-administer WIN. However, we did monitor estrous cycle phase via observation of vaginal cytology during the last 9 days of self-administration and averaged the infusions earned, active and inactive lever presses of rats during the last 5 days of self-administration on days that they were in a high estradiol phase (PRO) and a low estradiol phase (EMD). Using two-sample *t*-tests (PRO vs. EMD), we found no significant differences based on estrous cycle in infusions ($t_{(16)} = 0.639$, $p = 0.53$) or active lever presses ($t_{(16)} = 0.445$, $p = 0.66$; **Figures 3A,B**). Inactive lever presses did appear to be reduced during proestrus, but this only reached a trend level of significance ($t_{(16)} = 1.783$, $p = 0.09$; **Figure 3C**).

Adolescent Cannabinoid Exposure Does Not Acutely Affect Short-Term Spatial Memory

We next tested whether or not females would exhibit acute deficits in short-term spatial memory 24 h following the last self-administration session. Since previous research has indicated that adolescent experimenter administration of WIN, at doses substantially higher than those obtained during self-administration, can produce short-term memory deficits in males (Kirschmann et al., 2017), a separate cohort of rats was exposed to chronic WIN in adolescence using an experimenter-administration procedure. We compared a dose of WIN comparable to that obtained during self-administration (0.2 mg/kg/day) to the commonly used "high" dose of WIN (1.2 mg/kg/day). Neither self-administration, nor experimenter-administration of WIN at either dose, produced any short-term memory deficits in females. Both WIN and VEH self-administration groups exhibited equivalent discrimination of novel from familiar spatial locations (main effect of location ($F_{(1,13)} = 68.09$, $p < 0.001$), but no effect of treatment ($F_{(1,13)} = 0.08$, $p = 0.78$; **Figure 4A**). Similar results were observed under experimenter-administration conditions, with a main effect of location ($F_{(1,20)} = 43.19$, $p < 0.001$), but no effect of treatment ($F_{(2,20)} = 0.29$, $p = 0.75$; **Figure 4B**).

Working Memory Performance

All rats began training on the delayed-match-to-sample working memory task 10–11 days after the last drug exposure day, which followed extinction and initial reinstatement testing in the self-administration group. All groups received between 22 days and 25 days of training prior to final working memory assessments. We observed no differences between the self-administration groups across different phases of training (**Table 1**). Analysis of working memory performance once rats reached the final test phase, where the delays between sample and choice phases are longest, revealed no significant effects of treatment ($F_{(1,17)} = 0.01$, $p = 0.91$). However, we did observe the expected effect of delay, with performance accuracy significantly decreasing as the delay length was increased ($F_{(6,102)} = 44.99$, $p < 0.001$; **Figure 5A**). Thus, female rats that self-administered WIN during adolescence showed neither an improvement nor a detriment in working memory performance in adulthood under drug-free conditions.

Conversely, analysis of working memory performance in rats that received experimenter-administered WIN in adolescence indicated that exposure to the low, but not the high dose, produced working memory improvements in adulthood (see **Table 2** for training data). Analysis by rmANOVA identified the expected decrease in performance with increasing delay length ($F_{(6,126)} = 40.29$, $p < 0.001$), and a strong trend toward a treatment effect when all three treatment groups were included in the analysis ($F_{(2,21)} = 3.19$, $p = 0.06$). Due to the fact that the potential treatment effect appeared to be driven by low dose WIN exposure and differences are not expected at very short and very long delays, a separate two-way rmANOVA comparing the vehicle and low dose groups across 4–16 s delays was conducted, which indicated a significant improvement in working memory performance after adolescent exposure to low dose WIN ($F_{(1,14)} = 10.57$, $p = 0.006$; **Figure 5B**). Similar analysis

