



The insular cortex controls food preferences independently of taste receptor signaling

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The insular cortex (IC) contains the primary sensory cortex for oral chemosensation including gustation, and its integrity is required for appropriate control of feeding behavior. However, it remains unknown whether the role of this brain area in food selection relies on the presence of peripheral taste input. Using multielectrode recordings, we found that the responses of populations of neurons in the IC of freely licking, sweet-blind *Trpm5*^{-/-} mice are modulated by the rewarding postingestive effects of sucrose. FOS immunoreactivity analyses revealed that these responses are restricted to the dorsal insula. Furthermore, bilateral lesions in this area abolished taste-independent preferences for sucrose that can be conditioned in these *Trpm5*^{-/-} animals while preserving their ability to detect sucrose. Overall, these findings demonstrate that, even in the absence of peripheral taste input, IC regulates food choices based on postingestive signals.

Keywords: insular cortex, gustatory cortex, *Trpm5*, taste, food preference, postingestive reward

INTRODUCTION

The insula contains the primary gustatory cortex (GC), which encodes the oral chemosensory properties of food (Yamamoto et al., 1980; Cechetto and Saper, 1987; Ogawa et al., 1992; Hanamori et al., 1998b; Scott and Plata-Salaman, 1999; Katz et al., 2002; Rolls, 2006; Stapleton et al., 2006; Accolla et al., 2007). Furthermore, the insular cortex (IC), and particularly the GC, has been shown to participate in the regulation of feeding (Balleine and Dickinson, 2000; Cubero and Puerto, 2000; Stoeckel et al., 2008; Wagner et al., 2008). In rats, bilateral lesions of the GC reduce their ability to adequately modulate the incentive value of food outcomes, an effect that has been attributed to deficits in taste memory (Balleine and Dickinson, 2000). On the other hand, electrical stimulation of the IC induces robust flavor preferences, possibly due to modulation of the orosensory insular representation of that flavor (Cubero and Puerto, 2000). Thus, the involvement of the insula in

the regulation of feeding has primarily been attributed to its role in processing oral chemosensory information.

However, a more integrative role for the insula in feeding is suggested by the fact that, in humans (Small et al., 2001), as well as in rats (de Araujo et al., 2006), insular neuronal responses to food are inhibited by postingestive satiation. In addition, the effects of electrical stimulation of the insula in eliciting flavor preferences has also been attributed to the “imitation” of neural patterns evoked by reinforcing visceral information (Cubero and Puerto, 2000).

The transient receptor potential M5 (TRPM5) channel (Perez et al., 2002) is required for peripheral transduction of sweet, bitter, and umami tastants (Zhang et al., 2003). Mice lacking functional TRPM5 channels (*Trpm5*^{-/-}) have absent (Zhang et al., 2003) or vastly diminished (Damak et al., 2006) peripheral neural responses to sweet tastants and, in contrast to wild-type mice, do not show a preference for sweet tasting solutions in behavioral paradigms

that are dependent on orosensory responses (Zhang et al., 2003; de Araujo et al., 2008). We and others have shown that, in *Trpm5*^{-/-} animals, sucrose or glucose can be used to condition the development of spout preferences that are independent of orosensory input (de Araujo et al., 2008; Ren et al., 2010). Furthermore, in unconditioned *Trpm5*^{-/-} mice, sucrose evoked dopamine release in the ventral striatum (de Araujo et al., 2008). These behavioral and neurochemical responses, considered as a measure of food reward, were shown to depend on processes driven by caloric postingestive feedback, since they were absent when sucralose, a non-caloric sweetener, was used in place of sucrose (de Araujo et al., 2008). For these reasons, *Trpm5*^{-/-} mice are an ideal preparation to investigate a taste-independent role of the insula in the regulation of appetitive feeding. Here, we investigated the neural representation of the postingestive effects of sucrose in the dorsal IC of *Trpm5*^{-/-} sweet-blind mice, and determined whether integrity of the dorsal IC is necessary for expression of appropriate food selection behaviors when no taste input is present.

MATERIALS AND METHODS

ANIMALS

A total of 33 male mice were used. At the time of the experiments, animals were 3–6 months old. They were all homozygous for a partial deletion of the *Trpm5* gene (*Trpm5*^{-/-}; Zhang et al., 2003) on a C57BL/6 background, and were bred from mice generously donated by C. S. Zuker (UCSD, San Diego, CA, USA). Genotype was confirmed by PCR amplification of the *Trpm5* gene. Ten *Trpm5*^{-/-} mice were implanted with microelectrode arrays for neural recordings. Eight others were used for FOS immunoreactivity. Fifteen animals were used in lesion experiments of the IC (eight with IC lesions and seven with sham operations). All procedures were approved by the Duke University Institutional Animal Care and Use Committee.

STIMULI

Sucrose solutions (0.8M; Sigma-Aldrich, USA) were prepared daily at room temperature in deionized water. Deionized water was also used as a baseline stimulus. Whenever the terms “sucrose” or “water” are used, we imply 0.8 M sucrose solutions or deionized water, respectively.

BEHAVIORAL EXPERIMENTS

The behavioral component of all experiments involving licking was conducted in mouse-behavior chambers enclosed in a ventilated and sound attenuating cubicle (Med Associates Inc., St. Albans, VT, USA), as described previously (de Araujo et al., 2008). All experiments were conducted with naïve animals under a 20- to 22-h-long food and water deprivation schedule.

Conditioning to postingestive effects

A conditioning protocol that allows *Trpm5*^{-/-} mice to manifest tastant-independent preferences for sucrose was adapted from previous experiments (de Araujo et al., 2008). This protocol assesses the ability of sweet-blind animals to develop a preference for drinking from a sipper that is located in a position of the behavioral cage associated with the availability of 0.8 M sucrose. Briefly (also see Table 1), after side-preference was determined for each animal in

preliminary two-bottle tests where both sippers contained water, a pre-conditioning two-bottle sucrose vs. water preference test was conducted for 10 min. Thereafter, animals were exposed to a conditioning protocol, where 30 min *ad libitum* access to either water or sucrose was alternated for six consecutive days and, finally, to a post-conditioning two-bottle sucrose vs. water preference test conducted analogously to the pre-conditioning test. In all sessions, water sippers were located on the original bias side and sucrose sippers on the opposite side.

Two-bottle preference tests

In IC-lesioned and sham-operated animals, conditioned as described above, further sucrose vs. water two-bottle choice tests (10 min long) were conducted to verify taste-dependent preferences. To account for the effect of side-biases, mice were tested in each condition for four consecutive days with daily inversion of sucrose and water bottle positions (de Araujo et al., 2008), such that any consistent preference would depend on sensory factors, rather than a side-bias (de Araujo et al., 2008).

Preference measures

Two-bottle preference tests were analyzed by calculating the preference ratios (*P*) as $P(\text{Sipper 1}) = n(\text{Sipper 1}) / [n(\text{Sipper 1}) + n(\text{Sipper 2})]$ where *n*(.) denotes the total number of licks for a given stimulus during a session. Significance tests were based on one sample *t*-tests against 0.5, which is the reference value meaning indifference with respect to either sipper.

Water maze behavioral testing

In IC-lesioned and sham-operated animals, behavioral testing of spatial orientation was conducted in a Morris water maze (MWM) task, as described previously (Kee et al., 2007). Briefly, on each of six consecutive training days, mice received eight training trials divided in two blocks of four. At each trial they were placed in the water facing the wall in one of four possible different start locations (randomly chosen without substitution) and left to swim freely until they found the platform or 60 s had passed. Time to reach the platform was recorded in each trial and averaged across all trials for each animal in each day. One day after completion of training (day 7), spatial memory was tested in a probe trial where the platform was removed from the pool. The time spent searching in the correct quadrant of the maze (where the platform had been during training) was averaged across all animals and was compared to 15 s (one sample *t*-test), the time animals would be expected to swim in that quadrant if they were searching randomly.

FOS PROTEIN IMMUNOHISTOCHEMISTRY

Eight *Trpm5*^{-/-} mice were habituated to drink water from a single sipper in daily 30 min sessions. Once stable licking rates were obtained, animals were exposed to either water (four animals) or 0.8 M sucrose (four animals) in a single 30 min long session. To avoid unspecific effects associated with licking and/or different volumes ingested, consumption of sucrose was yoked to that of water. Two hours after the start of behavioral sessions, animals were deeply anesthetized with 100 mg/kg pentobarbital and perfused through the left ventricle with a saline flush (100 mL) followed by 4% paraformaldehyde in phosphate buffered saline

Table 1 | Protocol for postingestive conditioning.