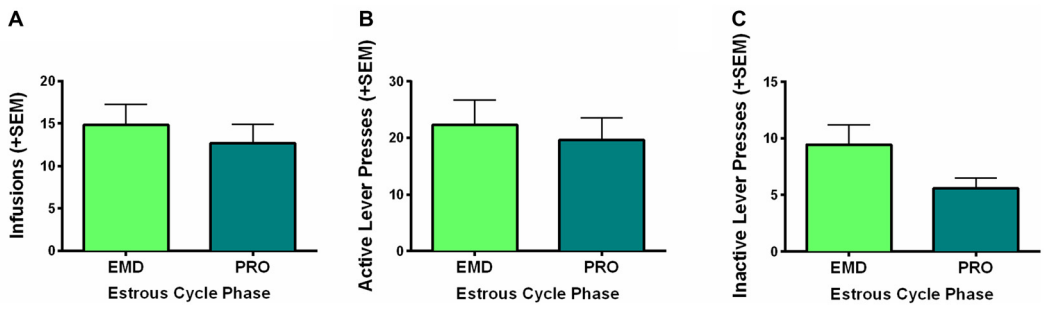


FIGURE 3 | Role of estrous cycle phase on WIN self-administration. Behaviors during the high estradiol phase of proestrus (PRO) were compared to behavior during the low estradiol phases of estrus, metestrus and diestrus (EMD). No significant differences were identified for **(A)** infusions earned, **(B)** active lever presses, or **(C)** inactive lever presses.

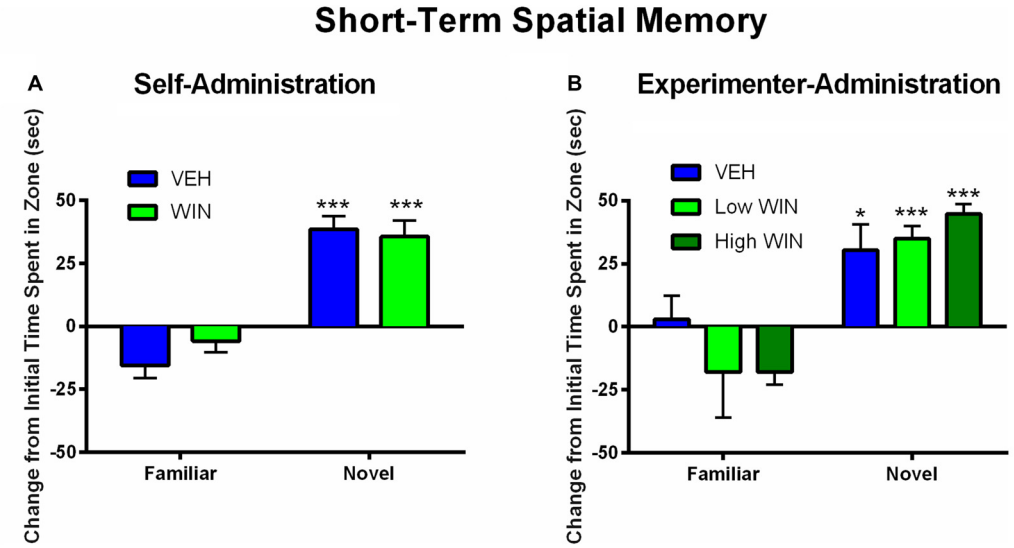


FIGURE 4 | Effect of adolescent WIN exposure on short-term spatial memory. **(A)** Self-administration of WIN did not result in any acute deficits in short-term spatial memory in an object location task in female rats tested 24 h after the last self-administration day. All rats showed a significant discrimination between the familiar and novel spatial locations, $^{***}p < 0.001$. **(B)** Experimenter-administration of either a low or high dose of WIN did not significantly alter short-term spatial memory 24 h after the last injection day. All groups exhibited significant discrimination between familiar and novel locations, indicating intact memory, $^{*}p \leq 0.05$, $^{***}p < 0.001$.