			Left side	Right side
Pre-test	Determination of side-bias	Two-bottle	Water	Water
			Bias side	Other side
Day 1	Pre-conditioning test	Two-bottle	Water	Sucrose
Day 2	Conditioning day 1	One-bottle	–	Sucrose
Day 3	Conditioning day 2	One-bottle	Water	–
Day 4	Conditioning day 3	One-bottle	–	Sucrose
Day 5	Conditioning day 4	One-bottle	Water	–
Day 6	Conditioning day 5	One-bottle	–	Sucrose
Day 7	Conditioning day 6	Two-bottle	Water	–
Day 8	Post-conditioning test	Two-bottle	Water	Sucrose

(PBS; pH, 7.4; 500 mL). Brains were post-fixed in the same fixative for 2 h, and then transferred to 30% sucrose with 0.02% sodium azide in PBS.

The fixed brains were then analyzed by a different experimenter that was blind to the treatment conditions. Free-floating serial 40 μm thick coronal sections of these brains were cut with a freezing-microtome and alternate sections were used for either FOS immunohistochemistry (Contreras et al., 2007; Kee et al., 2007) or thionin staining. Sections for immunohistochemistry were incubated in PBS with 1% H_2O_2 for 20 min, rinsed in PBS with 0.3% Triton-X100 (PBS-T), and then transferred to 10% normal swine serum (NSS) in PBS-T blocking solution for 2 h. Sections were then incubated in the primary antibody solution: rabbit anti FOS polyclonal antibody (Calbiochem, CA, USA) 1:10000 in PBS-T with 2% NSS. After 3 days at 4°C, sections were rinsed with PBS-T, incubated in 1:200 biotinylated swine anti-rabbit antibody for 1 h, rinsed with PBS-T, incubated for 1 h in 1:200 Vectastain ABC Elite kit (Vector Laboratories, CA, USA), rinsed with Tris buffer, and reacted for 3 min with a diaminobenzidine hydrochloride (DAB) solution containing 0.005% H_2O_2 in Tris buffer. After DAB staining, the sections were rinsed in PBS and serially mounted for counting of FOS-immunoreactive nuclei. IC boundaries were traced over the thionin-stained sections using the Paxinos and Franklin (2001) drawings as guidelines for regional boundaries, prior to counting of FOS-immunoreactive nuclei. Counting was performed manually using a microscope camera lucida and cells were counted bilaterally in each section.

STEREOTAXIC SURGERY FOR IC LESION, SHAM OPERATIONS, AND IMPLANTATION OF MULTIELECTRODE MICROARRAYS

Twenty-five *Trpm5*^{-/-} mice were anesthetized using 5% halothane followed by intramuscular injection of xylazine (5 mg/kg) and ketamine (75 mg/kg). Supplemental doses were administered whenever necessary. Craniotomies measuring $\sim 1 \text{ mm}^2$ were drilled at (AP = 0.9 mm, ML = ± 3.1 mm) relative to bregma. In eight animals a cannula was slowly lowered to ~ 2 mm below the brain surface to target the IC (Paxinos and Franklin, 2001) and 0.1 μL of a 20-mg/mL NMDA (*N*-methyl-D-aspartic acid; Sigma-Aldrich, USA) solution was manually injected into the IC (Corbit et al., 2002). After the injected solution had dispersed for 2 min, the

needle was removed and the contralateral side of the brain was subjected to the same procedure. In seven animals, the same surgical methodologies were repeated but NMDA was not injected (sham operation). In 10 other animals, a multielectrode microarray (16 channels) was implanted into the same area (see Figure 5). The side of implantation was balanced between left and right hemispheres. After surgery, animals were given ~ 1 week to recover before experimental testing was initiated.

NEURONAL RECORDINGS

Recordings of neural activity and timestamps of licking responses were performed simultaneously according to the procedure previously described (de Araujo et al., 2008). In two implanted animals, single-neuron activity could not be recorded. In the remaining eight mice, recordings were performed in each animal during a series of two or one-bottle tests, conducted in eight consecutive days (see Table 1). No assumption was made on the identity/stability of units recorded during different sessions.

HISTOLOGICAL CONFIRMATION OF IC LESION AND ELECTRODE TIPS PLACEMENT

In all animals, we followed a previously described histological method to identify the location of lesions or microwire implantation (de Araujo et al., 2008). Brain slices from lesioned and sham-operated animals were examined with a light microscope, and compared. Placement and extent of lesion was assessed by location of cannula tract and areas of gliosis (Corbit et al., 2002). In two mice, the insula lesion was only correctly placed on one side, while in one implanted animal the microwires were implanted outside of the insula, in the striatum. Data from these animals were excluded from the analyses.

ELECTROPHYSIOLOGY DATA ANALYSIS

All neuronal data analyses were performed with custom software written in Matlab (R14, MathWorks, Inc.) or using the Nex software (Nex Technologies, TX, USA).

Peri-event histograms

Peri-event histograms (PEHs) were constructed for each unit-stimulus pair using the PEH function of the Nex software (Nex Technologies, TX, USA). PEHs show the conditional probability

of observing a spike in a spike train at time t , on the condition that there is a reference event at time zero. The time axis was divided into bins and bin counts were normalized by the number of reference events. Analyses of responses in single cells were performed by constructing 1 s (± 500 ms) “PEHs” with 5 ms bins, using licks to sucrose or water as defining events. Bin values were expressed as impulses per second (i.e., normalized bin counts \times bin size in seconds), and a Gaussian filter (width = 3 bins) was applied to the resulting PEH. PEHs were constructed such that they would correspond to time intervals that occurred within licking clusters (see below).

Stimulus-specific single-neuron responses

Confidence limits for expected bin count in each PEH were calculated in the Nex software, using the assumption that the expected bin count has a Poisson distribution (Abeles, 1982). The count for each bin in a PEH was then compared to the respective confidence limits. A given unit was considered responsive to a stimulus when the values of at least three consecutive bins were outside the 95% confidence interval. Units that responded to only one of the two stimuli (water or sucrose) were considered to be “stimulus-specific,” while units that responded to neither or both stimuli were not considered “stimulus-specific.”

Mean population responses

For each neuron isolated during a given recording session, and for each licking cluster (see below) to sucrose, we first calculated the total number of spikes within this cluster normalized to cluster duration. These quantities were then averaged for each neuron across all clusters. Results were averaged across neurons, so that the resulting quantity was defined as the mean population firing rate response to sucrose, denoted FR^{SUC} . For water, the quantity FR^{H_2O} was analogously defined. Next, we defined the quantities $(FR^{SUC} - FR^{H_2O})_{PRE}$ and $(FR^{SUC} - FR^{H_2O})_{POST}$, taken as a measure of the differential mean population response to water and sucrose for each pre- and post-conditioning session respectively. Finally, for each recorded animal, we defined the quantity $\Delta FR = |(FR^{SUC} - FR^{H_2O})_{POST} - (FR^{SUC} - FR^{H_2O})_{PRE}|$ that represents the absolute value of the changes in the differential population responses to water and sucrose as a function of conditioning. These values were then correlated with a learning index (LI; see below for definition).

Learning index

The efficacy of the conditioning protocol was measured in each recorded animal by a quantity we denoted as the “LI.” This index provides a measure of the extent to which, after conditioning, $Trpm5^{-/-}$ animals increased their preference (P) for the sipper associated with sucrose. For each animal, LI was thus defined as $LI = (P_{Sucrose})_{POST} - (P_{Sucrose})_{PRE}$, where POST and PRE refer to post- and pre-conditioning test sessions respectively.

Determination of licking clusters

The analysis of licking patterns was performed as described previously (Davis and Smith, 1992; Gutierrez et al., 2006), i.e., pauses in licking longer than 0.5 s defined the end of a cluster. Cluster duration and average lick rates within clusters were used as controls for oromotor influences on neural activity.