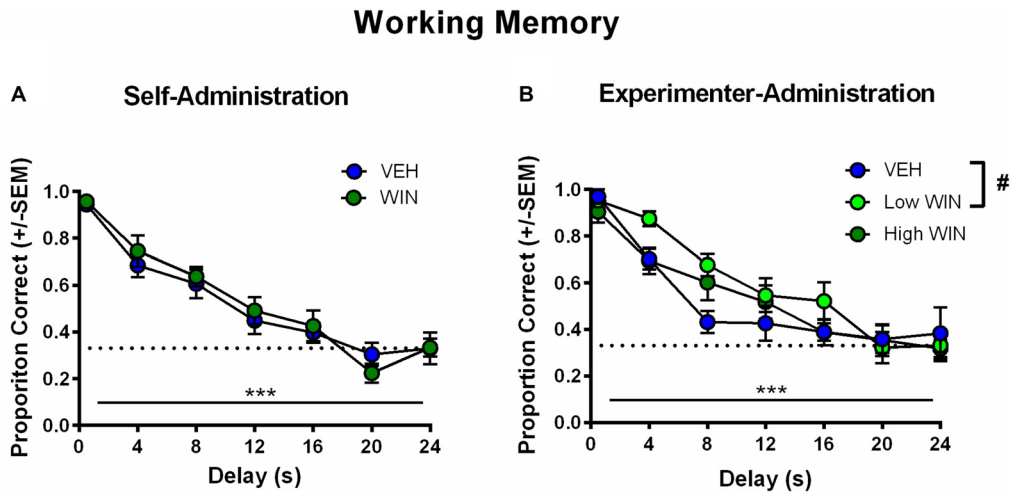


FIGURE 5 | Effect of adolescent WIN exposure on adult working memory. **(A)** Adult working memory performance in rats that self-administered WIN or VEH in adolescence. All working memory training and testing was conducted under drug-free conditions. As delay increased, performance decreased, $^{***}p < 0.001$. Adolescent WIN self-administration had no significant effect on working memory performance. **(B)** Adult working memory performance in rats that were exposed to WIN in adolescence via experimenter-administration (IP injection) of a low dose (0.2 mg/kg/day) or a high dose (1.2 mg/kg/day), or its vehicle. In all rats, as delay increased, performance decreased, $^{***}p < 0.001$. The low dose of WIN produced a strong trend toward improved working memory performance relative to vehicle, and separate analyses of delays from 4 s to 16 s, indicated a significant improvement in performance, $^{\#}p = 0.05$.

of the high WIN dose group did not reveal a significant treatment effect on performance ($F_{(1,14)} = 2.34$, $p = 0.15$).

DISCUSSION

The aims of the present study were to determine the long-term effects of self-administered cannabinoids in adolescence on adult cognitive function, and to explore the abuse liability of adolescent-onset cannabinoid use, in females. Additionally, we aimed to explore whether similar cognitive consequences arose from different routes of cannabinoid administration during adolescence. Similar to our previous findings in males, there were no detrimental effects of adolescent WIN self-administration on adult cognitive performance in females. Additionally,

experimenter-administration of a low dose of WIN in adolescent females resulted in improved performance in a delayed-match-to-sample working memory task in adulthood. Together, our data provide further support towards the notion that cannabinoid exposure under self-administration conditions, or at behaviorally-relevant doses, is less detrimental than originally suspected.

Analysis of Female Cannabinoid Self-Administration

The National Institutes of Health now requires the consideration of sex as a relevant biological variable in all new applications for funding. To our knowledge, our current study in females, in conjunction with our previously published findings in males (Kirschmann et al., 2017), is one of the first analyses of males and females in adolescent cannabinoid self-administration (but see work in adults by Fattore et al., 2007, 2010). We show here that adolescent female rats will self-administer the synthetic cannabinoid receptor agonist WIN55,212-2, and will attain a daily dose similar to, though slightly less than, the daily dose that what male rats attained in our prior study (∼0.15 mg/kg here in females vs. 0.22 mg/kg in males; Kirschmann et al., 2017). This is in contrast to rodent findings for other drugs of abuse such as cocaine, ethanol and opiates, where females take substantially higher amounts compared to males (e.g., Becker et al., 2007; Anker and Carroll, 2010; Becker and Koob, 2016; Bertholomey et al., 2016). However, our finding that females maintained a slightly lower daily dose of a cannabinoid receptor agonist than males is in line with human findings in which women report greater sensitivity to the subjective effects of inhaled or orally-administered cannabis (Cooper and Haney, 2014; Fogel et al., 2017). There were no significant differences in self-administration behaviors during the high estradiol phase (proestrus) compared to the low estradiol phases (estrus, metestrus and diestrus), indicating a lack of a role of estrous cycle in cannabinoid self-administration in adolescent females, at least to the degree to which we could assess estrous cycle in this study. In contrast, Fattore et al. (2007) found that ovariectomized adult females self-administered significantly less WIN compared to intact controls; however different strains and ages of rats were used in that study, and ovariectomy clamps estradiol at much lower levels than what occurs naturally during all phases of the estrous cycle, which could explain the difference in findings.

Female rats that self-administered WIN showed significant cue-induced lever pressing after a short abstinence period (10 days); and they exhibited even greater cue-induced lever pressing after an extended abstinence period (30 days), indicating an "incubation of craving" effect for a self-administered cannabinoid, similar to our previous findings in male rats (Kirschmann et al., 2017). However, female rats that self-administered the vehicle solution also exhibited increased cue-induced lever pressing after an extended period of "abstinence" (or lack of exposure to vehicle or cues). Few groups have ever examined incubation of craving in a vehicle group, particularly in females. The closest work may be that of Lee et al. (2013), and that of Werner et al. (2015), in which male rats trained to self-administer saline showed a lack of reinstatement for visual stimuli previously paired with infusion delivery; although in one case, if all reinstatement test days were collapsed, saline-trained animals did show a memory for the saline cue (Werner et al., 2015). Experiments with rodents responding for natural rewards (e.g., sucrose or food pellets) also have demonstrated reinstatement and incubation of craving for the cues previously paired with the natural rewards (e.g., Grimm et al., 2011; Darling et al., 2016; Dingess et al., 2017). Additionally, our paradigm utilized an audiovisual cue, and compound stimuli have themselves been shown to serve as conditioned reinforcers (Fuchs et al., 1998; See et al., 1999; Caggiula et al., 2002). Finally, adolescents have been shown to learn more about cues than adults (e.g., Meyer and Bucci, 2016). Thus, the increased cue-induced reinstatement after 30 days of abstinence in female rats that had previously self-administered the vehicle solution in adolescence is likely a reflection of compound cues serving as strong motivators of behavior in adolescent females, and makes it difficult to interpret if the incubation of craving observed in the WIN group is related specifically to the potential reinforcing property of WIN or to the compound cues. Future studies could examine if self-administration of other unit doses of WIN would produce greater self-administration and reinstatement in females, though the high level of responding in the vehicle group is still an important factor to consider when interpreting reinstatement and incubation of craving results across drug classes.

Cannabinoid Exposure in Adolescence Differentially Impacts Cognition

Similar to what we have shown in males, WIN self-administration did not cause acute deficits in short-term spatial memory in females. Contrary to findings in males (Abush and Akirav, 2012; Kirschmann et al., 2017), experimenter-administration of a high dose of WIN (1.2 mg/kg) in adolescence did not result in acute deficits in short-term spatial memory in females. This could reflect the differential sensitivity of females to cannabinoids; potentially, exposure to a higher or lower dose of WIN than was used in this study could have resulted in acute short-term memory deficits in females. We then examined the long-term effect of adolescent WIN exposure and we found a significant improvement in working memory in females that received the low dose (0.2 mg/kg) during adolescence. In contrast to what we previously reported in males, adolescent self-administration of WIN in females did not significantly impact adult working memory performance. It is not clear if this is due to sex differences in the impact of self-administered vs. experimenter-administered drugs, or if the lack of effect in the self-administration group is due to the fact that the females self-administered a lower dose of WIN (∼0.15 mg/kg/day) than males. Thus, a very tight dose-effect relationship may exist between adolescent WIN exposure and improvements in adult working memory performance. Nevertheless, under no conditions

by dose, sex, or route of administration have we observed long-lasting deficits in working memory after adolescent WIN exposure.