STATISTICAL ANALYSES

Unless otherwise stated, results from data analyses were expressed as mean \pm SE of the mean. Analyses were performed with custom software written in Matlab (R14, MathWorks, Inc.) or with Prism (GraphPad, San Diego). Analyses were two-way or one-way ANOVAs (with Bonferroni *Post hoc* tests), paired or unpaired two-sample t -tests, or one sample t -tests. Bonferroni–Holm’s corrections for multiple comparisons (Holm, 1979) were performed when several independent tests were used for the same dataset. Correlation analyses were performed using Pearson’s product moment correlation and proportions were compared using Fisher’s exact tests. The Kolmogorov–Smirnov test was used to check the goodness of fit with the normal distribution for each measure of behavior or neuronal activity.

RESULTS

IN THE ABSENCE OF PERIPHERAL TASTE INPUT, NEURONAL POPULATIONS IN IC ENCODE THE REINFORCING VALUE OF SUCROSE SOLUTIONS

In 7 $Trpm5^{-/-}$ mice, a multielectrode microarray comprising 16 electrodes was implanted unilaterally into the dorsal IC, where the GC is found (Cechetto and Saper, 1987; Ogawa et al., 1990; Stapleton et al., 2006; Accolla et al., 2007). Electrophysiological recordings of IC neuronal ensembles (average 6.8 neurons per ensemble) were conducted in eight recording sessions per animal. These recordings were performed while the animals were exposed to a conditioning protocol where the expression of preferences for sucrose depends solely on taste-independent, postingestive effects (de Araujo et al., 2008; see Materials and Methods and Table 1 for protocol).

During conditioning sessions, $Trpm5^{-/-}$ mice consumed significantly more sucrose than water (Figure 1A), indicating that these animals were sensitive to the postingestive effects of sucrose. Sipper-preference patterns observed during the post-conditioning sucrose vs. water tests were also indicative that the sweet-blind mice were sensitive to the postingestive effects of sucrose. Indeed, the consumption of water was unchanged while sucrose consumption significantly increased in the post-conditioning relative to the pre-conditioning testing sessions (Figure 1B). Accordingly, sucrose preference was significantly higher in post-conditioning than in pre-conditioning testing sessions (Figure 1C).

To assess the involvement of single IC neurons in the development of preferences for sucrose, we calculated in pre- and post-conditioning sessions the proportion of stimulus-specific neurons, i.e., neurons that responded selectively when the animal was licking for sucrose or for water (see Figure 2A and Materials and Methods). While the proportion of such neurons increased in the post-conditioning (23 out of 59 neurons, $\sim 39\%$) relative to the pre-conditioning sessions (14 out of 61 neurons, $\sim 23\%$), this difference was only borderline significant ($p > 0.07$; Fisher’s exact test). Nevertheless, we hypothesized that the variable individual propensities to sense the postingestive effects of sucrose were mirrored by the corresponding IC neural activity levels. Consequently, animals were divided according to their post-conditioning preference ratio for sucrose. Five mice displayed a preference ratio higher than 0.5 and were defined as “Learners,” while the remaining mice were classified as “Non-Learners” (Figure 1D). The proportions

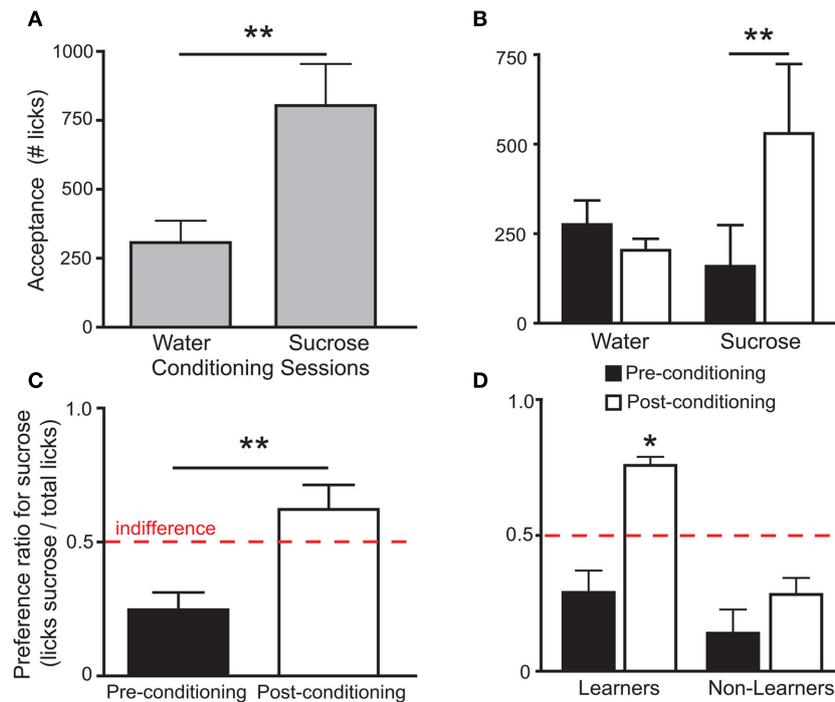


FIGURE 1 | Behavioral responses to sucrose and water in *Trpm5*^{-/-} mice before and after conditioning. In all figures, error bars indicate mean \pm SEM. **(A)** In the *Trpm5*^{-/-} animals where IC activity was recorded, average acceptance of sucrose during conditioning sessions (see **Table 1**) was higher than that of water (803 ± 152 and 308 ± 79 licks respectively; $t_6 = 5.1$, $**p < 0.003$, paired two-sample *t*-test). **(B)** In the same animals, when acceptance of sucrose and water was compared in pre-conditioning and post-conditioning preference tests, a significant effect was found for session (pre- vs. post-conditioning, $F_{1,12} = 4.9$, $p < 0.05$) but not for tastant (water vs. sucrose, $F_{1,12} = 0.5$, $p > 0.5$). However, the two factors also interacted significantly ($F_{1,12} = 10.7$, $p < 0.007$; two-way repeated measures ANOVA) and, while the consumption of sucrose increased from the pre-conditioning to the post-conditioning preference tests (159 ± 115 and 529 ± 194 licks, respectively; $t = 3.9$, $**p < 0.01$) the consumption of water was unchanged (276 ± 67 and 204 ± 32 licks, respectively; $t = 0.8$, $p > 0.05$, *Post hoc* Bonferroni). **(C)** In accordance with the acceptance

data, sucrose preference increased after conditioning (0.25 ± 0.06 to 0.62 ± 0.09 ; $t_6 = 5.3$, $**p < 0.002$, paired two-sample *t*-test). Red dashed line corresponds to 0.5 indifference level. While sucrose preference was significantly lower from the indifference ratio of 0.5 in the pre-conditioning test ($t_6 = 3.9$, $p < 0.02$), it was not in the post-conditioning test ($t_6 = 1.3$, $p > 0.2$; one sample *t*-tests vs. 0.5 with Bonferroni-Holm's correction for multiple comparisons). **(D)** In Learners (see text) preference for the sucrose sipper was not significantly different from 0.5 in the pre-conditioning session (preference ratio = 0.29 ± 0.08 ; $t_4 = 2.6$, $p > 0.05$), but was significantly higher than 0.5 in the post-conditioning test session (0.76 ± 0.03 ; $t_4 = 5.3$, $*p < 0.05$). In Non-Learners, preference in both pre and post-conditioning was low (0.14 ± 0.09 and 0.28 ± 0.05) and not significantly different from 0.5 ($t_1 = 4.1$, $p > 0.1$ and $t_1 = 3.6$, $p > 0.1$, respectively; one sample *t*-tests vs. 0.5 with Bonferroni-Holm's correction for multiple comparisons – note that only two animals were Non-Learners which hinders statistical significance).