Limitations and Conclusions

The present set of experiments does have some caveats. First, it is clear that adolescent females will respond for cues, regardless of the solution they receive IV. This limits our interpretation of the self-administration data on the degree to which WIN is reinforcing in and of itself. However, because adolescent females responding for IV WIN tended to show better discrimination between the active and inactive levers, there is some indication that WIN carries at least somewhat greater reinforcing value than the vehicle solution. Future work that includes a full dose-response curve and examines differences in responding with and without cues for both male and female adolescents in the same experiment would be warranted. Additionally, because our main question was focused on the long-term cognitive consequences of cannabinoid self-administration during adolescence, the fact that the vehicle group exhibited similar levels of responding during SA provides for a more closely-matched control group. All rats received equivalent exposure to the cues, and therefore the differences we observed in later working memory performance can be attributed to the WIN exposure itself. Second, because we did not include males and females in a singular experiment, definitive conclusions about sex differences in cannabinoid self-administration and effects of adolescent cannabinoid exposure on later cognitive performance cannot be made. Overall, the response rates are similar in the females of the current experiment and the males we previously tested (Kirschmann et al., 2017); a larger study that includes both sexes run simultaneously would be necessary to confirm this. Third, all of our females were food restricted for the duration of behavioral experiments. Though this was necessary to maintain motivation to complete the tasks, this could interact with our behavioral findings. In line with this, we did not conduct a full panel of cognitive assessments in our rats. Future work could aim to complete additional investigations of cognitive ability using paradigms that do not require food restriction to ensure task performance. Fourth, all of our experiments utilized the synthetic cannabinoid receptor agonist WIN55,212-2, not the main psychoactive component of marijuana, Δ^9-THC. Although WIN does act on the same receptors as THC, its pharmacological properties may be distinct from THC, and its chemical structure may be more comparable to the frequently-abused synthetic cannabinoids K2 and spice (Pertwee, 2010). Nevertheless, there is evidence for similar effects of experimenter-administered THC or WIN on short-term memory that are blocked by nonselective cannabinoid receptor antagonists (Hampson and Deadwyler, 2000; O'Shea et al., 2004, 2006; Schneider and Koch, 2007; Abush and Akirav, 2012; Renard et al., 2016), suggesting that any effects of THC on cognition are likely to be recapitulated by WIN. Finally, we acknowledge that conclusions about self- vs. experimenter-administration are limited, in that these groups experience different daily conditions. The animals receiving daily IP injections were not exposed to any behavioral chambers prior to the start of working memory training, whereas the animals self-administering entered the behavioral chambers daily during the course of adolescence. Future work could include animals that receive IP injections but are then allowed daily exposure to the behavioral chambers, to ensure that experience is equated prior to working memory training. Despite the IP females taking a longer amount of time to reach training criteria than SA females in earlier phases of working memory training, by the time delays were introduced, all animals were performing at similar levels (see **Tables 1**, **2**).

In conclusion, we report one of the first examinations of the effects of adolescent cannabinoid self-administration on cognitive function in females. We have found that adolescent cannabinoid exposure alone is not sufficient to produce long-lasting deficits in memory function. Our data are potentially consistent with human literature showing an increased sensitivity to cannabinoids in females, which may explain why females self-administered slightly less WIN, in contrast to other drugs of abuse. Finally, our data show some evidence for craving-like behavior after adolescent cannabinoid SA in females, suggesting the potential for abuse liability.

AUTHOR CONTRIBUTIONS

EKK and MMT contributed to the design of the experiments. DMM, EKK and CME were involved in data collection. EKK, DMM and MMT analyzed and interpreted the data with input from CME. EKK and DMM wrote the article with input from CME and MMT. All authors gave final approval for publication.