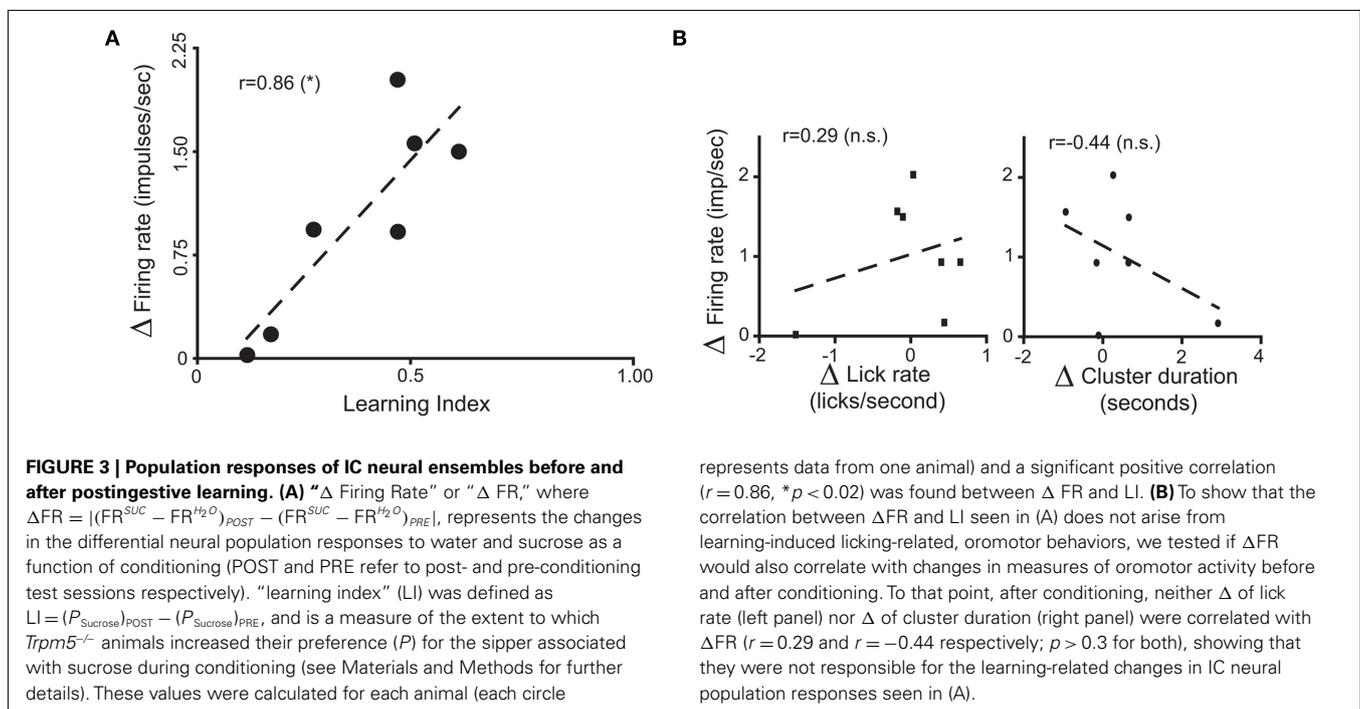
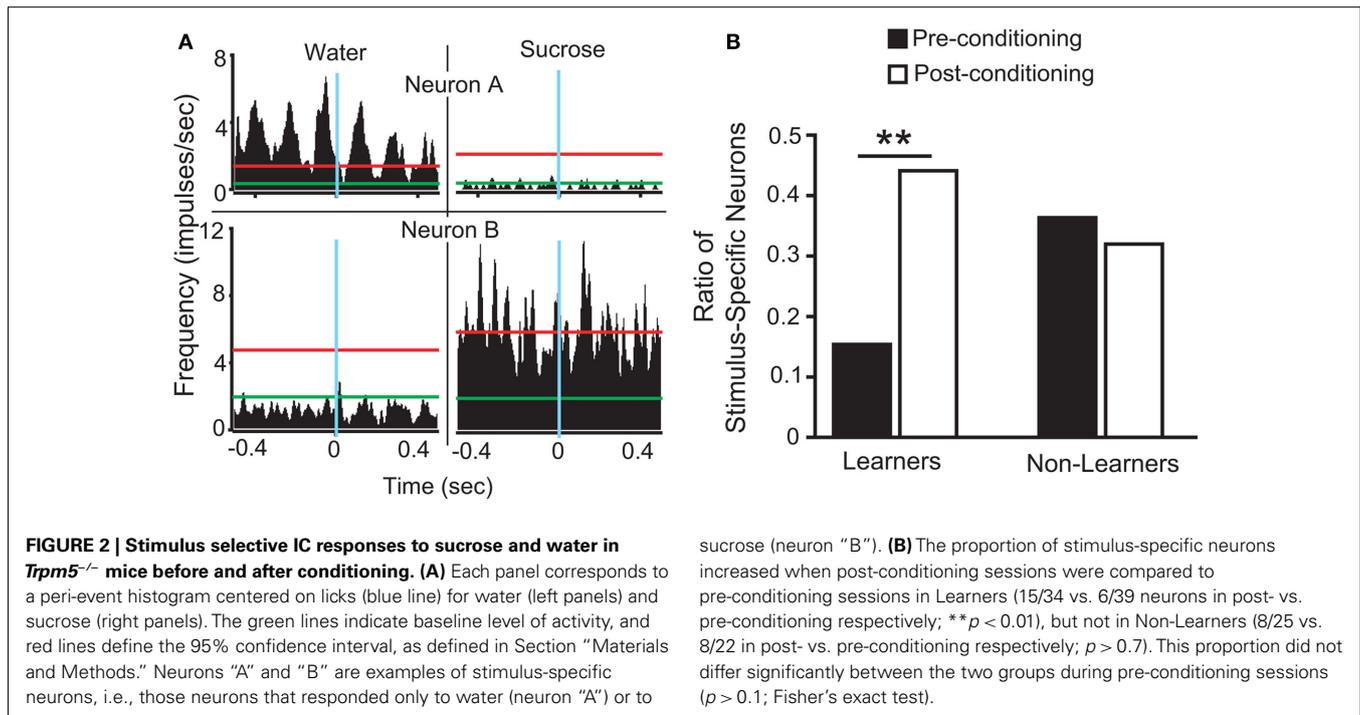
of stimulus-specific neurons in pre- and post-conditioning sessions were then compared separately for each of the two groups. In Learners, the stimulus-specific neurons occurred with greater frequency in post- relative to pre-conditioning sessions; an effect that was not observed in Non-Learners (**Figure 2B**).

Furthermore, for each animal, variation in the proportion of stimulus-specific neurons occurring in post-conditioning sessions (27, 33, 36, 36, 44, 50, and 75%) relative to pre-conditioning sessions (31, 9, 13, 44, 18, 17, and 33%) was calculated, showing that this difference (post-conditioning – pre-conditioning) was significantly different between Learners ($29.9 \pm 3.4\%$) and Non-Learners ($-6.1 \pm 2.6\%$; $t_5 = 6.1$, $p < 0.002$, unpaired two-sample *t*-test).

To further analyze changes in IC responses from pre- to post-conditioning, for each of the preference-testing sessions we calculated the mean population firing rate while animals licked for either stimulus and subtracted the within-session neuronal

population responses to water from those to sucrose. The absolute difference between the values thus obtained in pre- and post-conditioning sessions for each animal, denoted as “ Δ Firing Rate” [$\Delta FR = |(FR^{SUC} - FR^{H_2O})_{POST} - (FR^{SUC} - FR^{H_2O})_{PRE}|$, see Materials and Methods], represents the extent to which the relationship between sucrose and water population responses changed after conditioning. Each animal was also assigned a “LI,” [$LI = (P_{SUCROSE})_{POST} - (P_{SUCROSE})_{PRE}$, see Materials and Methods] that is a measure of the increase in sucrose preference during the post-conditioning relative to the pre-conditioning preference tests (see **Figure 1C**). As seen in **Figure 3A**, a significant positive correlation was found between ΔFR and the LI. Additionally, we verified that ΔFR differs significantly between Learners and Non-Learners (1.4 ± 0.2 and 0.09 ± 0.08 respectively; $t_5 = 3.7$, $p < 0.03$, unpaired two-sample *t*-test).