FUNDING

This study was supported by the National Institute of Health grant R21DA041775.

ACKNOWLEDGMENTS

The authors would like to acknowledge grant DA042029, and the NIMH Chemical Synthesis and Drug Supply Program for providing WIN55,212-2 for this study.

REFERENCES

Abush, H., and Akirav, I. (2012). Short- and long-term cognitive effects of chronic cannabinoids administration in late-adolescence rats. *PLoS One* 7:e31731. doi: 10.1371/journal.pone.0031731

Anker, J. J., and Carroll, M. E. (2010). Females are more vulnerable to drug abuse than males: evidence from preclinical studies and the role of ovarian hormones. *Curr. Top. Behav. Neurosci.* 8, 73–96. doi: 10.1007/7854_2010_93

Becker, M. P., Collins, P. F., and Luciana, M. (2014). Neurocognition in college-aged daily marijuana users. *J. Clin. Exp. Neuropsychol.* 36, 379–398. doi: 10.1080/13803395.2014.893996

Becker, J. B., and Koob, G. F. (2016). Sex differences in animal models: focus on addiction. *Pharmacol. Rev.* 68, 242–263. doi: 10.1124/pr.115.011163

Becker, J. B., Monteggia, L. M., Perrot-Sinal, T. S., Romeo, R. D., Taylor, J. R., Yehuda, R., et al. (2007). Stress and disease: is being female a predisposing factor? *J. Neurosci.* 27, 11851–11855. doi: 10.1523/JNEUROSCI.3565-07.2007

Bertholomey, M. L., Nagarajan, V., and Torregrossa, M. M. (2016). Sex differences in reinstatement of alcohol seeking in response to cues and yohimbine in rats with and without a history of adolescent corticosterone exposure. *Psychopharmacology* 233, 2277–2287. doi: 10.1007/s00213-016-4278-x

Buchy, L., Seidman, L. J., Cadenhead, K. S., Cannon, T. D., Cornblatt, B. A., McGlashan, T. H., et al. (2015). Evaluating the relationship between cannabis use and IQ in youth and young adults at clinical high risk of psychosis. *Psychiatry Res.* 230, 878–884. doi: 10.1016/j.psychres.2015.11.033

Caggiula, A., Donny, E., White, A., Chaudhri, N., Booth, S., Gharib, M., et al. (2002). Environmental stimuli promote the acquisition of nicotine self-administration in rats. *Psychopharmacology* 163, 230–237. doi: 10.1007/s00213-002-1156-5

Cooper, Z. D., and Haney, M. (2014). Investigation of sex-dependent effects of cannabis in daily cannabis smokers. *Drug Alcohol Depend.* 136, 85–91. doi: 10.1016/j.drugalcdep.2013.12.013

Darling, R. A., Dingess, P. M., Schlidt, K. C., Smith, E. M., and Brown, T. E. (2016). Incubation of food craving is independent of macronutrient composition. *Sci. Rep.* 6:30900. doi: 10.1038/srep30900

Deiana, S., Fattore, L., Spano, M. S., Cossu, G., Porcu, E., Fadda, P., et al. (2007). Strain and schedule-dependent differences in the acquisition, maintenance and extinction of intravenous cannabinoid self-administration in rats. *Neuropharmacology* 52, 646–654. doi: 10.1016/j.neuropharm.2006.09.007

Dingess, P. M., Darling, R. A., Derman, R. C., Wulff, S. S., Hunter, M. L., Ferrario, C. R., et al. (2017). Structural and functional plasticity within the nucleus accumbens and prefrontal cortex associated with time-dependent increases in food cue-seeking behavior. *Neuropsychopharmacology* doi: 10.1038/npp.2017.57 [Epub ahead of print].