Concerning the relationship between ΔFR and LI, we were concerned that the variation in ΔFR could reflect learning-induced

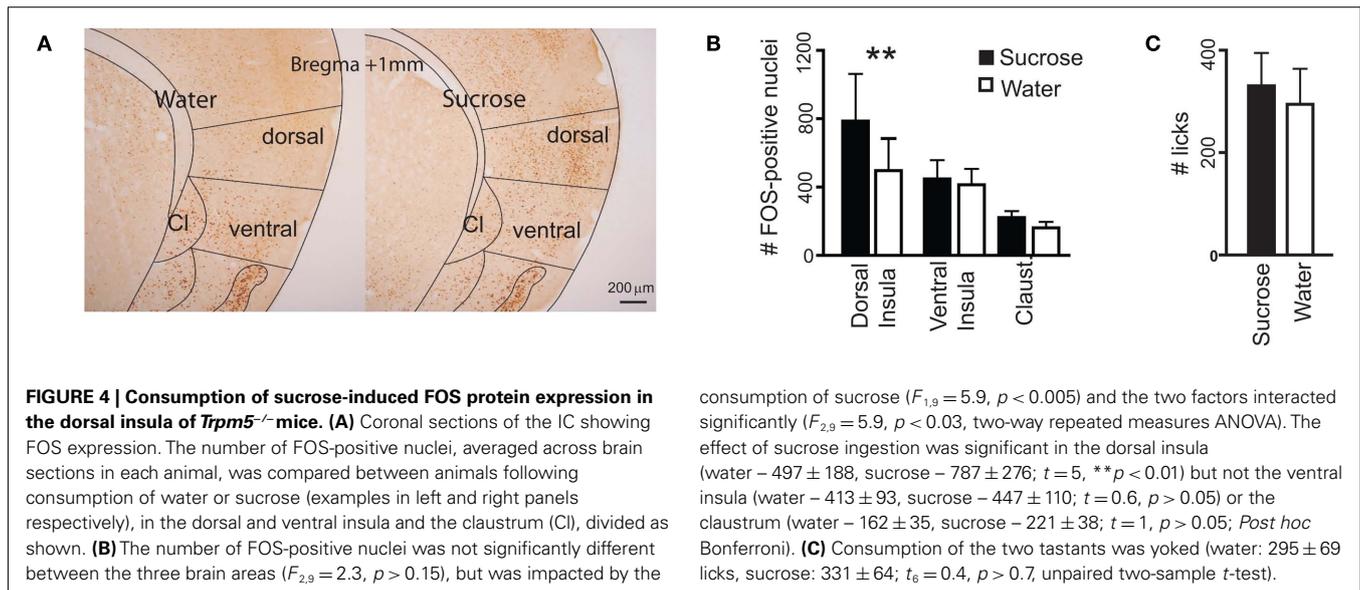


modifications in licking-related oromotor behaviors. To investigate this possibility, we tested if Δ FR would also correlate with changes in measures of oromotor activity, calculated analogously to the LI. Changes in lick frequency and licking cluster duration did not correlate with Δ FR (Figure 3B), thereby showing that these parameters cannot account for the relationship between Δ FR and the LI. In summary, these electrophysiological data showed that, in the absence of peripheral taste transduction events for sucrose,

IC neuronal populations reflect the postingestive reinforcing value of sucrose solutions.

POSTINGESTIVE RESPONSES TO SUCROSE ARE FOUND SPECIFICALLY IN THE DORSAL IC

The above electrophysiological results show postingestive-driven responses to sucrose in the dorsal insula, but do not provide information as to other IC locations where such responses could



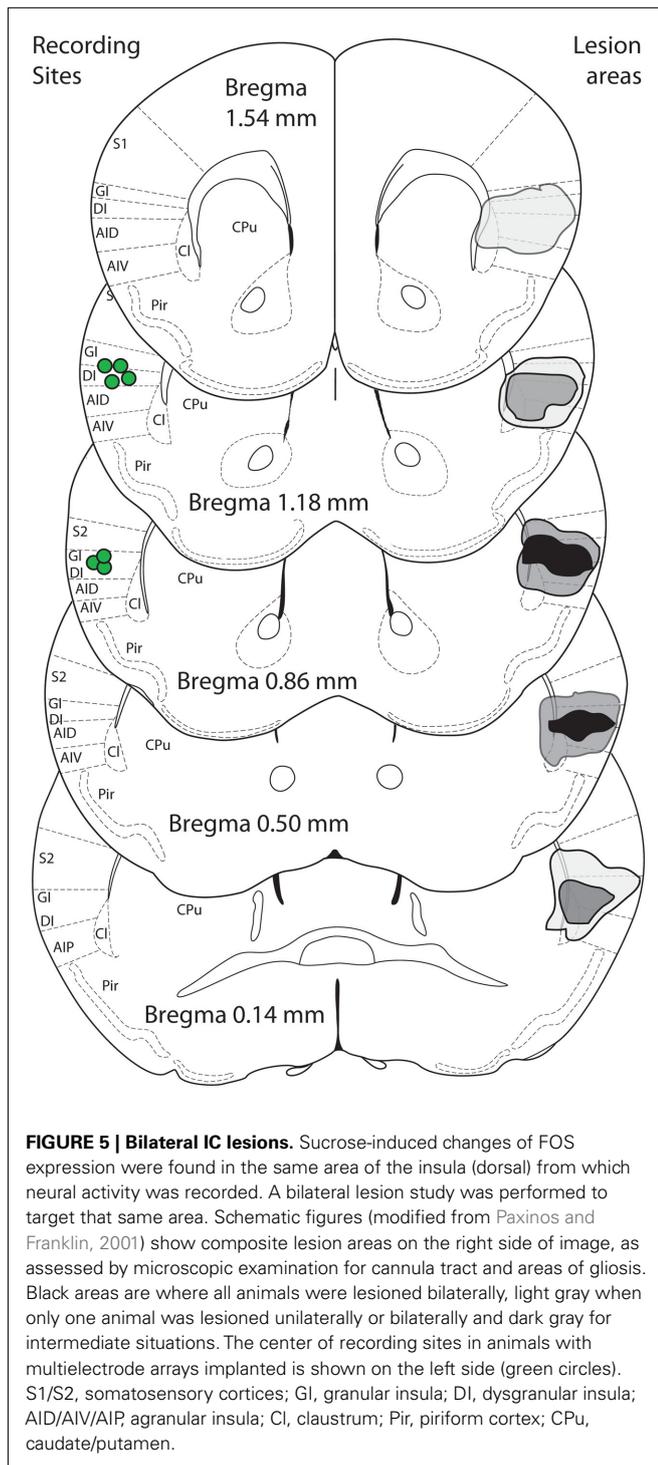
be found. To further investigate insular sites where post-ingestive reward may be represented, we measured patterns of FOS immunoreactivity in the IC of *Trpm5*^{-/-} mice (Figure 4A) following the ingestion of water or 0.8 M sucrose solutions ($n = 4$ mice for each tastant). In these KO mice, the ingestion of the sucrose solution induced a significantly greater amount of FOS protein synthesis in IC neurons than did the ingestion of the same volume of water (Figure 4B). Importantly, this effect was restricted to the dorsal zones of the IC. No significant differences were found in either the ventral insula or the claustrum (Figure 4B). Because the consumption of sucrose was experimentally yoked to that of water (Figure 4C), these effects were not accounted for by unspecific visceral stimulation associated with differential volumes of ingestion (e.g., gastric distention, Cechetto and Saper, 1987) or oromotor-related factors (e.g., licking-dependent somatosensory stimulation, Stapleton et al., 2006). Thus, as ascertained by FOS measurements, the sensitivity of the IC to post-ingestive factors in *Trpm5*^{-/-} mice is anatomically restricted to the dorsal zones.

THE IC IS NECESSARY FOR *TRPM5*^{-/-} MICE TO EXPRESS SIDE PREFERENCES CONDITIONED BY SUCROSE

We next inquired whether, in the absence of peripheral taste signaling, the IC is required for the development of preferences for sucrose. Following either bilateral lesions targeting the dorsal IC, induced with 2 μ g of *N*-methyl-D-aspartic acid, a glutamate receptor agonist ($n = 6$), or sham surgery ($n = 7$; Figure 5 and Materials and Methods), we analyzed the behavioral performance of *Trpm5*^{-/-} mice in the conditioning protocol (Table 1). We found that bilateral lesions to IC produced no behavioral effects on either pre-conditioning preference tests (Figure 6A) or conditioning sessions (Figure 6B). However, during post-conditioning preference tests, sham-operated animals not only consumed significantly more sucrose than water (Figure 6C) but also developed a significant preference for sucrose (0.76 ± 0.07 ; $t_6 = 4$, $p < 0.02$), whereas no such effects were observed in IC-lesioned animals (0.42 ± 0.1 ; $t_5 = 0.8$, $p > 0.4$; one sample *t*-tests vs. 0.5

with Bonferroni–Holm’s correction for multiple comparisons; Figure 6D).