Fattore, L., Cossu, G., Martellotta, C., and Fratta, W. (2001). Intravenous self-administration of the cannabinoid CB1 receptor agonist WIN 55,212–2 in rats. *Psychopharmacology* 156, 410–416. doi: 10.1007/s002130100734

Fattore, L., Spano, M. S., Altea, S., Angius, F., Fadda, P., and Fratta, W. (2007). Cannabinoid self-administration in rats: sex differences and the influence of ovarian function. *Br. J. Pharmacol.* 152, 795–804. doi: 10.1038/sj.bjp.0707465

Fattore, L., Spano, M. S., Altea, S., Fadda, P., and Fratta, W. (2010). Drug- and cue-induced reinstatement of cannabinoid-seeking behaviour in male and female rats: influence of ovarian hormones. *Br. J. Pharmacol.* 160, 724–735. doi: 10.1111/j.1476-5381.2010.00734.x

Fogel, J. S., Kelly, T. H., Westgate, P. M., and Lile, J. A. (2017). Sex differences in the subjective effects of oral Δ^9-THC in cannabis users. *Pharmacol. Biochem. Behav.* 152, 44–51. doi: 10.1016/j.pbb.2016.01.007

Fuchs, R. A., Tran-Nguyen, L. T. L., Specio, S. E., Groff, R. S., and Neisewander, J. L. (1998). Predictive validity of the extinction/reinstatement model of drug craving. *Psychopharmacology* 135, 151–160. doi: 10.1007/s002130050496

Gage, S. H., Hickman, M., and Zammit, S. (2016). Association between cannabis and psychosis: epidemiologic evidence. *Biol. Psychiatry* 79, 549–556. doi: 10.1016/j.biopsych.2015.08.001

Goldman, J. M., Murr, A. S., and Cooper, R. L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Res. B Dev. Reprod. Toxicol.* 80, 84–97. doi: 10.1002/bdrb.20106

Grimm, J. W., Barnes, J., North, K., Collins, S., and Weber, R. (2011). A general method for evaluating incubation of sucrose craving in rats. *J. Vis. Exp.* 57:e3335. doi: 10.3791/3335

Hampson, R. E., and Deadwyler, S. A. (2000). Cannabinoids reveal the necessity of hippocampal neural encoding for short-term memory in rats. *J. Neurosci.* 20, 8932–8942.

Jager, G., Kahn, R. S., Van Den Brink, W., Van Ree, J. M., and Ramsey, N. F. (2006). Long-term effects of frequent cannabis use on working memory and attention: an fMRI study. *Psychopharmacology* 185, 358–368. doi: 10.1007/s00213-005-0298-7

Kirschmann, E. K., Pollock, M. W., Nagarajan, V., and Torregrossa, M. M. (2017). Effects of adolescent cannabinoid self-administration in rats on addiction-related behaviors and working memory. *Neuropsychopharmacology* 42, 989–1000. doi: 10.1038/npp.2016.178

Lee, B. R., Ma, Y.-Y., Huang, Y. H., Wang, X., Otaka, M., Ishikawa, M., et al. (2013). Maturation of silent synapses in amygdala-accumbens projection contributes to incubation of cocaine craving. *Nat. Neurosci.* 16, 1644–1651. doi: 10.1038/nn.3533

Lefever, T. W., Marusich, J. A., Antonazzo, K. R., and Wiley, J. L. (2014). Evaluation of WIN 55,212–2 self-administration in rats as a potential cannabinoid abuse liability model. *Pharmacol. Biochem. Behav.* 118, 30–35. doi: 10.1016/j.pbb.2014.01.002

Marconi, A., Di Forti, M., Lewis, C. M., Murray, R. M., and Vassos, E. (2016). Meta-analysis of the association between the level of cannabis use and risk of psychosis. *Schizophr. Bull.* 42, 1262–1269. doi: 10.1093/schbul/sbw003

Meier, M. H., Caspi, A., Ambler, A., Harrington, H., Houts, R., Keefe, R. S. E., et al. (2012). Persistent cannabis users show neuropsychological decline from childhood to midlife. *Proc. Natl. Acad. Sci. U S A* 109, E2657–E2664. doi: 10.1073/pnas.1206820109