These findings suggested a highly specific role for the dorsal IC in the development of taste-independent side preferences conditioned by sucrose. In fact, during conditioning, both sham-treated and lesioned animals exhibited more licking during sucrose than water sessions. It is important to note that, in these conditioning sessions, mice had access to a single bottle, delivering either water or sucrose in alternate days. Thus, they only needed to detect sucrose in order to consume it differentially relative to water. We therefore concluded that the dorsal IC is not necessary for detection of the post-ingestive value of sucrose. However, during the post-conditioning preference-testing session, both water and sucrose were present, and the animals had to choose between the two tastants. Since *Trpm5*^{-/-} mice are taste-blind, in this last session sucrose and water were presented in the same positions as during the conditioning sessions (sucrose on the left and water on the right side or vice-versa), and animals had to use previously learned side cues to be able to demonstrate a preference for sucrose (de Araujo et al., 2008). During post-conditioning tests sham-treated mice, but not lesioned animals, displayed the expected higher preference for sucrose, suggesting that the dorsal IC was necessary for the association between sipper positions and the post-ingestive effects associated with the availability of sucrose. The importance of side cues for sham-treated animals to demonstrate a preference for sucrose was further confirmed in additional two-bottle sucrose vs. water preference tests, conducted across several days with alternation of bottle positions to eliminate side-bias. Under these conditions, preference for sucrose was not significant in either group (lesion: 0.57 ± 0.05 , sham: 0.58 ± 0.06 ; respectively $t_5 = 1.3$, and $t_6 = 1.2$, $p > 0.2$ for both, one sample *t*-tests vs. 0.5) and did not differ between them ($t_{11} = 0.1$, $p > 0.9$; unpaired two-sample *t*-test). Thus, the preference for sucrose initially expressed by sham-operated animals was disrupted, confirming that the development of such preferences in *Trpm5*^{-/-} mice is dependent on side associations while being independent of any particular orosensory cue (de Araujo et al., 2008).



An alternate explanation for the above results relates to possible differences in the duration of the experimental sessions. In fact, conditioning sessions were conducted for 30 min while preference sessions lasted only 10 min, raising the possibility that the differences in conditioning vs. preference sessions may result simply from differences in their duration. To test for this possibility, data for consumption during conditioning sessions was reanalyzed,

considering only the first 10 min for each session. No significant differences were found between sham and IC-lesioned animals (**Figure 7A**). Further comparisons were performed considering each sucrose or water conditioning day separately (i.e., days 1, 3, and 5 for sucrose and 2, 4, and 6 for water), to verify if there were different trends in consumption across conditioning. Again, no significant differences were found between sham and IC-lesioned animals (**Figures 7B,C**), a finding that further supports a more fundamental effect of dorsal IC lesions during preference tests, as argued above.

From the above experiments it follows that IC lesions disrupt the development of side preferences conditioned by sucrose. To eliminate the possibility that this disruption was due to impairments in spatial cognition (Bermudez-Rattoni et al., 1991), we tested the same animals in a MWM protocol (Kee et al., 2007). Animals were trained in the MWM for 6 days, a period with comparable duration to that of the postingestive conditioning. On the sixth day, no differences were found between the two groups in time to reach a hidden platform (**Figure 8A**). Furthermore, on a probe test conducted on the seventh day, both groups retained information on the spatial location of the submerged platform (**Figure 8B**). Thus, we conclude that animals with IC lesion had conserved spatial orientation in the MWM, and that IC integrity, while not necessary for the detection of the postingestive properties of sucrose, is required for such factors to condition side preferences in a two-bottle test.

DISCUSSION

In this study we have shown that the activity of neurons located in dorsal regions of the IC of sweet-blind *Trpm5*^{-/-} mice display a heightened sensitivity to taste-independent postingestive effects produced by caloric sucrose solutions. In addition, we found that changes in IC neuronal activity elicited by these taste-independent postingestive effects were mostly restricted to more dorsal regions of the IC, where the gustatory aspect of the insula is located. Finally, we have shown that focal lesions to dorsal insular areas, that were ineffective in disrupting the sensitivity to taste-independent postingestive effects of sucrose, do disrupt the ability of mice to associate a particular sipper position with the postingestive effects produced by the sucrose solutions. Overall, our data supports the concept that even in the absence of taste transduction, dorsal parts of the IC are critical for the formation of associations between environmental cues and postingestive effects.

Chemosensory responses in gustatory insular neurons are broadly tuned to multiple taste qualities, including sweet (Katz et al., 2002; Stapleton et al., 2006, 2007). However, in the absence of taste input, it remained unknown as to whether the rewarding postingestive properties of sucrose were represented in the insula. Here we have described adaptations in IC responses as sweet-blind *Trpm5*^{-/-} animals developed a preference for sucrose. This preference was established through a conditioning protocol that associates the contents of a particular sipper with its positive postingestive effects (de Araujo et al., 2008; Ren et al., 2010). In mice that developed a preference for sucrose, such neuronal adaptations occurred both at the single-neuron level, where they were expressed as an increased “discriminability” between sucrose and water responses (**Figure 2B**), and at the neural

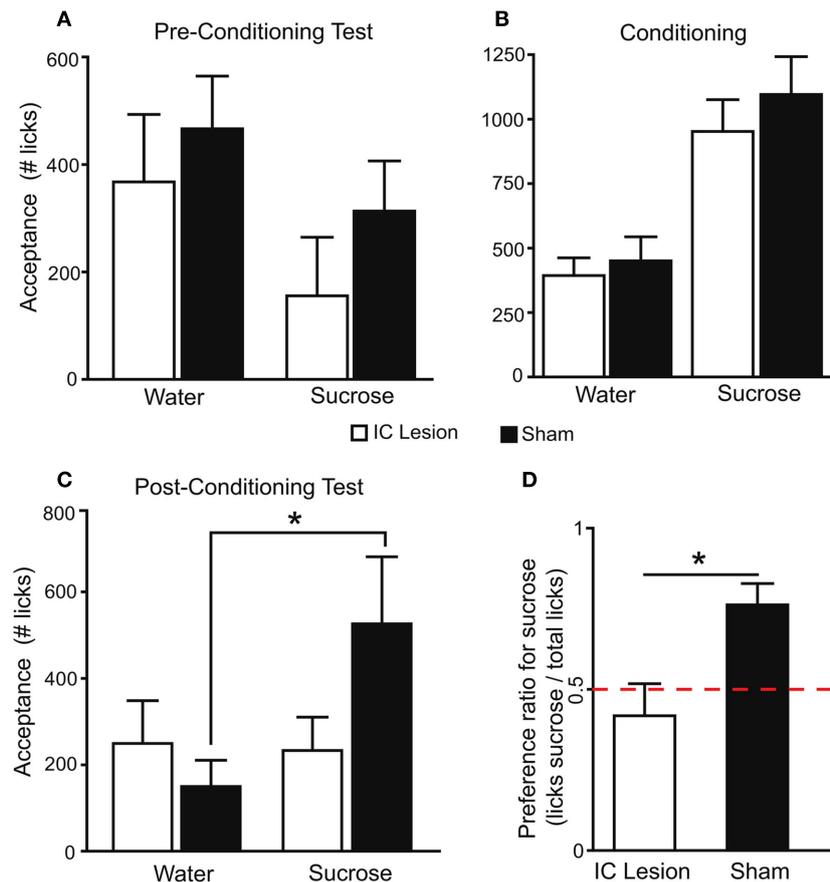


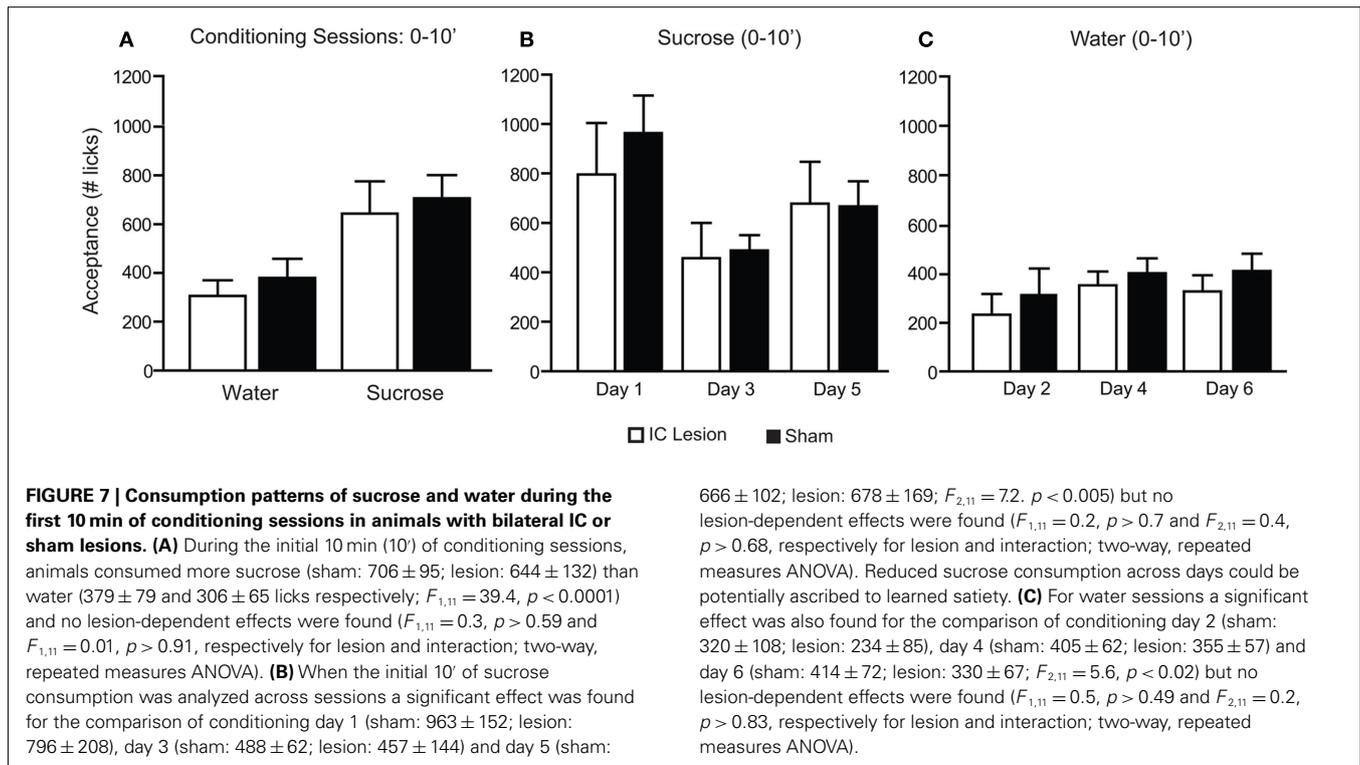
FIGURE 6 | The IC is necessary for the expression of taste-independent preferences conditioned by postingestive reward. (A) In pre-conditioning sucrose vs. water two-bottle preference tests, since sucrose was presented on the side opposite to bias, mice consumed more water (lesion: 368 ± 125 licks; sham: 466 ± 98) than sucrose (lesion: 156 ± 109 ; sham: 313 ± 93 ; $F_{1,11} = 11.3$, $p < 0.007$). However, lesion status had no effect ($F_{1,11} = 0.8$, $p > 0.37$) and the two factors did not interact ($F_{1,11} = 0.3$, $p > 0.59$; two-way, repeated measures ANOVA). (B) During the conditioning protocol, animals consumed more sucrose (sham: 1096 ± 147 ; lesion: 952 ± 123) than water (451 ± 93 and 393 ± 69 licks respectively; $F_{1,11} = 101.3$, $p < 0.0001$) and no lesion-dependent effects were found ($F_{1,11} = 0.4$, $p > 0.51$ and $F_{1,11} = 0.5$, $p > 0.48$, respectively for lesion and interaction, two-way, repeated measures

ANOVA). Thus, the two groups were equally able to detect the reinforcing postingestive effects of sucrose. (C) In post-conditioning tests, tastant had an effect on consumption ($F_{1,11} = 6.6$, $p < 0.03$) while lesion status did not ($F_{1,11} = 0.5$, $p > 0.48$). However, the two factors interacted significantly ($F_{1,11} = 7.8$, $p < 0.02$; two-way, repeated measures ANOVA). Data was then analyzed separately for each group. Sham-operated animals consumed more sucrose (526 ± 155 licks) than water (150 ± 61 ; $t_6 = 3.2$, $*p < 0.02$), while in the lesion group consumption did not differ (233 ± 77 and 250 ± 99 respectively; $t_5 = 0.2$, $p > 0.8$; paired two-sample t -tests). (D) Average preference for sucrose in the post-conditioning test differed significantly between groups ($t_{11} = 3$, $*p < 0.02$; unpaired two-sample t -test). Red dashed line corresponds to 0.5 indifference level.

population level, where they were expressed as changes in the difference between population responses to sucrose and water as a function of the behavioral sensitivity (LI) to the conditioning protocol (Figure 3A). Using an immunohistochemical approach, we confirmed the occurrence of taste-independent IC responses to sucrose and, furthermore, showed that these responses were restricted to the dorsal subdivision of the insula (Figure 4). Finally, using excitotoxic lesions (Figure 5) to explore the functional relevance of IC responses to sucrose in the absence of peripheral sweet taste transduction, we found that, after conditioning, animals with bilateral IC lesions did not develop a preference for sipper positions associated with sucrose availability (Figures 6C,D). These results define a new dimension in the insular representation of sugars, ascribing new functions to the IC that go beyond oral chemosensory representation. In particular, they

suggest a more fundamental role for the IC in food-reinforcement mechanisms that cannot be explained as arising from orosensory reward.

The *Trpm5*^{-/-} mice used in this study have a well defined deficit in the transduction of sweet, bitter, and umami tastants (Zhang et al., 2003). Indeed, their peripheral neural and behavioral responses to sweet tastants are essentially abolished (Zhang et al., 2003; de Araujo et al., 2008; Oliveira-Maia et al., 2009; Ren et al., 2010). Furthermore, we have shown that, even after being conditioned to the postingestive effects of sucrose, these animals do not express a preference for this tastant when they are tested in paradigms that depend on the detection of orosensory cues (see text and de Araujo et al., 2008). Thus, even if any such orosensory cues exist (e.g., osmolarity, viscosity), in this experimental paradigm these KO animals do not use them to guide their behavior.

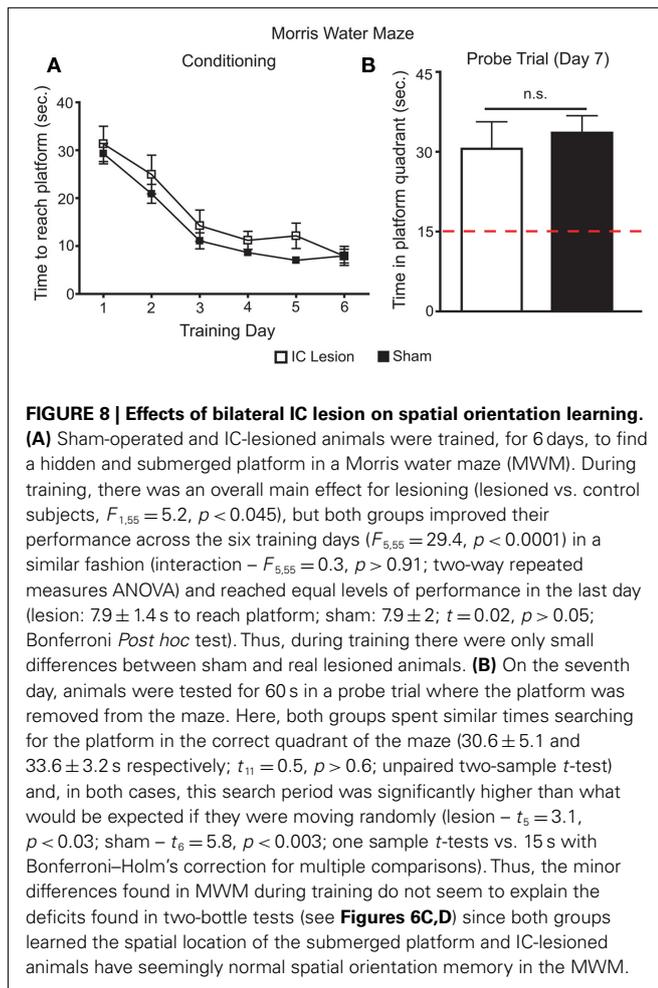


Insular cortex recordings performed in *Trpm5*^{-/-} mice before (pre) and after (post) conditioning demonstrated that, between these two periods, adaptations occurred in the neural responses to sucrose and water (Figures 2B and 3A). We interpreted these adaptations in the IC as reflecting postingestive-dependent learning. Two main factors support this interpretation: first IC responses to the two tastants were significantly changed after conditioning, and second that these adaptations co-varied with behavioral indices of the degree to which each animal developed a preference for sucrose (Figures 2B and 3A). The possibility that, in *Trpm5*^{-/-} mice, these IC responses could reflect orosensory factors is unlikely, not only because these animals do not exhibit behavioral and peripheral neural responses to sucrose (Zhang et al., 2003; de Araujo et al., 2008; Oliveira-Maia et al., 2009; Ren et al., 2010), but also because, as discussed above, both here and in a prior study (de Araujo et al., 2008), we have shown that, in *Trpm5*^{-/-} mice, the expression of conditioned preferences for sucrose is independent of orosensory factors. Finally, interpretations purely based on oro-motor factors such as lick rate and licking cluster duration were eliminated (Figure 3B).

Additional experiments were performed to confirm the relevance of IC responses for taste-independent postingestive learning. FOS immunostaining was used as an index of neuronal activation and confirmed the presence of IC neural responses to sucrose in *Trpm5*^{-/-} mice (Figure 4). These responses were obtained in animals where consumption of sucrose was yoked to that of water (Figure 4C), eliminating the possibility that they result from increased volume consumption, with unspecific effects resulting, for example, from stomach distention (Cechetto and Saper, 1987). Critical confirmation that IC responses were related to postingestive learning came from IC lesion experiments

demonstrating that the IC is necessary for *Trpm5*^{-/-} mice to show a post-conditioning preference for sucrose (Figures 6C,D).

In contrast to these results, a prior study with rats failed to demonstrate behavioral effects of bilateral insular lesions in a *flavor-nutrient* conditioning task (Touzani and Sclafani, 2007). We note that, because we were testing taste-independent responses, these experiments were conducted only in *Trpm5*^{-/-} mice. Thus, these results do not necessarily generalize to rats or possibly even to wild-type mice, even though the very localized expression pattern of *Trpm5* mRNA (Perez et al., 2002) renders the latter possibility less likely. Several other factors may have contributed to the difference between the Touzani and Sclafani (2007) study and our results. In this regard, similar discrepancies have been described relative to the effects of IC lesions on flavor and taste aversion learning, and several reasons have been used to rationalize these inconsistent results. These include the nature of stimuli (taste vs. olfactory) and location of lesion (Kiefer et al., 1982; Mackey et al., 1986; Kiefer and Morrow, 1991; Yamamoto et al., 1995; Cubero et al., 1999; Fresquet et al., 2004; Inui et al., 2006; Roman et al., 2006). Relative to the effects of IC lesions on appetitive conditioning, Touzani and Sclafani (2007) performed experiments involving conditioning to a distinctive flavor, and the possibility remains that the presence of olfactory and/or taste cues may have influenced the ability of lesioned animals to form associations between the solutions and their nutritive value. Furthermore, their IC lesions were centered in the agranular, more ventral division of the rat insula (Touzani and Sclafani, 2007) which, according to our FOS expression analyses in mice, was not recruited by the postingestive-related effects produced by sucrose intake (see Figure 4).



In fact, the post-sucrose consumption increase in IC FOS immunostaining in *Trpm5*^{-/-} mice was restricted to the dorsal sub-division of the insula, an area where electrophysiological measurements were also performed, and that was targeted in the IC lesion experiment (Figure 5). This area includes a more dorsal granular area, where visceral responses have been identified (Cechetto and Saper, 1987; Barnabi and Cechetto, 2001), and an immediately ventral dysgranular area, where taste responses have been reported in rats (Yamamoto et al., 1988; Lundy and Norgren, 2004). That said, the definition of such distinct functional subdivisions of the insula has been debated since single-neurons throughout the insula can respond to multiple sensory modalities, namely taste, visceral, and nociceptive stimuli (Cechetto and Saper, 1987; Hanamori et al., 1997, 1998a,b) and taste responsive neurons have also been described in the granular cortex (Ogawa et al., 1992; Accolla et al., 2007). Thus, the exact identities of the neural networks involved in the taste-independent post-ingestive responses in the dorsal IC that are described here remain to be elucidated.

As mentioned, the primary GC is located in the IC (Cechetto and Saper, 1987; Accolla et al., 2007) and, besides its role in encoding the chemosensory properties of tastants (Rolls, 2006; Stapleton

et al., 2006; Accolla et al., 2007), it is required for associations to be formed between taste and malaise (Braun et al., 1972; Lorden, 1976; Yamamoto et al., 1980; Bermudez-Rattoni et al., 1991; Accolla and Carleton, 2008). Thus, in conditioned taste aversion (CTA) paradigms, pharmacological manipulations (Bermudez-Rattoni et al., 1991; Gutierrez et al., 1999), protein synthesis inhibition (Rosenblum et al., 1993) or irreversible lesions (Braun et al., 1972; Lorden, 1976; Yamamoto et al., 1980) in the GC disrupt the formation of a “memory trace” linking a conditioned taste cue to ensuing visceral malaise. Here we have identified two new functions for the IC in the integration of post-ingestive sensory information. First, we showed that the IC can represent positive post-ingestive outcomes related to the caloric value of a sucrose solution (Figures 2–4) and second, that this brain area plays a relevant role in the modulation of learned behavior toward positive post-ingestive outcomes, even in the absence of orosensory taste input (Figure 6).

While our results support the concept that post-ingestive reward is represented in the IC, it is important to mention that, in *Trpm5*^{-/-} mice with bilateral IC lesions, unconditioned responses to sucrose were conserved. In fact, during conditioning sessions, both lesioned and sham-operated mice consumed more sucrose than water (Figures 6B and 7), showing that the IC is not necessary for detection of the reinforcing post-ingestive effects of sucrose. Clearly, other brain areas must participate in this process (de Araujo et al., 2008; Touzani et al., 2008; Oliveira-Maia et al., 2011). Nevertheless, the effects of IC lesion on post-ingestive dependent conditioning seem to reflect a deficit in the capacity to associate between post-ingestive effects and the side of the behavioral box where they were obtained (Figures 6C,D). Our results thus contribute additional evidence toward the view that a primary role of the IC in CTA (Braun et al., 1972; Lorden, 1976; Yamamoto et al., 1980; Accolla and Carleton, 2008), and possibly other taste-guided learning (Cubero and Puerto, 2000), is the association between stimuli (such as spout-side and post-ingestive reward).

A broad role for the insula in the representation of aversive outcomes, even in the absence of gustatory-related stimulation, has previously been extensively reported (Phillips et al., 1997; Ploghaus et al., 1999; O’Doherty et al., 2003; Wicker et al., 2003; Simmons et al., 2004; Singer et al., 2004; Contreras et al., 2007). In fact, aversion-related representation in the IC has been suggested as a potential explanation for the effects of insula lesions on addictive behaviors (Contreras et al., 2007; Naqvi et al., 2007) and affective decision-making (Clark et al., 2008). Insular representation of positive emotions has also been described, mostly in association with food-related stimuli (Small et al., 2001; de Araujo et al., 2006; Stoeckel et al., 2008; Wagner et al., 2008), but is not as well established (Jabbi et al., 2007). One study demonstrated that hypocretin transmission in the dorsal insula regulates the reinforcing effects of nicotine infusions (Hollander et al., 2008). Here we further show that the same area of the insula is involved in the representation of a positive outcome that, while being food-related, occurs independently of oral chemosensation.

In summary, we have shown that insular IC neurons are modulated during feeding and can exert control on

feeding behaviors even when no oral chemosensory inputs are present. This novel finding could underlie the reorganization of neuronal representations of taste cues in the GC following changes in internal state (Buresova et al., 1979; Accolla and Carleton, 2008; Grossman et al., 2008). Additionally, our findings indicate that the IC performs previously unidentified functions in representing the rewarding post-ingestive consequences of consuming calorie-dense foods, possibly underlying the proposed involvement of the insula in pathological feeding behaviors (Stoeckel et al., 2008; Wagner et al., 2008).

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