Meyer, H. C., and Bucci, D. J. (2016). Age differences in appetitive Pavlovian conditioning and extinction in rats. *Physiol. Behav.* 167, 354–362. doi: 10.1016/j.physbeh.2016.10.004

Mokrysz, C., Landy, R., Gage, S., Munafo, M., Roiser, J., and Curran, H. (2016). Are IQ and educational outcomes in teenagers related to their cannabis use? A prospective cohort study. *J. Psychopharmacol.* 30, 159–168. doi: 10.1177/0269881115622241

Moss, H. B., Chen, C. M., and Yi, H.-Y. (2014). Early adolescent patterns of alcohol, cigarettes, and marijuana polysubstance use and young adult substance use outcomes in a nationally representative sample. *Drug Alcohol Depend.* 136, 51–62. doi: 10.1016/j.drugalcdep.2013.12.011

O'Shea, M., McGregor, I. S., and Mallet, P. E. (2006). Repeated cannabinoid exposure during perinatal, adolescent or early adult ages produces similar longlasting deficits in object recognition and reduced social interaction in rats. *J. Psychopharmacol.* 20, 611–621. doi: 10.1177/0269881106065188

O'Shea, M., Singh, M. E., McGregor, I. S., and Mallet, P. E. (2004). Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. *J. Psychopharmacol.* 18, 502–508. doi: 10.1177/0269881104018004007

Pertwee, R. G. (2010). Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. *Curr. Med. Chem.* 17, 1360–1381. doi: 10.2174/092986710790980050

Renard, J., Rushlow, W. J., and Laviolette, S. R. (2016). What can rats tell us about adolescent cannabis exposure? Insights from preclinical research. *Can. J. Psychiatry* 61, 328–334. doi: 10.1177/0706743716645288

Salas-Wright, C. P., Vaughn, M. G., Perron, B. E., Gonzalez, J. M. R., and Goings, T. C. (2016). Changing perspectives on marijuana use during early adolescence and young adulthood: evidence from a panel of cross-sectional surveys. *Drug Alcohol Depend.* 169, 5–10. doi: 10.1016/j.drugalcdep.2016.09.026

Schneider, M. (2008). Puberty as a highly vulnerable developmental period for the consequences of cannabis exposure. *Addict. Biol.* 13, 253–263. doi: 10.1111/j.1369-1600.2008.00110.x

Schneider, M., and Koch, M. (2003). Chronic pubertal, but not adult chronic cannabinoid treatment impairs sensorimotor gating, recognition memory and the performance in a progressive ratio task in adult rats. *Neuropsychopharmacology* 28, 1760–1769. doi: 10.1038/sj.npp.1300225

Schneider, M., and Koch, M. (2007). The effect of chronic peripubertal cannabinoid treatment on deficient object recognition memory in rats after neonatal mPFC lesion. *Eur. Neuropsychopharmacol.* 17, 180–186. doi: 10.1016/j.euroneuro.2006.03.009

See, R. E., Grimm, J. W., Kruzich, P. J., and Rustay, N. (1999). The Importance of a compound stimulus in conditioned drug-seeking behavior following one week of extinction from self-administered cocaine in rats. *Drug Alcohol Depend.* 57, 41–49. doi: 10.1016/s0376-8716(99)00043-5

Spear, L. P. (2000). The Adolescent brain and age-related behavioral manisfestations. *Neurosci. Biobehav. Rev.* 24, 417–463. doi: 10.1016/s0149-7634(00)00014-2

Takahashi, R. N., and Singer, G. (1979). Self-administration of delta 9-tetrahydrocannabinol by rats. *Pharmacol. Biochem. Behav.* 11, 737–740. doi: 10.1016/0091-3057(79)90274-0

Werner, C. T., Milovanovic, M., Christian, D. T., Loweth, J. A., and Wolf, M. E. (2015). Response of the ubiquitin-proteasome system to memory retrieval after extended-access cocaine or saline self-administration. *Curr. Vasc. Pharmacol.* 40, 3006–3014. doi: 10.1038/npp.2015.156

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 and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership