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CHEMOSENSORY LEARNING AND MEMORY

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Milagros Gallo and Edmund Rolls

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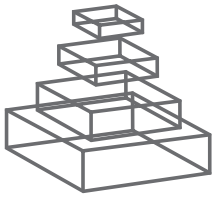

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Cover image provided by Ibbl sarl, Lausanne CH

ISSN 1664-8714
ISBN 978-2-88919-072-0
DOI 10.3389/978-2-88919-072-0

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CHEMOSENSORY LEARNING AND MEMORY

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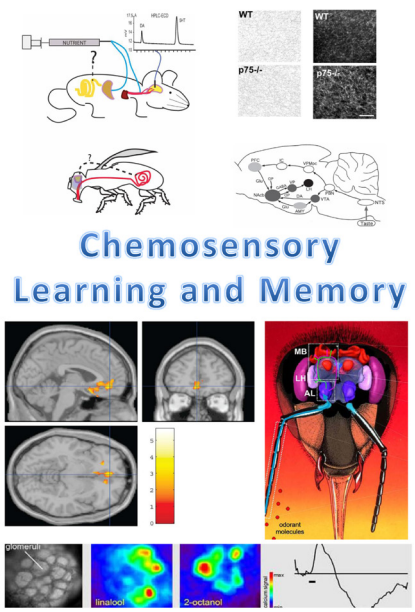

The contribution of research in the chemosensory field to advancing knowledge on learning and memory mechanisms has a long tradition. At the middle of the twentieth century, behavioural data provided evidence that taste and olfactory cues led to robust long-lasting memories after single learning episodes. The peculiar features of some of these types of learning, such as conditioned taste aversion in mammals, were a challenge for learning theory at the time, which was modified in order to integrate the new findings.

In the following decades, the reliability of the behavioural models favoured the application of anatomical, neurophysiological and pharmacological techniques prompting great progress in the identification of the specific neural circuits involved in taste and olfactory learning, thanks to the use of a variety of invertebrate and vertebrate models. In spite of the previous views that considered chemosensory learning as simple models of learning, based on its phylogenetic and ontogenetic universality, at present the systems-level approach is revealing the need to focus on the interactions between a variety of sensory, rewarding, cognitive, emotional and motor systems for a full understanding. The great impact on the field of the more recent developments in molecular biology and human neuroimaging techniques are also remarkable. Nowadays understanding the brain processes involved in learning and memory requires a wider approach to the experience-dependent neural plasticity that includes new phenomena such as adult neurogenesis and epigenetics. In fact, research on plasticity in the olfactory system is important in both areas. Moreover, the realms of chemosensory learning

and memory have expanded to shield light on social, clinical and applied issues, thus creating a wide multidisciplinary scene.

In this context, this Research Topic is aimed to offer an updated scene of the present knowledge and questions raised in a rapidly expanding field by gathering views obtained with different species from invertebrate to humans and various techniques.

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SYSTEMS NEUROSCIENCE

EDITORIAL
published: 25 October 2012
doi: 10.3389/fnsys.2012.00073

Chemosensory learning and memory

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The aim of this issue is to present an updated view of present knowledge and questions raised in the rapidly expanding field of chemosensory (taste and olfactory) learning. Taste is a powerful primary (unlearned) reinforcer, and topics such as olfactory-taste and visual taste association learning are covered in this issue. But the reinforcing properties of taste can themselves be modified, for example, by post-ingestive consequences, for example, in taste aversion learning, and this type of learning is also covered in this issue.

In fact, research on the chemosensory systems has played an important role in advancing knowledge of the brain mechanisms of learning and memory. A well-known example is conditioned taste aversion (CTA). Since the time it was discovered (García et al., 1955), the unique nature of CTA presented a challenge to the contemporary learning theory, and CTA contributed to present theoretical views of learning. CTA learning also became a useful tool for researchers on the neural mechanisms of learning and memory. Jan Bures was a leader in research on the brain mechanisms of CTA in Europe for several decades. We would like to dedicate this special issue to Jan Bures, who passed away on August 24, 2012, in Prague. The field of chemosensory learning is greatly saddened by the news (http://www.ctalearning.com/announcements.asp). He, together with his wife Olga Buresova, was a pioneer during the seventies in applying reversible brain inactivation techniques in order to identify the specific role of the areas forming the CTA circuits. Among other findings, he discovered the critical role of the parabrachial area in taste-visceral signal association, and the relevance of cortico-pontine connections in taste processing (Bures et al., 1998). In addition to his outstanding scientific contributions, Jan was a wonderful colleague and mentor for us and many of the contributors to the present issue and we will never forget him.

The papers forming this issue are representative of the long history and great development of the field thanks to the use of different species and a variety of technical and theoretical approaches. The widely ranging review by Yamamoto and Ueji (2011) of flavor learning including both learned food preferences and aversions, and the paper by Scott (2011) reviewing classic knowledge on the brain mechanisms of CTA, highlight the advances in the field during the last decades. Wider and more complex brain systems than previously thought contribute to flavor learning, with age-dependent interactions between areas such as the insular cortex, amygdala, hippocampal, thalamic, and reward systems (Gámiz and Gallo, 2011). The evidence reported by Neseliler et al. (2011) using an *in vivo* genetically modified rodent model of hypercholinergic innervation is an example of the value of new approaches to support the hypothesis linking acetylcholine and CTA. As Guzmán-Ramos and Bermúdez-Rattoni (2011) describe, major research advances have been made on the cascade of molecular changes involved in the consolidation of CTA taking place during the post-acquisition period.

Remarkable progress has also been made in the field of food preferences. de Araujo (2011) provides a review that includes data obtained both in rodents and Drosophila on the role of taste and energy-sensing systems receiving gastrointestinal and post-absorptive signals in the formation of long-lasting preferences mediated by dopamine release. The elegant experimental work using a variety of techniques reported by Oliveira-Maia et al. (2012) adds evidence on this topic demonstrating the role of the insular cortex in detecting the postingestive effects of sucrose intake.

Closely linked to taste learning in detecting chemical molecules is olfactory learning. As Sandoz (2011) shows in his review, the honeybee has been a model for applying behavioral, neurophysiological and neuroanatomical techniques to research on olfactory learning. Two separate models of the role in olfactory learning of the rat olfactory bulb (Auffarth et al., 2011) and the human glomerulus (Schaefer and Margrie, 2012) are presented.

Finally, Rolls (2011) reviews evidence from primates including humans on the value of taste as a primary reinforcer and the role of the orbitofrontal cortex in building olfactory-taste, and visual-taste associations. He also shows how top–down cognition and attention influence taste and olfactory processing in ways that must involve learning, and also considers the cortical mechanisms involved in taking decisions about olfactory and taste stimuli.

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Received: 05 October 2012; accepted: 05 October 2012; published online: 25 October 2012.

*Citation: Gallo M and Rolls E (2012) Chemosensory learning and memory. Front. Syst. Neurosci. **6**:73. doi: 10.3389/fnsys.2012.00073*

frontiers in
SYSTEMS NEUROSCIENCE

MINI REVIEW ARTICLE
published: 05 September 2011
doi: 10.3389/fnsys.2011.00076

Brain mechanisms of flavor learning

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Once the flavor of the ingested food (conditioned stimulus, CS) is associated with a preferable (e.g., good taste or nutritive satisfaction) or aversive (e.g., malaise with displeasure) signal (unconditioned stimulus, US), animals react to its subsequent exposure by increasing or decreasing ingestion to the food. These two types of association learning (preference learning vs. aversion learning) are known as classical conditioned reactions which are basic learning and memory phenomena, leading selection of food and proper food intake. Since the perception of flavor is generated by interaction of taste and odor during food intake, taste and/or odor are mainly associated with bodily signals in the flavor learning. After briefly reviewing flavor learning in general, brain mechanisms of conditioned taste aversion is described in more detail. The CS–US association leading to long-term potentiation in the amygdala, especially in its basolateral nucleus, is the basis of establishment of conditioned taste aversion. The novelty of the CS detected by the cortical gustatory area may be supportive in CS–US association. After the association, CS input is conveyed through the amygdala to different brain regions including the hippocampus for contextual fear formation, to the supramammillary and thalamic paraventricular nuclei for stressful anxiety or memory dependent fearful or stressful emotion, to the reward system to induce aversive expression to the CS, or hedonic shift from positive to negative, and to the CS-responsive neurons in the gustatory system to enhance the responsiveness to facilitate to detect the harmful stimulus.

Keywords: conditioning, taste, odor, aversion, preference, brain

Food and fluid intake is one of the most essential behaviors since animals require adequate nutrients and reject toxins for their survival. Although energy homeostasis is a basis of regulating food and fluid intake, actual ingestive behavior in animals including humans is controlled by innate and learned flavor preference and/or aversion. Animals have innate predispositions to accept some (sweet tasting) and reject other (bitter tasting) foods, and also they acquire feeding responses on the basis of the orosensory properties and postingestive consequences of foods. Animals learn to prefer the flavor of foods and fluids that are associated with positive postingestive nutritional consequences. On the other hand, if animals consume an unfamiliar food or fluid and experience visceral discomfort or malaise, they easily learn to avoid the flavor at subsequent exposures. First, brain regions related to processing of taste information is briefly summarized.

CENTRAL PATHWAYS OF TASTE INFORMATION

Central gustatory pathways have been well studied in monkeys and rodents especially in rats. **Figure 1** shows a schematic diagram of some of the gustatory and related pathways in the rat. Branches of the facial (chorda tympani and greater superficial petrosal), glossopharyngeal, and vagus (superior laryngeal) nerves, which synapse with receptor cells in the taste buds, convey taste messages to the first relay nucleus, the rostral part of the nucleus of the tractus solitarius (NTS). The second relay nucleus for ascending taste inputs is the parabrachial nucleus (PBN) of the pons. The third relay station is the parvocellular part of the ventralis posteromedial thalamic nucleus (VPMpc). This thalamic nucleus sends taste information to the insular cortex (IC). In monkeys, however, ascending fibers of neurons in the gustatory area of the NTS directly reach the VPMpc, bypassing the PBN (Beckstead et al., 1980).

The neural pathway of the brain reward system has also been studied (Wise, 2002). As shown in **Figure 1**, the essential components are the ventral tegmental area (VTA) of the midbrain which is the origin of the mesolimbic dopamine system, the nucleus accumbens (NAcb) of the ventral forebrain which is an essential interface from motivation (e.g., palatability) to action (e.g., eating), and the ventral palladum (VP) situated between the NAcb and lateral hypothalamus known as the feeding center.

It is not fully understood how the taste system interacts with the reward and feeding system. The amygdala including the central nucleus (CeA) and basolateral nucleus (BLA), the prefrontal cortex (PFC) including the ventrolateral (or anterior sulcal) and dorsomedial cortices and the IC are the candidates for the interfaces between the two systems. The IC sends axons to the PFC (Shi and Cassell, 1998), and the dorsomedial PFC neurons actually respond to gustatory stimuli (Karadi et al., 2005). Among other structures, the PFC is interconnected with the feeding-related subcortical areas such as the VTA (Kosobud et al., 1994) and NAcb (Brog et al., 1993). Behavioral studies have shown that the PFC is associated with various mechanisms in the central feeding control, including conditioned taste aversion (CTA; Hernadi et al., 2000; Karadi et al., 2005).

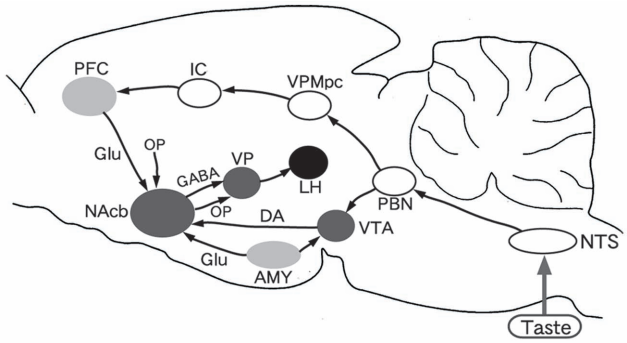

FIGURE 1 | Diagram of connections from the taste system to reward system and feeding center in the rat brain. NTS, nucleus of the tractus solitarius; PBN, parabrachial nucleus; VPMpc, parvocellular part of the ventralis posteromedial thalamic nucleus; IC, insular cortex; PFC, prefrontal cortex; AMY, amygdala; VTA, ventral tegmental area; NAcb, nucleus of accumbens; VP, ventral pallidum; LH, lateral hypothalamic area. DA, dopamine; GABA, γ-aminobutyric acid; Glu, glutamic acid; OP, opioids.

FLAVOR PREFERENCE

There are at least two types of learning-based flavor preference: attenuation of neophobia and conditioned flavor preference which consists of conditioned odor preference and conditioned taste preference. When an animal ingests a harmless new substance or liquid, it shows neophobia, or a small intake with caution toward the novel food, and it increases the consumption at subsequent exposures after learning that the food is safe to consume (Bures et al., 1998). Through this process of the attenuation of neophobia (or learned safety), food can be classified as familiar and safe. The IC is suggested to be important in recognition whether the taste is familiar or novel (Gutiérrez et al., 2003; Bahar et al., 2004b). More recent studies suggested that attenuation of neophobia depends not only on the IC (Rodriguez-Ortiz et al., 2005), but also hippocampus and perirhinal cortex (De la Cruz et al., 2008).

When ingestion of novel food (even if it is neutral or mildly aversive) is associated with preferable oral sensations or positive postingestive consequences, the food becomes hedonically positive and preferred on the basis of its taste and odor as cue signals (Sclafani, 2001). Several pairings are required for this effect in contrast to one paring for the aversion learning. Although this phenomenon is collectively called conditioned flavor preference, if you focus on either taste or odor as a cue signal, this learning is referred to as conditioned taste preference and conditioned odor preference, respectively.

Conditioned odor preference can be established when an odor is associated with highly palatable taste (Sakai and Yamamoto, 2001; Sclafani et al., 2001) as well as it is associated with positive postingestive effects (Sclafani and Nissenbaum, 1988). Our ongoing study (Ueji and Yamamoto, 2011) is testing whether weanling (3-week-old) rats can acquire conditioned odor preference, the results being compared with those obtained in young adult (8-week-old) rats. During the acquisition phase of learning, one of the following solutions was presented to each rat for 15 min daily across six consecutive days. Half of the rats in each age group received an unsweetened grape-flavored solution on odd-numbered days and a sweetened (with 2, 20, or 30% sucrose) cherry-flavored solution on even-numbered days. The remaining rats received a sweetened grape-flavored solution on odd-numbered days and an unsweetened cherry-flavored solution on even-numbered days. In the following test session, each rat received the preference test with unsweetened cherry- and unsweetened grape-flavored solutions simultaneously for 15 min daily across four consecutive days. Both 3-week-old and 8-week-old rats showed a significant preference for the odor (cherry or grape) previously experienced with 2% sucrose compared with that previously presented in water. When the concentration of sucrose is increased from 2 to 30%, the 3-week-old rats showed a significant aversion for the odor associated with 30% sucrose (**Figure 2**), while 8-week-old rats again showed preference for the odor associated with this strong sucrose, suggesting a hedonic shift from positive to negative with increasing the concentration of sucrose in weanling rats. Our study suggests that aversion to high concentration of sucrose is due to its oral sensation (e.g., osmotic effects) rather than its postingestive caloric effects. Interestingly, both preference and aversion learning acquired at the age of 3 weeks was preserved when retested at the age of 20 weeks (**Figure 2**). These results suggest that weanling experience of food strongly affects the feeding behavior in adulthood.

Concerning the brain regions responsible for this type of taste-associated odor preference, Sakai and Yamamoto (2001) demonstrated that rats with lesions in the amygdala showed rapid extinction of preference to the saccharin-associated odor. However, rats with lesions in the IC showed retention of learning similar to that of the control rats. Rats with lesions in the sulcal prefrontal or cingulated cortices showed an intermediate disruptive effect on preference to the saccharin-associated odor. The results also suggested that the odor was associated with the hedonic aspect of taste. Although their results suggested the importance of the

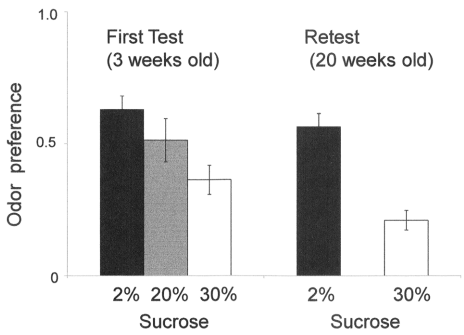

FIGURE 2 | Preference to odor which was previously associated with three concentrations of sucrose solutions. The odor associated with 2% sucrose was significantly preferred compared with the odor associated with water. The odor associated with 30% sucrose was significantly avoided, and the preference for the odor associated with 20% was similar to that associated with water. The ordinate denotes the preference ratio obtained from the conventional two-bottle preference method as described in the text.

amygdala especially in the retention process of the odor learning, the results also showed that the amygdala-lesioned rats could acquire this learning, which means that the acquisition phase of this learning may involve different parts of the brain in parallel, with either being able to create this type of association learning.

More recently, Desgranges et al. (2010), using a sucrose conditioned odor preference as a flavor experience in rats, demonstrated that the neuronal population activated by both odor and taste strongly increased in the BLA, but not in the IC by using the compartmental analysis of temporal activity with fluorescence *in situ* hybridization (catFISH) for Arc mRNA. Their results suggest that this greater odor–taste convergence in the BLA is based on the recruitment of a new population of previously silent neural units that acquired the ability to respond to both chemosensory inputs after repeated odor–taste association.

Conditioned taste preference is established when the taste of food is associated with positive postingestive consequences. A representative procedure for this learning is seen in an article by Touzani and Sclafani (2007): rats were trained with distinctive taste stimuli (conditioned stimulus, CS) paired with intragastric infusion of maltodextrin (16%; unconditioned stimulus, US). The CS solutions contained 0.03% sucrose octaacetate + 0.2% saccharin (bitter–sweet) and 2% NaCl + 0.2% saccharin (salty-sweet). It is common to use mildly aversive taste for this type of learning. Although the central neural mechanism of association of taste with postingestive reward is not fully understood, lesion studies suggested that the PBN (Sclafani et al., 2001) and LH (Touzani and Sclafani, 2001) play important roles in conditioned taste preference. Although the amygdala is essential for preference learning when the primary cue is a flavor (both gustatory and olfactory components), it is not critical in taste preference learning (Touzani and Sclafani, 2005). The IC is also not essential for conditioned taste preference and conditioned odor preference (Touzani and Sclafani, 2007).

FLAVOR AVERSION

In contrast to the flavor preference, when ingestion of a novel food, even if it has preferable taste or odor, is associated with unfavorable postingestive effects or malaise, the food becomes hedonically negative and is avoided and elicits aversive behavior on basis of its taste and odor. This flavor aversion can be divided into conditioned odor aversion and conditioned taste aversion.

Conditioned odor aversion can be acquired in experimental animals by pairing drinking of water with an odor and an intraperitoneal injection of malaise-inducing LiCl (e.g., Inui et al., 2006). However, animals acquire little aversion to an odor CS, when delivered close to the liquid, with a long CS–US delay, a condition in which aversion to a taste CS can occur (Inui et al., 2006). Animals, however, can acquire strong aversions to the odor CS paired with delayed malaise when it is presented with a taste stimulus as a combined stimulus as the CS. This phenomenon is referred to as taste-potentiated odor aversion (TPOA; Rusiniak et al., 1979). Although direct evidence has not been revealed for the brain mechanism of TPOA, lesion-experiments by Inui et al. (2006) suggested the importance of the amygdala in the formation of TPOA: lesions of the amygdala disrupted both odor and taste aversions, whereas lesions of the thalamic taste area or IC disrupted taste aversion but attenuated only odor aversion. Fernandez-Ruiz et al. (1993) and Desgranges et al. (2009) also reported that the lesions of the IC disrupted the acquisition of aversion to a taste CS without affecting the aversion to an odor CS. Desgranges et al. (2008) suggested that the BLA is necessary for acquisition, consolidation, and retrieval of conditioned odor aversion. Taking into account that there exist neurons that receive convergent inputs of taste and odor (Desgranges et al., 2010) and taste and visceral inputs (Barot et al., 2008), we think that there are neurons that have convergent inputs of taste, odor, and visceral information in the BLA.

CONDITIONED TASTE AVERSION

Conditioned taste aversion is established when the taste of food (CS) is followed by malaise (US). This association learning between the CS and US is quickly established, and animals remember the taste for a long time, and reject its ingestion at subsequent exposures (Garcia et al., 1955; Bures et al., 1998). After the acquisition of CTA to the CS, the taste quality may not change, but the hedonic aspect changes drastically from positive to negative. On the view of a number of previous researches, behavioral and neural characteristics of CTA can be elucidated by the following five items: (1) alertness (novelty of CS), (2) association between CS and US, (3) avoidance, (4) aversion (hedonic shift from positive to negative), and (5) augmentation of responses to the CS (see **Figure 3**).

ALERTNESS (NOVELTY OF CS)

It is well documented that strong CTA can be acquired when the CS is novel rather than familiar (Bures et al., 1998). Novelty plays a key role in alerting animals to be cautious toward the food (neophobia). In their investigation of the role of the cholinergic system in the IC, Miranda et al. (2000) found that novel tastes significantly elevated acetylcholine (Ach) levels, whereas familiar tastes did not. Furthermore, inactivation of the nucleus basalis magnocellularis, which is the origin of cholinergic projections to IC, before presentation of a novel taste blocked the increase in Ach release and impaired CTA acquisition. On the basis of these findings, Clark

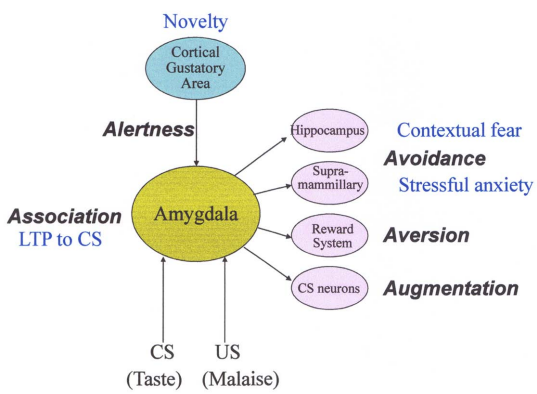

FIGURE 3 | Behavioral and neural characteristics of conditioned taste aversion. CS, conditioned stimulus; US, unconditioned stimulus; LTP, long-term potentiation. See text for details.

and Bernstein (2009) tried to enhance the salience of a familiar CS (saccharin) by infusing carbachol, a direct cholinergic agonist, before CTA and found that rats were able to acquire CTA to familiar saccharin. They also found that familiar CS associated with illness after carbachol, but not vehicle, induced significant elevation of Fos-like immunoreactivity in the amygdala. These results support the notion that Ach activity in the IC provides a critical signal of taste novelty that facilitates CTA acquisition. Thus, familiarity information is stored in the IC and is sent to the subcortical taste relay stations (Yamamoto et al., 2009).

ASSOCIATION BETWEEN CS AND US

Long-term potentiation occurs in the BLA in response to a single electrical stimulation of the PBN. When we used fairly a large electrode, both gustatory and general visceral routes were stimulated, and activity of mass neurons was recorded as evoked potentials. After repetitive stimulation of the PBN, the evoked potential to single stimulation of the PBN was potentiated by more than 50% of the original response (Yamamoto and Yasoshima, 2007). Once a paring of the CS and US occurs, the established long-term potentiation to the CS is the basis of the aversive learning. In fact, Yasoshima et al. (2006) showed by the Fos-like immunoreactivity analysis that sucrose CS induced strong activation of BLA neurons to re-exposure to sucrose after the acquisition of CTA.

Concerning the role of amygdala in CTA, a number of studies have dealt with the functions of the amygdalar subnuclei in the formation of CTA. Although the studies have yielded inconsistent behavioral results, overall electrolytic or excitotoxic lesions show little, if any, involvement of the CeA in CTA (Yamamoto et al., 1995; Morris et al., 1999). Our previous lesion–behavioral studies (Yamamoto et al., 1995) showed that lesions of the CeA had little effects on CTA, and lesions of the BLA severely impaired CTA. Consisting with these findings, a recent study shown below has demonstrated evidence that BLA is a site for CS–US convergence.

Using the catFISH imaging analysis, Barot et al. (2008) provided evidence that, during CTA acquisition, CS and US information converges exclusively on a subset of neurons in the BLA, but not in the IC, when presentation of the stimuli is effective in promoting learning (novel CS–US paring) but not effective when the same stimuli are presented in an ineffective manner (familiar CS–US pairing or backward CS–US pairing). On the basis of their findings, they have proposed a model in which potentiation of US responses by "novel" CS presentation is key to coincidental activation and its sensitivity to "temporal order" (CS–US pairing but not US–CS paring). The existence of neurons receiving convergence of information from pathways mediating CS and US and showing strong and prolonged activation is the basis of association memory formation and is critical for subsequent plasticity.

AVOIDANCE

Yasoshima et al. (2005, 2007) found that the supramammillary nucleus and thalamic paraventricular nucleus were activated by retrieval of the CS after the acquisition of CTA in the overall survey of the brain with the Fos-like immunoreactivity technique. These two regions are suggested to be involved in the expression of anxiety and psychological stress (Wirtshafter et al., 1998; Bubser and Deutch, 1999), and Yasoshima et al. (2005) have suggested that the supramammillary nucleus is activated by memory-elicited discomfort during retrieval of CTA.

Since lesions of hippocampus induce essentially no effect on the acquisition itself of CTA (Yamamoto et al., 1995), CTA is generally accepted as "non-hippocampal" learning, Considering the well-documented facts that the hippocampus is concerned with context fear learning, the hippocampus may modulate CTA in some respects. In line with this notion and on the basis of their previous finding that environmental and temporal contexts can modulate taste aversion learning (Moron et al., 2002), Gallo and her colleagues (Manrique et al., 2009a,b) have studied modulation of taste aversion learning by the time of day (morning 9:00 or evening 19:00) in rats of different ages ranging from 32-day-old to 25-month-old and the role of hippocampus in such a modulation. Their results suggest that the ability to form segregated representations of a complex experience is impaired in aging and abolished by lesions of the dorsal hippocampus.

AVERSION (HEDONIC SHIFT FROM POSITIVE TO NEGATIVE)

Yasoshima et al. (2006) also found that the BLA, extended amygdala and NAcb were also activated during the retrieval phase of CTA. The latter two regions belong to the reward system. CS information from the BLA reaches the NAcb directly or via the extended amygdala (Groenewegen et al., 1999; Shammah-Lagnado et al., 1999, 2001). The *r*-aminobutyric acidergic (GABAergic) neurons in the NAcb send axons to the VP as the main output target (Zahm et al., 1985). The CS induced strong activity in the BLA where little activity was induced by the CS in control animals, suggesting a key role of the BLA in the formation of CTA.

The reward system may be involved in aversive reactions to the CS after the acquisition of CTA. To elucidate the role of the VP in the expression of CTA, Inui et al. (2007) examined the effects of microinjections of a $GABA_A$ receptor antagonist, bicuculline, on the intake of CS in a retrieval test. They showed that the blockade of $GABA_A$ receptors in the VP by microinjections of bicuculline disrupted the expression of CTA (**Figure 4B**), and have suggested that this is due to elimination of aversive responses to the CS. This finding suggests that the GABAergic neurotransmission in the VP is involved in expression of aversive responses to CS, we actually confirmed the increase of the level of extracellular GABA release in the VP using microdialysis technique (**Figure 4A**; Inui et al., 2009). Using a newly developed manganese-enhanced MRI technique, Inui et al. (2011) actually demonstrated an activated pattern in projective neurons from the NAcb to VP by the presentation of a learned aversive taste stimulus inducing rejective behaviors in the retrieval of CTA. We conclude from these findings that the CS presentation after acquisition of CTA increases the extracellular GABA release in the VP through the activation of the NAcb receiving inputs from the amygdala, inducing the expression of aversive responses to the CS and the inhibition of consumption of the CS.

The suggestion that the increase of GABA level in the VP induces expression of aversive responses is based on our previous finding (Shimura et al., 2006). Microinjections of muscimol, a $GABA_A$ receptor agonist, significantly decreased the consumption of water, saccharin, and quinine solutions in rats (**Figure 4C**). Interestingly, the rats showed strong aversive taste reactivity, such

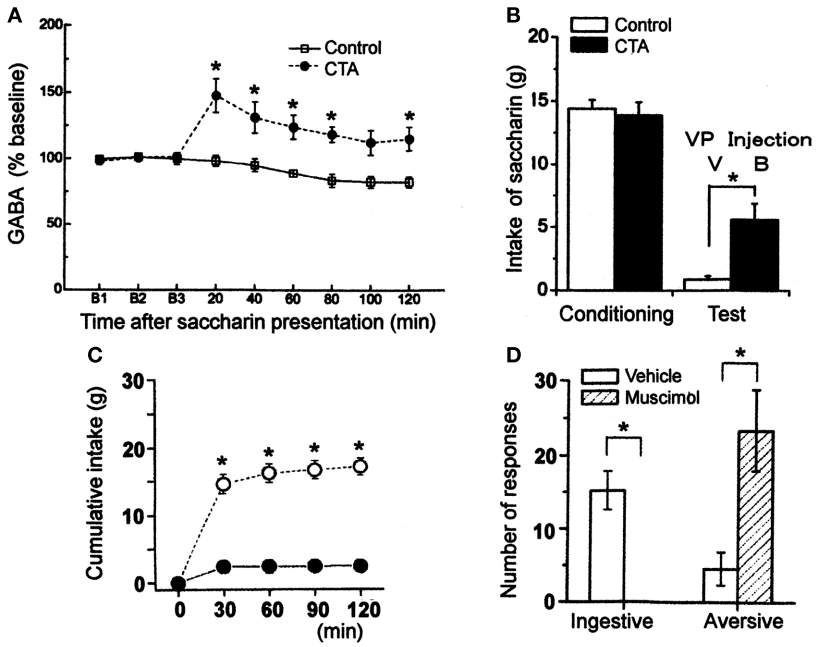

FIGURE 4 | Functional importance of GABA in the ventral pallidum (VP). (A) Intraoral infusion of saccharin solution after CTA acquisition increased GABA release in the VP. **(B)** The intake of saccharin in the vehicle-injected control group (V) was very small after CTA acquisition, but it increased significantly after microinjection of bicuculline [50 ng **(B)**]. **(C)** Muscimol (100 ng) injected into the VP suppressed the intake of saccharin which was significantly different from the intake after vehicle injection at each time point. **(D)** Mean ± SEM number of ingestive and aversive taste reactivity responses to intraoral infusion of 5 mM saccharin after microinjection of muscimol or vehicle in the VP. $*p < 0.05$. (Modified from Shimura et al., 2006; Inui et al., 2007, 2009.)

as chin rubbing, gaping, forelimb flailing, and head shaking, and decreased ingestive reactivity, such as tongue protrusions and rhythmic mouth movements, after the voluntary intake of fluids or the intraoral infusion of normally preferred water or saccharin solution (**Figure 4D**). Thus, the VP is suggested to participate in aversive aspects of ingestive behavior through robust GABAergic neurotransmission. Increased GABAergic transmission in the VP might activate various brain sites responsible for the aversive taste reactivity, including the parvicellular subdivision of the intermediate nucleus of the NTS (iNTSpc), a region strongly activated in association with CTA expression (Schafe et al., 1995). The iNTSpc, which is proved to receive direct projection from the amygdala (Spray and Bernstein, 2004), might receive indirect inputs from the VP to exert aversive reactions by exposure to a conditioned aversive taste.

AUGMENTATION

Perceived intensity of the CS becomes stronger after the acquisition of CTA. Shimura et al. (1997) recorded neuronal responses to taste stimuli from the PBN of anesthetized rats. Animals were separated into two groups: the CTA group that had acquired a taste aversion to 0.1 M NaCl (CS) by paired presentation of an i.p. injection of LiCl (US), and the control group without CTA experience. Taste-responsive neurons in the CTA group showed larger responses to NaCl than in the control group. Tokita et al. (2004, 2007) found that the enhanced responses to the CS were observed exclusively in amiloride-sensitive NaCl-best neurons, but neither in amiloride-insensitive NaCl-best nor any other best neurons. They have suggested that amiloride-sensitive components of NaCl-best neurons play a critical role in the recognition of the distinctive taste of NaCl. Not only PBN neurons, but CGA neurons (Yamamoto et al., 1989; Yasoshima and Yamamoto, 1998) and amygdalar neurons (Yamamoto and Fujimoto, 1991; Yasoshima et al., 1995) exhibit enhanced responses to the CS after CTA acquisition. Augmentation of CS responses enables the animal to facilitate detecting and avoiding the harmful substance.

The aversive memory is stored in the IC, amygdala, and others in the long term after consolidation process accompanying protein synthesis which is derived from augmented activation of relevant neurons (e.g., Shema et al., 2007, 2011). Consolidated memory regains the labile state when retrieved, which is known as reconsolidation. Extinction, a decline in the frequency or intensity of the conditioned response following the withdrawal of reinforcement, is not loss of the original memory, but is a new learning accompanying consolidation and reconsolidation. Consolidation, reconsolidation, and extinction have been studied in taste aversion learning (e.g., Berman and Dudai, 2001; Koh and Bernstein, 2003; Bahar et al., 2004a; Garcia-delaTorre et al., 2010). These are important issues to be more clarified in the future.

ACKNOWLEDGMENTS

This article was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (21500808 to Takashi Yamamoto and 23500986 to Kayoko Ueji).

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

Received: 26 June 2011; accepted: 15 August 2011; published online: 05 September 2011.

Citation: Yamamoto T and Ueji K (2011) Brain mechanisms of flavor learning. Front. Syst. Neurosci. ***5****:76. doi: 10.3389/fnsys.2011.00076*

frontiers in
SYSTEMS NEUROSCIENCE

HYPOTHESIS AND THEORY ARTICLE
published: 23 November 2011
doi: 10.3389/fnsys.2011.00087

Learning through the taste system

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Taste is the final arbiter of which chemicals from the environment will be admitted to the body. The action of swallowing a substance leads to a physiological consequence of which the taste system should be informed. Accordingly, taste neurons in the central nervous system are closely allied with those that receive input from the viscera so as to monitor the impact of a recently ingested substance. There is behavioral, anatomical, electrophysiological, gene expression, and neurochemical evidence that the consequences of ingestion influence subsequent food selection through development of either a conditioned taste aversion (CTA) (if illness ensues) or a conditioned taste preference (CTP) (if nutrition). This ongoing communication between taste and the viscera permits the animal to tailor its taste system to its individual needs over a lifetime.

Keywords: taste, learning, conditioned taste aversion, conditioned taste preference, rat

INTRODUCTION

Taste is an intermediary between the external and internal worlds. It is located at the interface of these two vastly different environments, and thus is charged with making the final decision about what, from an uncontrolled and often hostile chemical surround, should be incorporated into the highly controlled biochemical environment within.

Taste is the beginning of a long chemosensory tube that extends from palate to intestines, with receptors along that length that are sensitive to the products liberated by digestion. Those on the palate are not unique to taste; the same receptors often occur elsewhere in the body. What is unique is that those serving taste gather their information before the irrevocable decision to swallow has been made, and so can influence that decision. Hence, whereas the distribution of information from other chemical sensors may be limited to the gastrointestinal (GI) tract, or may be conveyed through the vagus only to the hindbrain, that from taste receptors is projected through the brainstem to the thalamus and multiple cortical sites as well as to ventral forebrain areas. This vast distribution through the central nervous system permits the control of somatic and autonomic reflexes, a cognitive evaluation, and hedonic appreciation.

An apt metaphor for taste is a Janus head, mounted on an ancient city gate, one face turned outward to assess the traffic beyond the walls, warning of approaching toxins and alerting gatekeepers to the availability of nutrients; the other turned inward to monitor the city's changing needs and to adjust its decisions of what passes through the city walls to satisfy them (Scott, 1987).

This view of the role of gustation as a visceral (internal) as well as somatic (external) sense defines its learning capacity. Taste is exquisitely well suited to learn from visceral consequences (satiety, nausea); it is less inclined to learn from those that are somatic (tones, lights, and shocks). As Garcia noted in describing the development of a taste aversion following a meal, no other aspect of the event was implicated in having caused nausea: not his dinner companions, the table settings, or the background music, only the visceral component represented by the taste of the meal (Garcia et al., 1985).

Taste learning, then, is largely a matter of conditioning. The realm of conditioning can be broadly divided into those events that one can do something about, and those that one cannot. The former typically demands operant conditioning to manipulate the environment to one's satisfaction, using somatic senses to gather information and striated muscles for action; the latter more commonly demands classical conditioning to prepare for the inevitable, often using smooth muscles. Gustatory learning serves as a special case of classical conditioning, with taste representing the conditioned stimulus (CS), and the visceral sequelae of ingestion, the unconditioned stimulus (US). It has been proposed that the visceral response alters the reward value of the taste, and that this new value then guides the animal's behavior to either seek or avoid that taste (Rolls, 2005).

Such learning can occur in an appetitive or aversive direction, with the establishment of either conditioned taste aversions (CTAs) or preferences (CTPs).

Aversions are more robust. It is of greater urgency to the animal to avoid a chemical that has sickened it than to develop a preference for one among many that have proven to provide nutrition. The CTA is readily established with a single pairing of the gustatory CS and the visceral US, even with a CS-US interval for several hours. It is not impaired by placing the animal under deep anesthesia or rendering it comatose before the US is delivered. Indeed, the predisposition of an animal to develop a CTA is so striking that the investigators first suspected it to be an artifact, a suspicion laid to rest only by an exhaustive series of studies and arguments (Revusky, 1977).

As easily as it is created, a CTA is notoriously difficult to extinguish (Nolan et al., 1997). Having been poisoned is clearly an experience not to be forgotten. The CTA also has a robust impact on behavior, often suppressing subsequent acceptance of the CS to less than 10% of preconditioned levels, even in animals motivated to consume by moderate deprivation. With such a

dramatic impact on behavior, the CTA has a half-century history as a rich topic of research. Thousands of studies were conducted during the 1960s and 1970s on behavioral variables such as the distinctiveness and novelty of the taste, the nature and severity of the nausea, the amount of time between them and how that time was spent. With these clearly defined, the CTA could be employed by researchers as a tool for altering taste acceptability, creating a profound reduction in acceptance of the CS, from which generalization gradients of both quality and intensity could be determined to reveal the relative similarities among taste qualities.

In parallel, behavioral neuroscientists began to investigate the mechanisms by which this extraordinary learning process occurred, using rats in nearly all studies. They performed lesions of taste pathways and relays to determine which areas of the nervous system were required in order to develop and retain a CTA. There followed electrophysiological investigations of the impact of a CTA on taste processing, immunohistochemical studies of gene expression elicited by a CTA, and microdialysis experiments on the neurochemical consequences of the experience.

The modest counterpart of the CTA—the CTP—has received less attention. A CTP can be established rather quickly by pairing a novel taste with recovery from a dietary deficiency, most notably the provision of thiamine to animals on a thiamine-deficient diet (Rodgers, 1967; Capretta, 1977). More commonly, however, the impact of a CTP on behavior is revealed only gradually over days of continuous pairing of taste with nutrition, though that impact can reach levels equal to those of a CTA in the opposite direction, i.e., approaching 100% preference (Sclafani and Nissenbaum, 1988). The electrophysiological and neurochemical concomitants of a CTP are also more subtle than those of a CTA. Yet, the CTP may have played a larger role in defining human culture than the CTA, for while the latter is powerful, it is idiosyncratic to the individual. The CTP, by contrast, is often shared by members of a culture where certain foods are available. It is typical of a culture's cuisine that there are a few piquant tastes (the CS) accompanied by carbohydrate loads (the US). The gustatory CS comes to be favored by association with the nutritional US, and the cuisine, with all its cultural trappings and traditions, tends to bind its consumers together as part of their cultural identity.

In the paragraphs that follow, I will review some of the work on the mechanisms of CTAs and CTPs that have come from our laboratory and those of our colleagues.

CONDITIONED TASTE AVERSION: LESION STUDIES

The ingredients of the CTA—taste and visceral distress—are represented widely across the CNS. Investigators have performed lesions of areas that receive such inputs in an effort to define which are crucial to the creation and to subsequent retention of a CTA. Results implicate the **area postrema** in acquisition (Rabin et al., 1983a), but not retention (Rabin et al., 1983b). They reveal that loss of the **parabrachial nuclei (PBN)** causes the most profound disruption on both creating a CTA (with lesions of the lateral division) (Spector et al., 1992; Yamamoto et al., 1994) and maintaining a previous aversion (medial division) (Sakai et al., 1994). They implicate the **ventromedial globus pallidus** in both acquisition and retention (Hernadi et al., 1997). Electrolytic lesions of the **basolateral amygdala** disrupt the creation and retention of a CTA (Yamamoto et al., 1995), but NMDA lesions, which spare fibers of passage, do not (Chambers, 1990); thus the amygdala remains a likely participant in CTA formation, but the axons that pass through it may be of greater import, since leaving them intact sustains the learning. Lesions of prefrontal cortex have yielded conflicting results, and its role in CTAs remains uncertain. Those in **insular cortex (IC)**, however, reliably degrade CTA acquisition (Braun et al., 1982; Bermudez-Rattoni and McGaugh, 1991) and have an even larger impact on retention. Thus, the cast of participants in creating and retaining a CTA as demonstrated by these fixed, permanent lesions range from the deepest recesses of the brain stem through ventral forebrain to the neocortex.

Greater insight may be gleaned from lesions that are reversible, or that combine the loss of more than one area. Ivanova and Bures (1990a,b) temporarily disabled regions of the brainstem with microinjections of tetrodotoxin (TTX). They reported that TTX injected in the PBN up to one day in advance or four days following training blocked the consolidation of a CTA without affecting the rejection threshold for quinine. Thus, taste processing per se remained intact, but the associative functions required for learning the aversion were disrupted by inactivation of the PBN.

The crucial role of PBN in mediating associative taste learning was reinforced by Clark and Bernstein (2009). These investigators performed asymmetrical, unilateral lesions of the PBN and IC, and thereby disrupted CTA acquisition. However, when both lesions were made in the same hemisphere, leaving the contralateral hemisphere intact, learning proceeded normally. Thus, communication between PBN and IC is essential for CTA learning.

CONDITIONED TASTE AVERSION: ELECTROPHYSIOLOGY

The effect of a CTA is to reverse the behavioral reaction to a previously preferred taste to one of the revulsion. The rejection response is organized in caudal brainstem (Norgren and Grill, 1982) and released in stereotypical fashion upon encountering an inherently aversive stimulus or one to which a CTA has been formed. The nucleus of the solitary tract (NST), the first central relay for taste, is likely to be involved in CTA formation. Here gustatory and visceral afferents converge (Norgren, 1981) yet do not directly overlap, communicating instead via the adjacent reticular formation, offering a close association between signals from the two necessary components of a CTA.

Chang and Scott (1984) took single neuron recordings from the NST of rats that were (1) unconditioned (tasted the saccharin CS with no subsequent nausea), (2) pseudoconditioned (experienced the nausea US with no preceding taste), or (3) conditioned (the taste of the saccharin CS was paired with LiCl-induced nausea US). The recordings revealed that sweet-oriented NST neurons gave exaggerated responses to the saccharin CS in conditioned rats, and that the increase was due to a sharp spike of activity that peaked at about 900 msec following stimulus delivery (**Figure 1**). This is reminiscent of the phasic burst of activity elicited by aversive quinine. Moreover, the neural response profile to saccharin in conditioned rats was more similar to those of

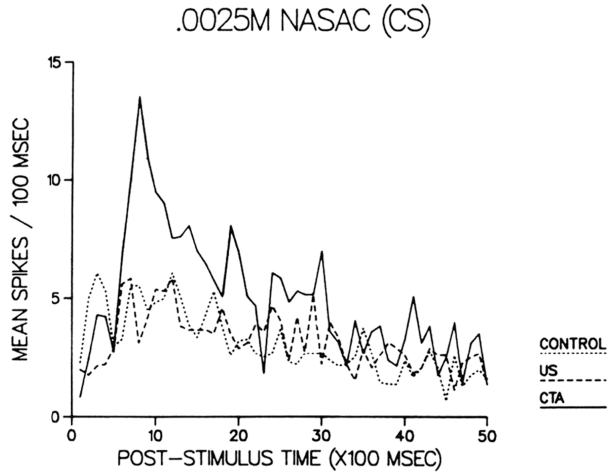

FIGURE 1 | Post-stimulus time histograms of the mean responses among sweet-sensitive neurons in the nucleus of the solitary tract to the conditioned stimulus: 0.0025 M sodium saccharin. Activity is shown for control (dotted line), pseudoconditioned (dashed line), and conditioned (solid line) rats. The enhanced activity during the first three seconds of the evoked response in conditioned animals may represent the increased salience of the sodium saccharin taste and its aversive quality.

aversive stimuli. We concluded that the sensory code for the saccharin CS (and, to a lesser extent, for other sweet stimuli) was altered at the first central taste relay by conditioning, and that such a modification could explain not only the behavioral reaction, but also the immunohistochemical, and neurochemical consequences of a CTA described below. Such a modification of the taste signal might also reveal why the cephalic phase insulin release from the pancreas, a parasympathetic reflex elicited by sweet taste, is blocked after a CTA has been created to that taste (Louis-Sylvestre and LeMagnen, 1980). The altered neural message would lose the capacity to innervate the vagal efferents responsible for stimulating pancreatic β cells.

In a subsequent experiment, we created, and then fully extinguished a CTA to saccharin (Nolan et al., 1997), then recorded responses to saccharin and other stimuli in these recovered rats and in unconditioned controls (McCaughey et al., 1997). The mean responses to all stimuli were no different in the two groups of rats, nor was there any significant difference between the neural response profiles to any taste. However, the neural activity was not completely restored to the preconditioning state. There remained a clear vestige of the conditioning experience in an attenuated burst given to the CS by the sweet-sensitive subgroup of neurons. The burst was no longer associated with conditioned behavior—which was fully extinguished—though it may have served as a permanent marker for a once-salient CS that can abet subsequent reacquisition of the aversion.

Curiously, lesions in gustatory NST do not interfere with either the acquisition or retention of a CTA (Grigson et al., 1997; Shimura et al., 1997a). Even if the electrophysiological effects recounted above were only advisory to a more commanding CTA nucleus or circuit, the blockade of taste information at this obligatory synapse would appear to prevent any subsequent learning. The lack of an impact may reflect the inadequacy of lesions to fully compromise a functioning relay, particularly when they must spare adjacent areas that control vital reflexes, including respiration.

In the parabrachial nucleus, the creation of a CTA to NaCl resulted in an elevated response to that stimulus in sodium-specific taste cells, in agreement with the responses to the saccharin CS in the NST described above (Shimura et al., 1997b). The same was found in the IC (Yamamoto et al., 1989) and amygdala, where an exaggerated response to the CS is often expressed as inhibition (Yasoshima et al., 1995). Finally, in the hypothalamus of naïve rats, the taste of saccharin activates areas associated with feeding and inhibits those for satiety; after the saccharin is paired with nausea, these roles are reversed (Aleksanyan et al., 1976). Thus, the impact of a CTA is demonstrable in the electrophysiological activity of neurons across the widely dispersed regions that process taste activity, and that impact is appropriate to guide the aversive reaction to the CS that follows conditioning.

CONDITIONED TASTE AVERSION: GENE EXPRESSION

The formation of long-term memories requires the expression of immediate early genes and the synthesis of their associated proteins (McGaugh, 2000). The gene *c-fos* has been shown to be expressed and the associated Fos protein synthesized in a variety of species as a basis for modifying the neural activity associated with learning (Sanyal et al., 2002). *c-fos* expression, then, can serve as a useful index of conditioning.

Houpt et al. (1994) demonstrated that sucrose elicited *c-fos* expression in the NST after it had been paired with an intraperitoneal injection of LiCl. This was not simply a response to the aversive taste that sucrose had become, for quinine did not induce the same expression in unconditioned rats (Houpt et al., 1996). However, this index of learning in NST was blocked when the amygdala was impaired, demonstrating the importance of centrifugal fibers in mediating the conditioning process (Schafe and Bernstein, 1996).

The results of gene expression are more detailed in the PBN. Yamamoto and his colleagues have shown that *c-fos* is induced in cells in the medial division of PBN by hedonically positive tastes associated with ingestion, and in neurons in more lateral divisions by aversive tastes. Saccharin activates medial cells in naïve rats, but after the saccharin has been paired with a LiCl injection, its taste induced *c-fos* expression in the lateral division (Yamamoto et al., 1994). Moreover, when *c-fos* expression was blocked in PBN, CTA learning was impaired, just as it is when the PBN is lesioned (Yasoshima et al., 2006). Thus, a functioning PBN, capable of modifying its responses to a taste stimulus as a consequence of experience, is crucial to learning to avoid toxins.

In forebrain, Bernstein and Koh (2007) addressed the issue of which areas reacted to novel tastes differently from those that were familiar, since only the former serve as effective conditioned stimuli. They used *c-fos* expression to identify the central nucleus of the amygdala (CNA) and IC as two such sites. The next question was which of these also responded to the US, the second of the necessary elements of a CTA. Only the CNA expressed *c-fos* to a LiCl US, implicating this nucleus as

a crucial nexus for making the association. Yet while IC, which had responded to gustatory novelty, did not show increased *c-fos* expression to the US alone, its neurons did respond to pairing of the CS and US, reinforcing its role in the associative process, perhaps through communication with the PBN and amygdala (Ferreira et al., 2006).

Gene expression, of course, is a precursor of protein production, and it is assumed that the protein is the basis for forming the associative memory. Accordingly, when protein synthesis was inhibited by administration of anisomycin into the IC, a CTA was not formed (Rosenblum et al., 1993). More specifically, the administration of oligodeoxynucleotide (ODN) antisense to *c-fos* in the amygdala blocked CTA acquisition (Lamprecht and Dudai, 1996). Again, the two critical forebrain regions—IC and amygdala—are implicated, and the capacity of their neurons to synthesize proteins in general, and Fos in particular, is essential to associative learning.

Finally, Bernstein and her colleagues (Barot et al., 2008; Chung et al., 2011) used a direct visualization approach to identify neurons that responded both to the CS and US, reasoning that this afforded them *prima facie* data from which to support the association. They found such cells only in the basolateral nucleus of the amygdala, and only under normal training conditions, i.e., CS followed by US.

The bulk of evidence from gene expression studies, then, focuses on three structures as being central to the acquisition and retention of CTAs: the PBN, the amygdala (both central and basolateral nuclei), and the IC.

CONDITIONED TASTE AVERSION: NEUROCHEMISTRY

Experiencing the conditions under which a CTA is created is stressful. It is unsurprising, then, that when the CS to which a CTA has been developed is subsequently presented, reminiscent of that experience, plasma corticosteroid levels rise (Smotherman et al., 1976). Yet, the CTA does not depend on this adrenal index of stress, for adrenalectomized rats acquired CTAs as readily as controls (Ader et al., 1978).

A more specific measure of the impact of a CTA is seen in the levels of neurochemicals associated with reward or aversion, particularly in the limbic system. Reward is associated with increased dopamine (Hoebel, 1984) and reduced acetylcholine (Rada et al., 1991) in the nucleus accumbens, and lowered serotonin levels in the paraventricular hypothalamus (Stanley et al., 1989). This relationship has been demonstrated both through microdialysis, where dopamine levels in the accumbens rose following a sweet taste, and conversely through reverse microdialysis in which dopamine administered to the accumbens increased sucrose consumption (Hajnal and Norgren, 2001).

The taste of saccharin evoked dopamine release in the rat's accumbens, in accord with its reinforcing value. However, if the saccharin had been paired with nausea to create a CTA, the same stimulus caused a reduction in dopamine, and instead a release of acetylcholine in accumbens (Mark et al., 1991), and serotonin in the hypothalamus (West et al., 1991), denying the neurochemical basis for reward.

CONDITIONED TASTE PREFERENCE

When subjects develop a CTA, there is little doubt that they are subsequently repulsed by the taste. Not only do they avoid it, they also show the well-defined mimetic reflexes of aversiveness: gaping, head-shaking, and chin-rubbing. All that follows—the blockade of parasympathetic reflexes, the alteration of the afferent signal and its projection to brain areas associated with avoidance, the reversal of neurochemical release from rewarding to aversive—is in accord with this powerful experience.

The impact of a CTP is more subtle. The behavior changes only over days of training, though the CS does finally reach asymptotically high levels of acceptance, and is quite resistant to extinction. Mimetic responses of rats, however, do not change as the CS wins acceptance, raising the question of whether the hedonic quality of the CS has increased (Sclafani, 1991). Two types of evidence argue that it does. Giza et al. (1997) recorded electrophysiological responses from the NST of rats that had been conditioned to prefer either of two formerly aversive chemicals. In each case, the gustatory code was modified to reflect a less aversive quality, though the effect was less pronounced than that seen in the opposite direction upon creation of a CTA (Chang and Scott, 1984). Secondly, the taste for which a preference was acquired now elicited a heightened dopamine release in the nucleus accumbens, a clear neurochemical marker of reward (Mark et al., 1994).

CONCLUSION

Learning through the taste system is intimately allied with GI consequences. The animal knows two facts: what the chemical was (taste), and what it did (GI). This information permits it to tailor its chemical selection to full individual advantage over a lifetime. The learning process draws on responses that extend from the viscera through caudal brainstem, to ventral forebrain and cortex, implying an ancient system, much like the control of feeding itself. It is only a marginally conscious process, for CTAs can be learned while comatose, and most people cannot recall the occasion upon which they developed a food aversion (Bernstein, 1985). Conditioned aversions and preferences provide the operative link between the chemical and biochemical environments.

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

Received: 28 July 2011; accepted: 10 October 2011; published online: 23 November 2011.

Citation: Scott TR (2011) Learning through the taste system. Front. Syst. Neurosci. 5:87. doi: 10.3389/fnsys.2011.00087

frontiers in
SYSTEMS NEUROSCIENCE

REVIEW ARTICLE
published: 26 September 2011
doi: 10.3389/fnsys.2011.00079

Post-learning molecular reactivation underlies taste memory consolidation

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It is considered that memory consolidation is a progressive process that requires post-trial stabilization of the information. In this regard, it has been speculated that waves of receptors activation, expression of immediate early genes, and replenishment of receptor subunit pools occur to induce functional or morphological changes to maintain the information for longer periods. In this paper, we will review data related to neuronal changes in the post-acquisition stage of taste aversion learning that could be involved in further stabilization of the memory trace. In order to achieve such stabilization, evidence suggests that the functional integrity of the insular cortex (IC) and the amygdala (AMY) is required. Particularly the increase of extracellular levels of glutamate and activation of *N*-methyl-D-aspartate (NMDA) receptors within the IC shows a main role in the consolidation process. Additionally the modulatory actions of the dopaminergic system in the IC appear to be involved in the mechanisms that lead to taste aversion memory consolidation through the activation of pathways related to enhancement of protein synthesis such as the Protein Kinase A pathway. In summary, we suggest that post-acquisition molecular and neuronal changes underlying memory consolidation are dependent on the interactions between the AMY and the IC.

Keywords: conditioned taste aversion, glutamate, dopamine, molecular reactivation, memory consolidation

INTRODUCTION

Memories of aversive events are more likely to persist over time. The strong and long–lasting representation of these memory traces may depend on the molecular mechanisms required for the consolidation process. A good example of a long–lasting memory trace representation is the conditioned taste aversion (CTA), a learning model where animals associate a novel taste (conditioned stimulus, CS) with gastric malaise (unconditioned stimulus, US), decreasing the CS intake in further presentations. This kind of learning can be acquired with only one-trial and the CS can be separated from the US by many hours, which allows a temporal resolution of the mechanisms involved in the gustatory stimulus acquisition and the further association with the gastric malaise (Naor and Dudai, 1996; Yamamoto et al., 1998; Welzl et al., 2001). Since this task involves the recognition and avoidance of toxic and potentially deadly food, the efficient consolidation and storage of this information makes CTA a good model to study the molecular mechanisms through which memories are established. Overall, these mechanisms would have to promote synaptic plasticity in the structures that are related to acquisition and storage of the information; among these, the insular cortex (IC) and the amygdala (AMY) are key structures involved in acquisition and consolidation of CTA (Bermudez-Rattoni, 2004).

POST-ACQUISITION ACTIVATION AND TASTE MEMORY CONSOLIDATION

Formation of long-term memories is based on molecular and structural changes that allow neuronal networks to stabilize and support long-term storage. It has been proposed that this consolidation process relies on memory trace reactivation seen as neuronal post-learning activity in absence of sensory stimulation. Trace reactivation theory establishes that the expression of patterns of activity in neural ensembles during an experience should be spontaneously re-activated during subsequent periods of behavioral inactivity: even during post-training wakefulness, indicating that information can be maintained and processed concurrently within relevant cortical sites (Hoffman and McNaughton, 2002). For instance, these authors made simultaneous neural recordings in the macaque neocortex, i.e., posterior parietal cortex, motor cortex, somatosensory cortex, and dorsal prefrontal cortex after a sequential reaching behavior, the simultaneous analysis revealed that cells in all four areas exhibited similar firing related to the task, and interestingly those cells tended to be coactive afterward. According to this hypothesis, memory consolidation relies on reactivation and reorganization of newly acquired information. After the initial encoding of sensorimotor experience, a series of cellular, molecular, and systems-level alterations develop over time, engaging reactivation patterns in neocortical structures during awareness and sleep periods, stabilizing the initial memory representation, converting it into a long–lasting memory trace (Smith, 2001; Robertson et al., 2004; Walker and Stickgold, 2004; Stickgold, 2005; Ellenbogen et al., 2006; Gais et al., 2007; Rasch and Born, 2007). Particularly in CTA learning, there is evidence suggesting that such post-learning activities may be part of the trace consolidation mechanisms. For instance, single unit recording in the basolateral nucleus of the amygdala (BLA) showed an increase

of activity 30 min after CS–US pairing (Yamamoto and Fujimoto, 1991), and similar results were obtained from single unit recordings in the IC, where 20–30 min after CS–US pairing IC neurons had an increment of excitability (Yasoshima and Yamamoto, 1998). These reports suggest that the association between the CS and US could induce post-acquisition changes that create a long–lasting activation of these structures that may potentiate synaptic efficacy even after stimulation has ceased.

CTA CONSOLIDATION: INSULAR CORTEX AND AMYGDALA

Many studies have proven that two temporal lobe structures, the IC and the amygdala (AMY) are highly involved in taste memory formation. These studies have demonstrated either by lesions or by administration of several neurotransmitters antagonists before the CS presentation, that CTA is affected or impaired in one or both structures. Although, these effects could be evaluated performing a long-term memory (LTM) test, it is unclear if the affected stage was the acquisition or the consolidation of the memory trace. In order to evaluate this, a short-term memory (STM) test may clarify the role of some neurotransmitter systems in the different memory stages. For instance, the administration of scopolamine, a muscarinic antagonist, into the IC before CTA acquisition affected both STM and LTM indicating that the LTM effect is attributable to an impairment of the actual memory trace formation (Naor and Dudai, 1996; Ferreira et al., 2002). Conversely, blockade of the *N*-methyl-D-aspartate receptors (NMDAr) before CTA training impairs only LTM leaving STM intact, indicating that the activity of these receptors is required for memory consolidation (Ferreira et al., 2002; Bermudez-Rattoni, 2004). Another neurotransmitter system that has been involved in CTA memory consolidation is the dopaminergic system. Disruption of dopamine projections in the IC by the administration of a catecholaminergic toxin (6-hydroxydopamine) before CTA training, impairs acquisition of this task (Fernandez-Ruiz et al., 1993) and the blockade of the D1 type receptors before CTA training impairs LTM (Berman et al., 2000). Accordingly, we have seen by using *in vivo* microdialysis that the first presentation of taste stimuli, like saccharin or quinine, induces a significant increase of dopamine release but not glutamate within the IC, suggesting a differential role of these neurotransmitters in taste processing (**Figures 1A,B**). The dopaminergic increment is thought to be related to the novelty of the stimulus, since the presentation of water did not induce any significant changes in dopamine release and both saccharin (0.1% v/v, sweet) and quinine (0.005% v/v, bitter) solutions, being different taste modalities, yet novel stimuli, induced a dopamine increase (Guzman-Ramos et al., 2010). Some evidences show similar results in other structures receiving dopaminergic aferences such as nucleus accumbens (Bassareo and Di Chiara, 1997; Feenstra et al., 2000) and prefrontal cortex (Bassareo and Di Chiara, 1997; Feenstra et al., 2000; Rossetti and Carboni, 2005; De Leonibus et al., 2006) during the exposure to novel stimuli. In this regard, it has been considered that dopaminergic responses are not only related to the rewarding quality of the stimuli, but also to their salience (Ljungberg et al., 1992; Ungless, 2004). For instance, a salient novel gustatory stimulus is important for the animals, since it can produce either favorable or aversive consequences. Hence, dopamine increase may be a suitable signal that triggers the mechanisms to store relevant information. In CTA training, we have addressed whether the dopaminergic signal related to the CS presentation was involved in the acquisition or the consolidation of the memory trace. To do so, we blocked the D1 receptors before the CS–US exposure and performed STM and LTM tests. Interestingly, pre-trial treatment only impaired LTM leaving STM intact; and when the D1 receptors were blocked just after the CS presentation, neither STM nor LTM were impaired (**Figure 2**), indicating that the dopaminergic action within the IC during the CS processing is involved specifically on CTA memory consolidation (Guzman-Ramos et al., 2010).

From this evidence we could say that both STM and LTM storage mechanisms are triggered during training. Muscarinic receptors are involved in STM, whereas D1 and NMDA receptors are activated to further consolidate the memory trace.

PROTEIN SYNTHESIS INVOLVED IN POST-ACQUISITION ACTIVITY FOR TASTE MEMORY CONSOLIDATION

Among the molecular differences between STM and LTM mechanisms is the dependence on protein synthesis (Davis and Squire, 1984; Martin et al., 2000; Dudai, 2004). The consolidation process

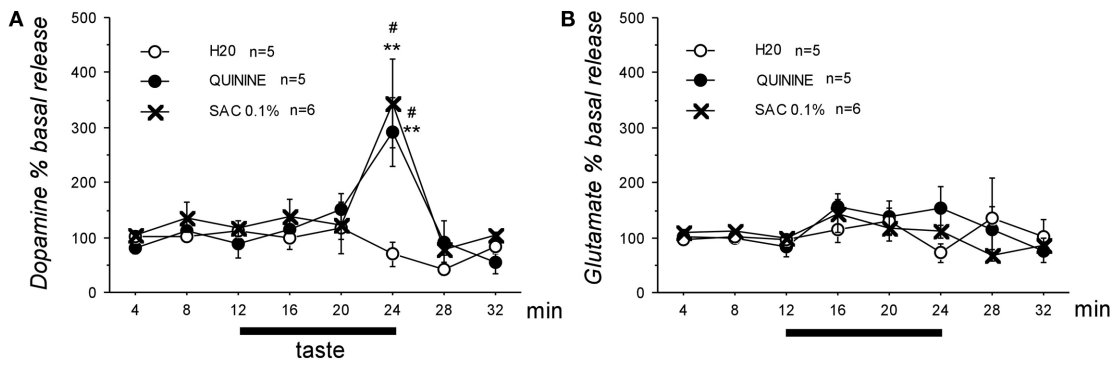

FIGURE 1 | (A) Dopamine and **(B)** glutamate release in the IC during the exposure to novel gustatory stimuli (saccharin 0.1%, quinine 0.005%) or water. Fractions of 4 μL, the first three samples are baseline release before taste stimulation. Data is shown as mean ± SEM; $*p < 0.05$ and $**p < 0.01$ vs. same fraction of control group and $\#p < 0.05$ vs. baseline fractions. A significant increase of dopamine is shown in the IC of groups that were exposed to novel taste stimulation.

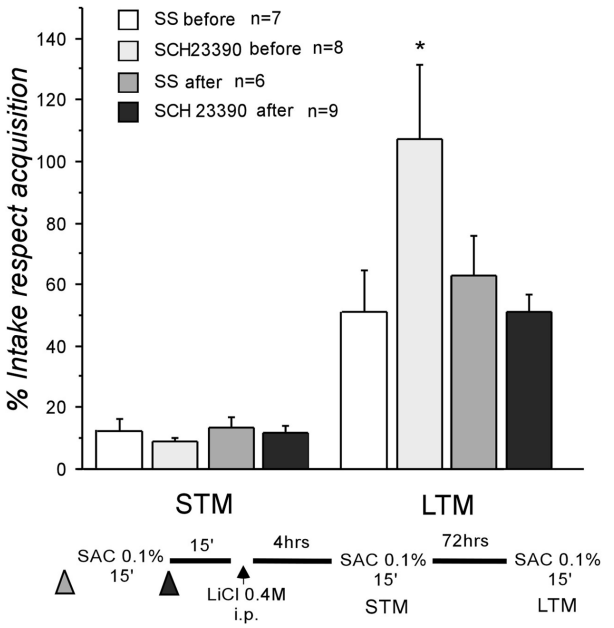

FIGURE 2 | Short- and long-term memory (STM and LTM) effects of the intra-cortical infusion of SCH23390, a D1 receptors antagonist, into the IC. "SCH23390 before" group and a control group that received saline solution injections (SS before) were injected 15 min prior CTA training (gray triangle) that consisted in the presentation of a saccharine solution (0.1%) during 15 min, followed by an i.p. LiCl administration (0.4 M, 7.5 mL/kg). "SCH 23390 after" and SS after groups received the injections immediately after the saccharin exposure (black triangle). All the groups showed aversion in the STM test (4 h after conditioning), whereas in the LTM test (72 h) only the group that was injected with the D1 antagonist shows an impairment of consolidation. Saccharin consumption during the memory tests are expressed as percentage of consumption during acquisition stage ± SEM. $*p < 0.05$.

is thought to be a progressive stabilization that requires time and involves synaptic plasticity based on the synthesis of new proteins that allow neuronal changes underlying the memory trace storage. Hence, one of the most popular approaches to study the role of any brain structure in memory consolidation has been for many years the administration of protein synthesis inhibitors. Administration of anisomycin in the IC before and after the CS presentation has an effect on LTM (Rosenblum et al., 1993), similarly, the administration of this drug into the central subnucleus of the amygdala (CeA), but not in the BLA affects LTM for CTA (Bahar et al., 2003; De la Cruz et al., 2008; Garcia-DeLaTorre et al., 2009).

As an example of protein expression in the IC and the amygdala related to the CTA consolidation process is the protein c-fos. This protein is an immediate early gene product that regulates the transcription of "late response genes" contributing to long-term neuronal changes (Herdegen and Leah, 1998; Walton et al., 1999). In CTA training, the CS and US association elicits an increase in c-fos expression in the CeA and in the IC (Yamamoto et al., 1997; Wilkins and Bernstein, 2006). Furthermore, the local administration of an antisense oligonucleotide (ASO) in the AMY or in the IC impaired CTA LTM seen in the 24-h test (Yasoshima et al., 2006). Consolidation of CTA is also related to the synthesis of brain-derived neurotrophic factor (BDNF), a neuronal growth factor that has been involved in plasticity-related events such as long-term potentiation (LTP; Messaoudi et al., 2002; Bramham and Messaoudi, 2005) and memory formation of several tasks (Mizuno et al., 2000; Bekinschtein et al., 2008; Ma et al., 2011). Taste aversion learning induces an increase of BDNF expression in the CeA and the IC whereas the inhibition of this expression by local administration of BDNF ASO affects LTM but not STM (Ma et al., 2011). Another protein involved in CTA consolidation is CREB, it has been reported that the administration of ASO of CREB into the AMY before CTA training produced significant deficits on LTM measured 3–5 days after conditioning, however, STM remained intact (Lamprecht et al., 1997). These particular examples provide evidence that some proteins engaged in synaptic plasticity and memory consolidation are mainly related to the consolidation of CTA CS–US association and not only to CS or US exposure, indicating a role in the stabilization of the memory trace formed by the stimuli pairing.

NEUROTRANSMITTERS INVOLVED IN POST-ACQUISITION ACTIVITY FOR TASTE MEMORY CONSOLIDATION

It has been proposed that long-term stabilization of memory may need reactivation of the biochemical pathways that were initially active during training in order to sustain the levels of proteins required for the ongoing consolidation process. This hypothesis is supported by evidence showing that NMDA receptor synthesis and activity is required for memory consolidation. In this regard, Cui et al. (2005) demonstrated by using a specific inducible knockout mouse that CTA impairments are evident when the NR1 subunit of the NMDAr in the forebrain regions was decreased from 1 up to 3 weeks after CTA training,. These results suggest that a prolonged glutamatergic/NMDAr activity is engaged in CTA LTM formation (Shimizu et al., 2000; Cui et al., 2005). Similarly, memory impairments for taste aversion memory have been demonstrated by the blockade of NMDAr activity at 30, 60, or 120 min after CTA acquisition (Gutierrez et al., 2003). Altogether these results are in accordance with the cellular consolidation theory stating that molecular changes that underlie consolidation might occur within hours or even days after the post-trial stage. Within these molecular changes neurotransmitters release has been scarcely studied. Recently, we have reported by using *in vivo* microdialysis that the CS–US pairing in CTA training induces a significant concomitant increase of glutamate and dopamine within the IC. As we can see in **Figure 3**, while monitoring CTA training, saccharin exposure induces a dopamine increase and the LiCl administration induces a glutamate increase in the IC. Interestingly, about 40 min after the association of both stimuli, dopamine and glutamate extracellular levels showed again a significant and transient increase. Nonetheless, this phenomenon was not related to the CS or US presentation alone, since saccharin followed by NaCl administration, or water followed by LiCl did not produce any of the post-acquisition neurotransmitters release as observed after the saccharin–LiCl association. Furthermore, the backward conditioning, which involves the same stimuli that were associated, failed to induce such post-learning changes in dopamine and glutamate. Altogether, these results indicated that only the forward association of the stimuli induced post-trial increments of

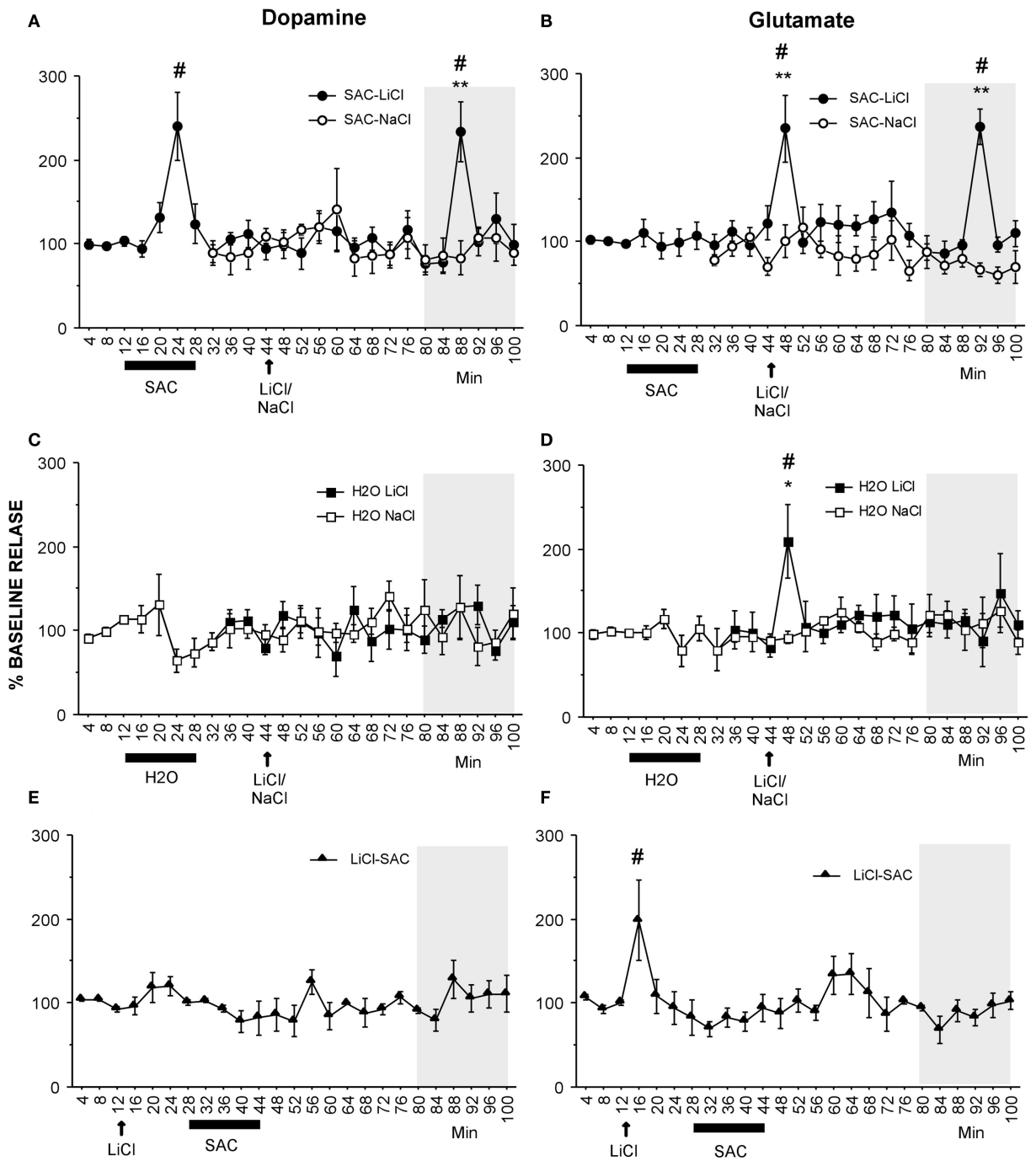

FIGURE 3 | Extracellular dopamine and glutamate levels in the IC increase concomitantly in the post-acquisition period of CTA training. **(A)** Dopamine monitoring: SAC-LiCl, conditioned group ($n = 10$) received 0.1% saccharin solution followed by 0.4 M LiCl i.p. injection (7.5 mL/kg); SAC-NaCl, non-conditioned group ($n = 7$) received 0.1% saccharin solution followed by 0.4 M NaCl i.p. injection (7.5 mL/kg); the CS elicited a dopamine increase in both groups but only the conditioned group showed a post-acquisition increase in the 88-min fraction. **(B)** Glutamate responses monitoring in conditioned and non-conditioned groups, the US elicited an increment in the SAC-LiCl group due to the LiCl injection but only the conditioned group showed a post-acquisition increase in the 92-min fraction. **(C)** Dopamine responses of control groups to stimuli: H_2O–LiCl group ($n = 5$) received tap water followed by 0.4 M LiCl i.p.; H_2O–NaCl group ($n = 6$) received tap water followed by 0.4 M NaCl i.p.; Dopamine levels are significantly different during saccharin exposure that during water exposure and showed no post-acquisition increments. **(D)** Glutamate responses to the LiCl and NaCl injection showed no post-acquisition changes, only the one related to the US. **(E)** Dopamine responses during backward conditioning: LiCl-SAC ($n = 7$), received 0.4 M LiCl i.p., and later, a 0.1% (wt/vol) saccharin solution; there is no post-acquisition increment. **(F)** Glutamate response during the backward conditioning. Graphics expressed as means of % baseline release ± SEM. $*p < 0.05$, and $**p < 0.01$ vs. control group and $\#p < 0.05$ vs. baseline release (Guzman-Ramos et al., 2010).

glutamate and dopamine. These neurochemical signals appear to be related specifically to the consolidation process, since blockade of the NMDAr in the post-acquisition stage impairs only long, but not STM and the simultaneous blockade of the dopaminergic D1 receptors and the NMDA induces a greater impairment in CTA consolidation, suggesting a synergic role of these neurotransmitter systems (Guzman-Ramos et al., 2010).

A considerable amount of evidence indicates that D1 receptors activity can modify the strength of synaptic transmission, potentiating NMDAr conductance by means of NR1 subunit phosphorylation through PKA, enhancing the excitability of neurons and leading to a cooperative action that may strengthen the memory trace formation (Cepeda et al., 1998; Wang and O'Donnell, 2001; Jay, 2003; Tseng and O'Donnell, 2003, 2004; Hallett et al., 2006). The role of the dopaminergic system in synaptic plasticity and memory consolidation has been proven, particularly through the activation of cAMP/PKA/CREB pathway involving protein synthesis induction (see Jay, 2003). Similarly, dopamine has also been related to the persistence of the LTM trace, since the intrahippocampal infusion of a D1 agonist 9 h after training in a one-step inhibitory avoidance task makes a weak training last longer, indicating that dopaminergic signal can modulate the maintenance of LTM storage through a post-acquisition activity (Rossato et al., 2009).

Further mechanisms are involved in the long-term maintenance of the CTA memory trace, for instance, the cortical activity of PKM ζ, a Protein Kinase C (PKC) isoform that is persistently active after the consolidation period, is necessary for persistence of memory for several weeks and even after 3 months from the CTA training; the blockade of this enzyme in the IC impairs LTM in an apparently irreversible way (Shema et al., 2007, 2009). Moreover, the over expression of PKM ζ in this brain structure about 2 weeks after training, enhances LTM performance in the CTA tests (Shema et al., 2011). On the other hand, the post-acquisition blockade of PKM ζ in the BLA has no effect on LTM maintenance, but PKM ζ inactivation 15 min after CS presentation, in the CS–US interval, produces a significant effect on taste aversion tested 2 days after conditioning (Gamiz and Gallo, 2011). This implies that CTA memory trace maintenance is dependent on the activity of this enzyme within the IC, and its role on the AMY may be related to the acquisition stage.

AMYGDALA AND INSULAR CORTEX INTERACTION ON CTA LONG-TERM STORAGE

The amygdala and the IC have reciprocal projections (Pitkanen, 2000; Price, 2003); and some data suggests that the BLA projection to the gustatory cortex is important for the taste aversion memory stabilization. For instance, it has been demonstrated that tetanic stimulation of BLA induces LTP in the IC, increasing the neuronal response to low frequency stimulation (Escobar et al., 1998b; Jones et al., 1999). Thus, LTP induction in the BLA–IC projection before CTA acquisition enhances this task retention by making the extinction process slower (Escobar and Bermudez-Rattoni, 2000). In agreement with the role of NMDAr on memory consolidation, it was demonstrated that such potentiation depends on the NMDAr activity in the IC, since intra-cortical administration of antagonists of this receptors impair both CTA and LTP induction in the BLA–IC pathway (Escobar et al., 1998a,b). Conversely, the possibility that amygdala NMDA activation could improve taste aversive memory has been demonstrated. Thus, BLA administration of glutamate before the gastric malaise induction during CTA training enhances aversive taste memory formation, and local administration of an NMDAr antagonist in the IC 1 h after conditioning impaired taste aversion memory enhancement (Ferreira et al., 2005). This suggests that the interaction among amygdala and IC through the glutamatergic system could contribute to the CTA memory trace establishment and consolidation even in post-acquisition stages. In this regard, amygdala post-acquisition activity seems to be required for CTA consolidation since the reversible inactivation of this structure by tetrodotoxin (TTX, a voltage-sensitive sodium channel blocker) 15 min and up to 1.5 h after CS–US pairing attenuates CTA memory, such attenuation is inversely proportional to the time interval between the acquisition and the intra-amygdalar injection of TTX (Roldan and Bures, 1994).

The amygdala functional integrity is also required for aforementioned post-acquisition neurochemical changes seen in the IC related to previous CS–US association; thus reversible post-acquisition blockade of the amygdala with bilateral TTX infusion hindered post-acquisition glutamate and dopamine increments in the IC and impaired CTA consolidation (Guzman-Ramos et al., 2010). Accordingly, these results indicate that amygdala activation is associated to IC post-acquisition activity. In addition, it would be possible that the amygdala needs to be re-activated after the acquisition of the task to outline the memory trace, which is consistent with the evidence that post-training brain activity is related to previous learning experience (Peigneux et al., 2006; Eschenko and Sara, 2008; Lansink et al., 2008; Marrone et al., 2008). Particularly in the amygdala, single unit recordings of spontaneous activity revealed that the firing rate of BLA neurons increased gradually after inhibitory avoidance training (tone paired with footshock), peaking at 30–50 min post-shock (Pelletier et al., 2005), a time frame that goes in accordance with the neurochemical reactivation herein described and with the effect of amygdalar post-acquisition manipulations that enhance memory. There is ample literature showing that emotionally arousing experiences have been related to increase in stress hormones such as glucocorticoids, exerting their central effect in the amygdala through the activation of β-adrenergic receptors (Ferry et al., 1999; Ferry and McGaugh, 2000; Roozendaal, 2000; McGaugh and Roozendaal, 2002). In this regard, CTA memory retention is enhanced after post-acquisition administration of corticosterone into the BLA and the IC (Miranda et al., 2008). Taken together, these results support the idea that keeping emotional experience in the long-term requires amygdala activity not only during the acquisition period through stimuli signaling, but through post-learning stages.

The post-acquisition engagement of the amygdalar activity has been related to the idea of spontaneous oscillatory activity in this structure that is generated by emotionally arousing conditions. Thus, neuronal recordings in freely moving animals have revealed that during these kind of experiences the firing rate of the BLA neurons increases and it is synchronized trough a theta frequency (4–7 Hz; Pelletier and Pare, 2004). Since theta activity dominates

during the learning period (Pare and Collins, 2000; Seidenbecher et al., 2003) the main consequence of the amygdalar oscillations is to produce temporal windows of neuronal discharging that facilitates the interaction among the structures that synchronized during the acquisition period (Pare et al., 2002), as could be the case of IC and AMY during CTA. This way, theta frequency activity would enhance the depolarization of afferent structures generating a neurochemical reactivation and promoting synaptic plasticity. For instance, it has been reported that CTA training produces an increment in BLA–IC functional connectivity seen as an increased correlation in the activity of simultaneously recorded neurons of these structures (Grossman et al., 2008). Therefore, plastic changes underlying memory trace consolidation, may need reactivation of particular biochemical pathways to sustain the levels of proteins that are required for the consolidation of memory (Wang et al., 2006).

POST-TRAINING MOLECULAR CHANGES INVOLVED IN MEMORY CONSOLIDATION

In order to consolidate a memory trace the activation of several intracellular pathways must be triggered modulating protein synthesis and synaptic plasticity. For instance, the activation of the extracellular responsive kinase 1–2 (ERK1–2) in the IC is required for long- but not STM of CTA (Berman et al., 1998). However the implications of this kinase in post-acquisition stages of this task have not been assessed. Interestingly, a fear conditioning task induces two waves of ERK1–2 activation in the lateral amygdala and the BLA, the fist 60 min after conditioning and the second one about 6 h post-acquisition (Trifilieff et al., 2006). Similarly, a 60-min increase in ERK1–2 activity after paired presentation of tone and shock has been reported, and this effect is absent with the presentation of the CS or the US alone, or an unpaired tone-shock presentation (Schafe et al., 1999); which implies that the learning experience generates delayed reactivations that could be involved in the consolidation process. In a similar way, PKA activity is required for long-term stabilization of CTA memory, the inhibition of PKA in the IC during the post-acquisition stage impairs long- but not STM (Guzman-Ramos et al., 2010), in accordance with the dopamine and glutamate reactivations that are shown in this structure. Hence, dopaminergic modulation may facilitate the reactivation of PKA in aversive associative tasks, for instance the blockade of D1 receptors in the hippocampus after 3 or 6 h impair one-step inhibitory avoidance consolidation, and that the inhibition of PKA on the same temporal patterns renders the same effect (Bernabeu et al., 1997). As mentioned, the activation of kinases has a modulatory effect on protein synthesis and particularly expression of IEG like c-fos. In this regard, there is evidence of IEG expression after post-acquisition reactivation related to previous learning; that is, rats trained in the odor discrimination task had more c-fos expression than unpaired control rats in their prelimbic cortex, ventrolateral orbital cortex, and BLA (Tronel and Sara, 2002). Similarly, in a one-trial learning paradigm in which mice learned to enter a dark compartment to escape from an aversively illuminated area showed more Arg 3.1/Arc mRNA expression 15 min and 4.5 h post-training detected specifically in the learning group when compared to the control or the retrieval groups (Montag-Sallaz and Montag, 2003).

This kind of monitoring of protein expression after learning should provide patterns about the timeline of the required neuronal changes that underlie memory stabilization in the long-term. In this regard, CTA consolidation and some related protein expression waves has been reported, the IEG HZF-3 increases in the BLA at 1 and 3 h after CTA conditioning and this up-regulation is not present with the presentation of the flavor or the malaise induction only, supporting the idea of a specific role in the associative learning (Ge et al., 2003). Recent reports showed an interesting temporal dichotomy in the expression of BDNF in the IC and nuclei of the amygdala. Thus, from 2 to 6 h after CTA conditioning there was a significant BDNF increase within the CeA, and from 4 to 6 h an increase was observed in the IC and in the BLA, these up-regulations were related to the association of the stimuli, since the CS or the US alone and a delayed paring of CS–US did not induce BDNF increases (Ma et al., 2011).

CONCLUSION

As we have seen, many of the post-acquisition molecular changes in the amygdala or in the IC overlap shortly after conditioning, form 45 min to 1 h, and other waves of activity in at least 6 h, suggesting that there is a time frame where neurochemical changes trigger receptors and kinases activation leading to increased expression of proteins required for memory consolidation. Whether such reactivations are occurring repeatedly is still unclear, but several reports have indicated reactivation activity within a time frame that goes in accordance with cellular consolidation theory (Dudai, 2004). Thus, the neuronal changes caused by up-regulation of protein synthesis within the learning-engaged structures may occur within few hours or even days, as the Cui et al. (2005) have suggested. Such protein synthesis induction appears to be related to spontaneous activity after the exposure to the information (i.e., CS–US association). These kinds of mechanisms involved in the progressive stabilization of the information may be related to the salience of such information, or what we have been calling "emotional memories," which may be of life-saving importance for the animal. Such is the case of CTA learning where a specific flavor may be toxic and could have deadly consequences in the future. The relevance of the information may induce mechanisms that reinforce the memory trace in an efficient way to prompt retrieval and adequate behavioral change. We propose that this could be achieved by post-acquisition reactivation signals during post-training wakefulness. As mentioned, simultaneous neural recordings in the macaque neocortex, revealed that cells in all four areas exhibited firing related to the task (sequential reaching behavior), and those cells tended to be coactive afterward (Hoffman and McNaughton, 2002). Another example is the sequential replay in hippocampal place cells, where population activity in the hippocampus was recorded while rats ran back and forth on a linear track for a water reward at each end. During the run, each neuron's firing was tuned to a particular location along the track, which was stable from lap to lap. These locations define a temporal sequence of place-cell firing on the timescale of seconds. During awake period immediately after the spatial task, the same neurons fired again on the timescale of hundreds of milliseconds, but in the reverse temporal order, which may serve to propagate information from the rewarded location backward along incoming

trajectories (Foster and Wilson, 2006; Diba and Buzsaki, 2007). It is suggested that such replay might constitute a general mechanism of learning and memory because this is more readily observable in a new environment than a familiar one (Foster and Wilson, 2006). We propose that in CTA memory the post-acquisition activity involves amygdalar spontaneous reactivation that triggers neuronal changes within the IC since it can engage into oscillatory activity related to emotional learning promoting the facilitation of the neuronal interactions strengthening the memory trace.

ACKNOWLEDGMENTS

Supported by CONACYT 060478 and DEGAPA-UNAM IN216709-3, and the Department of Psychology Texas A&M University.

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

Received: 27 July 2011; accepted: 31 August 2011; published online: 26 September 2011.

Citation: Guzmán-Ramos K and Bermúdez-Rattoni F (2011) Post-learning molecular reactivation underlies taste memory consolidation. Front. Syst. Neurosci. ***5****:79. doi: 10.3389/fnsys.2011.00079*

frontiers in
SYSTEMS NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 01 December 2011
doi: 10.3389/fnsys.2011.00097

Genetically induced cholinergic hyper-innervation enhances taste learning

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Acute inhibition of acetylcholine (ACh) has been shown to impair many forms of simple learning, and notably conditioned taste aversion (CTA). The most adhered-to theory that has emerged as a result of this work – that ACh increases a taste's perceived novelty, and thereby its associability – would be further strengthened by evidence showing that enhanced cholinergic function improves learning above normal levels. Experimental testing of this corollary hypothesis has been limited, however, by side-effects of pharmacological ACh agonism and by the absence of a model that achieves long-term increases in cholinergic signaling. Here, we present this further test of the ACh hypothesis, making use of mice lacking the p75 pan-neurotrophin receptor gene, which show a resultant over-abundance of cholinergic neurons in sub-regions of the basal forebrain (BF). We first demonstrate that the p75−/− abnormality directly affects portions of the CTA circuit, locating mouse gustatory cortex (GC) using a functional assay and then using immunohistochemisty to demonstrate cholinergic hyper-innervation of GC in the mutant mice – hyper-innervation that is unaccompanied by changes in cell numbers or compensatory changes in muscarinic receptor densities. We then demonstrate that both p75−/− and wild-type (WT) mice learn robust CTAs, which extinguish more slowly in the mutants. Further testing to distinguish effects on learning from alterations in memory retention demonstrate that p75−/− mice do in fact learn stronger CTAs than WT mice. These data provide novel evidence for the hypothesis linking ACh and taste learning.

Keywords: p75 knockout mouse, cholinergic system, conditioned taste aversion, taste learning

INTRODUCTION

The cholinergic system is implicated in the performance of many survival behaviors, notably including feeding (Bermudez-Rattoni, 2004). Perturbations of the basal forebrain (BF), the primary source of cortical and limbic system acetylcholine (ACh, Hecker and Mesulam, 1994; Semba, 2004), impair both expression of naïve preferences for one taste over another (Pratt et al., 2007), and the learning of new preferences in paradigms such as conditioned taste aversion (CTA, whereby animals learn to dislike/avoid tastes associated with gastric distress, Gutierrez et al., 1999b; Gonzalez et al., 2000; Semba, 2000; Bermudez-Rattoni, 2004). Similarly, pharmacological antagonism of muscarinic cholinergic synapses within parts of the taste system that receive input from sub-regions of BF, including gustatory (insular) cortex (GC) and basolateral amygdala (BLA), also hinder CTA learning (Naor and Dudai, 1996; GC, Berman et al., 2000; Gutierrez et al., 2003).

This work, and microdialysis studies suggesting that presentation of a new taste causes release of ACh in GC (Miranda et al., 2000), form the basis of a powerful theory implicating ACh as the signal for taste novelty (Miranda et al., 2000; Ranganath and Rainer, 2003; Jeewajee et al., 2008; Nunez-Jaramillo et al., 2008), and thus as a vital part of strong CTA (Bermudez-Rattoni, 2004). These studies would be much strengthened, however, by the complimentary data, showing that enhancement of cholinergic activity in the taste system improves taste learning. At least one study has in fact suggested that cholinergic agonism allows learning to otherwise ineffective stimuli (Clark and Bernstein, 2009), but related data concerning normal learning are difficult to collect and interpret, both because the reduction of consumption typically used to measure CTA suffers from a floor effect (making enhanced learning difficult to detect) and also because cholinergic agonism can result in seizures and profoundly disrupt pathways that may be unrelated to CTA (Olney et al., 1983; Naor and Dudai, 1996). What would be highly useful in this regard is a model organism with chronic, non-traumatic elevations of cholinergic function; while developmental cholinergic manipulation would, like all other methods of cholinergic manipulation, have the potential to cause secondary effects in other systems, it would be simple in this model organism to test the corollary hypothesis of the ACh theory, namely that increases in cholinergic function improve taste learning.

Perturbed function via non-traumatic developmental processes (i.e., in which neurons are not suddenly removed from a system accustomed to their presence) can be achieved in mice by genetic

manipulation of gene expression. One such mouse model provides a particularly useful phenotype for studies of cholinergic function: the p75 knockout mouse contains a targeted deletion of the p75 low affinity, pan-neurotrophin receptor gene, which, in the normal adult brain, is selectively expressed in BF cholinergic neurons (Hartikka and Hefti, 1988). Although the loss of this receptor has a range of effects, the most notable of these is increased numbers of cholinergic neurons (and decreased numbers of GABAergic neurons) in BF (Van der Zee et al., 1996; Naumann et al., 2002; Lin et al., 2007). If this increase in cholinergic neuron number in fact results in a taste system that is "hyper-cholinergic," then the cholinergic theory of taste novelty would predict that p75−/− mice should condition more strongly than normal mice.

Here, we performed this test, first by functionally defining the GC in mice and demonstrating that the p75−/− phenotype results in reliable cholinergic hyper-innervation of this region, and then by showing significantly supra-normal learning in the mutants. Our examinations of these mice therefore provide novel evidence for the hypothesis that ACh controls taste associability.

MATERIALS AND METHODS

SUBJECTS

Adult male and female wild-type (WT; 57BL/6J) and strain-matched p75−/− mice (Lee et al., 1992) from the Jackson Laboratory (Bar Harbor, ME, USA) served as subjects in this study. All animals were maintained on a 12 h light/12 h dark cycle. Animals were housed individually with free access to standard food pellet and maintained on a 23.5 h water deprivation schedule for the duration of training and experimentation. All the behavioral experiments were carried out between 12:00 and 4:00 pm.

CORTICAL CANNULATION

Adult mice were initially anesthetized via intraperitoneal (ip) injections of a ketamine/xylazine cocktail (ketamine, 100 mg/kg; xylazine, 10 mg/kg) and placed in a stereotaxic frame. General anesthesia throughout the surgery was maintained using isoflurane inhalant (0.5% isoflurane at 0.5 l/min oxygen). A scalp midline incision was made, after which the scalp was retracted and the skull leveled. Two small holes were drilled in the skull so that guide cannulae (23-gage, 10 mm in length) could be lowered into putative GC under stereotaxic guidance (AP: +1.48 mm and ML ± 3.10 mm relative to bregma; DV −1.8 mm from the surface of the brain). Cannulae were stabilized using Vetbone and dental acrylic. Stainless steel stylets (30-gage, 10 mm in length) were inserted into the guide cannulae to ensure patency.

MUSCIMOL DOSE RESPONSE

Mice were allowed to recover for a minimum of 7 days following surgery. After recovery, mice were maintained on a 30 min/day water restriction protocol for 6 days in the home cage, to ensure stable intake of water. The last three of these days, water intake (g) from the cage lick-spout was measured, and the average water intake for each mouse was calculated.

On test day, mice received infusions of one of five different doses of muscimol (MP Biomedicals, LLC, Ohio 1, 0.75, 0.5, 0.25, and 0 μg/μl, diluted in 0.3 μl saline). Fifteen minutes after the infusion, mice were given 30 min access to water through a lick-spout in their home cages. The effect of muscimol on the water intake of each mouse was then quantified [infusion day (g)/average water intake (g) × 100]. Motor behavior and water consumption were compared with mice that received saline infusion.

Our results show 0.5 μg/μl muscimol to be the smallest concentration that resulted in a significant change in the water intake of the mice [$t(7) = 2.644$, $p = 0.033$, two-tailed]. In accordance with these results, we used 0.5 μg/μl muscimol in 0.3 μl saline for identifying GC in the CTA protocol.

DRUG DELIVERY

Mice were secured in experimenter's hand and infusion cannulae, connected via polyethylene tubes to 10 μl Hamilton syringes in an infusion pump (Harvard Apparatus, Massachusetts, MA, USA), were inserted to ∼0.2 mm beyond the bottom of the previously implanted guide cannulae. Muscimol (dosing determined as described above) was bilaterally infused into the GC at a rate of 0.15 μl/min for 2 min (for a total of 0.3 μl). Infusion cannulae were left in place for an additional minute to allow for diffusion from the tip of the injecture (Stone et al., 2005), after which they were removed slowly, to ensure that negative pressure did not suck infusate up into the guide cannula.

IDENTIFYING GUSTATORY CORTEX

Adult mice were adapted to water restriction as described above. On the training day, mice received intra-cranial infusions of either 0.5 μg/μl muscimol in 0.3 μl saline or saline alone, and were returned to their home cages. Fifteen minutes later, mice were given 30 min of *ad lib* access to a novel palatable solution (100 mM NaCl). Immediately afterward, they were given intraperitoneal injections of LiCl (0.15 M, 2% body weight), which induced gastric malaise. All animals received 15 min access to water 2 h after the termination of the training session unless indicated otherwise; by this time, mice were observed to be drinking normally, which led us to conclude that there was no need to interpolate a rest day between training and test: thus, 24 h after training, mice were once again given 30 min of *ad lib* access to 100 mM NaCl in a testing session. Because basal consumption was highly variable, and because p75−/− mice as a group drank slightly but significantly more than WT mice (see **Table 1**), the acquisition of CTA was quantified in terms of a normalized comparison between NaCl solution intake in the training and testing sessions [(NaCl intake on the testing day/NaCl intake on the infusion day) × 100]. Subsequent testing (see below) demonstrated that the observed differences in basal consumption had by themselves little impact on learning.

BETWEEN-STRAIN COMPARISON OF CONDITIONED TASTE AVERSION

Mature adult WT and p75−/− mice (43 mice for high-LiCl and 48 mice for low-LiCl experiments) were adapted to the water deprivation protocol for 6 days and then given the CTA protocol as described above; **Table 1** provides more details on the groups of adult mice (genders and strains) used in these experiments. No muscimol was administered in these experiments, and 10 mM saccharin was used instead of NaCl. For the "low-LiCl" experiments, the concentration of intraperitoneally administered injection of LiCl was reduced to 1% of body weight. To more completely characterize the induced aversions, testing sessions were

Table 1 | Breakdown of groups used in behavioral tests.

Genotype	Gender	Number	Average intake (g)	Std. error mean
LOW-LICL				
p75 KO	Female	13	1.806	0.10772
p75 KO	Male	8	1.981	0.09082
Total		21	1.873	0.07597
WT	Female	17	1.369	0.08021
WT	Male	10	1.539	0.12271
Total		27	1.432	0.06836
Total		48	1.625	0.05945
HIGH-LICL				
p75 KO	Female	19	1.952	0.09463
p75 KO	Male	3	1.779	0.015212
Total		22	1.928	0.08424
WT	Female	1	0.936	N/A
WT	Male	20	1.454	0.07284
Total		21	1.429	0.07355
Total		43	1.684	0.0675

repeated for 5 more days (six testing sessions in all); across this period, the induced aversion gradually faded, allowing evaluation of extinction of learning.

HISTOLOGY

Mice were anesthetized with isoflurane followed by an injection of a ketamine/xylazine/acepromazine cocktail. After deep anesthesia was achieved, the mice were perfused with ice-cold saline followed by ice-cold 80–100 ml of 4% paraformaldehyde (PFA), 0.1 M phosphate buffer, pH 7.4. Brains were rapidly removed, post-fixed overnight at 4˚C in PFA alone, and maintained in 30% sucrose at 4˚C until sectioning for cannula placement, ChAT staining, or NeuN staining.

Identification of gustatory cortex

A subset of mice implanted with cannulae received fluorescent muscimol (0.5 μg/μl, BODIPY, TMR-X conjugate, Invitrogen, CA, USA delivered through the method described above) prior to perfusion, to visualize the diffusion of muscimol. From these animals, 100 μm coronal slices were cut starting either at the corpus callosum intersection or at the first appearance of cannulae tracks (whichever was more anterior). PBS-soaked sections were imaged immediately after slicing, through the 4× objective on an Olympus IX-81 inverted fluorescence microscope (Allen et al., 2008). The image of the whole coronal brain section was captured using an Orca-ER digital CCD camera (Hamamatsu, Japan) and Volocity software (PerkinElmer, Waltham, MA, USA). Images were then overlaid on figures from a mouse brain atlas (Paxinos and Franklin, 2001) using Adobe Illustrator CS3. This allowed for identification of mouse GC, based on the location of effective muscimol infusion sites and of effective spread of the infused muscimol.

The diffusion of effective infusions of fluorescent muscimol (i.e., those that blocked CTA) was estimated (and plotted on a lateral view of the mouse brain) to be around the diameter of the cannulae (635 μm). Diffusion was roughly elliptical, due to the presence of the cannulae themselves, averaging 635 μm in the horizontal axis and 228 μm dorsal–ventral axis.

ChAT and NeuN staining

Twelve consecutive coronal slices (40 μm thick) through GC, beginning where the corpus callosum first intersects anteriorly (corresponding to +1.10 relative to bregma) were cut on a vibratome and collected in PBS. Every third slice was used for the following immunostaining procedure. Fifteen consecutive coronal slices (40 μm thick) through gustatory thalamus, beginning where the corpus callosum first intersects caudally (corresponding to −2.46 relative to bregma) were cut on a vibratome and collected in PBS. Starting from the fifth slice (corresponding to −2.30 relative to bregma), every third slice was used for the following immunostaining procedure.

Sections were gently agitated for 30 min at room temperature in a preblock solution containing 0.1% NP-40 with 10% donkey serum in PBS, and incubated overnight in primary antibody solution diluted in the preblock solution. The primary antibodies used were mouse anti-neuron-specific protein NeuN (1:1000; Chemicon, Temecula) for the identification and quantification of the neuronal somas and goat anti-choline acetyltransferase (ChAT, 1:1000; Chemicon) for detecting cholinergic cell bodies and fibers. The sections were also stained with a rabbit anti-human p75 neurotrophin receptor (p75, 1:1000; Promega, Madison, WI, USA) to confirm that the p75−/− mice did not express this receptor.

After being washed three times in PBS (20 min each), the sections were incubated in secondary antibodies diluted in preblock solution (Rhodamine-, Cy5-, and FITC-conjugated secondary antibodies respectively; Jackson ImmunoResearch, West Grove, PA, USA) for 3 h. After another three washes, the sections were mounted on Superfrost Plus (Fisher Scientific) slides in *n*-propyl gallate. The sections were kept at 4˚C in the dark until imaged.

Whole brain slices were imaged using the 4× objective on an OlympusIX-81 inverted fluorescence microscope fitted with fluorescein, rhodamine, and Cy5 filters. Images for each whole coronal brain section were captured using an Orca-ER CCD digital camera (Hamamatsu, Japan) and Volocity software (Improvision, Lexington, KY, USA). Slice locations were matched to corresponding images from the mouse atlas (Paxinos and Franklin, 2001).

Uncompressed 16-bit gray-scale images were made of NeuN- and ChAT-stained slices at 2 μm intervals using an automated focus drive. Imaging was done at 20× magnification resulting in images of 436.20 μm × 332.35 μm regions of GC, and 872.4 μm × 332.35 μm regions of gustatory thalamus. Images were exported to ImageJ (NIH, USA) for analysis. From each series of 20 images in a stack, the six images were used to z-project the stack into a single image. Using the Z-projection through the maximum intensity values for each image stack ensured that all the fibers and cell somas were present in one image, allowing analysis of the total number of cells, and of fiber density and length, averaged across slices and across mice.

ChAT density analysis

Images were first converted to 8-bit in ImageJ and the Feature J software plug-in was used to detect axons using Hessian-based

matrices (Grider et al., 2006). The resulting eigen-image was converted into a binary image by thresholding using the Isodata algorithm implemented in Image J. The calculated threshold was adjusted as needed (in increments of not more than 10 pixels) to optimally detect all axons with high background signals. The fraction of the thresholded image covered with the fibers was calculated using the Analyze Particles tool. The thresholded images were then skeletonized (i.e., the width of each fiber was reduced to a single pixel) and axonal length was re-quantified in isolation from potential artifacts of staining intensity.

NeuN staining

For automated counting of neuron number, the watershed algorithm was implemented on thresholded images and particle analysis were carried out using the analyze particles tool. Only particles bigger than 150 square pixels were included in the automated count. Manual counting analysis of cells was performed on a subset of the data, using the cell counter plug-in on the original image. The cell counts obtained manually and with the automated method from 12 different images were found to be closely correlated. Therefore, the automated counting was deemed satisfactory, and carried out on the other images.

QUANTITATIVE REAL-TIME PCR

RNA was prepared from GC of WT and p75−/− mice using the RNeasy mini kit (Qiagen, Valencia, CA, USA). RNA was reverse transcribed into cDNA using MMLV-reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers [46]. Real-time PCR was performed in triplicate for each sample using a Rotor-Gene 3000 (Qiagen, Valencia, CA, USA). Primer sets were designed using BLAST (NIH, USA). Primer sets used in this study were:

GAPDH f 5′-AACT TTT GGC ATT GTG GAA GG-3′, GAPDH-r 5′-GCA TGC AGG GAT GAT CT-3′, M1f 5′-CAT GGA GTC CCT CAC ATC CT-3′, M1-r 5′-TGT ATT TGGT GGA GCT TTT GG-3′, M2-f 5′-TAC CCA GTT AAG CGG ACC AC-3′, M2-r 5′-CCC GTC TTC CAC AGT CCT TA-3′, M4-f 5′-ATC GAG ATC GTA CCT GCC AC-3′, M4-r 5′-AAT GGC AAA GAT TGT CCG AG-3′.

The two ΔΔCT method was used for real-time PCR analysis (Livak and Schmittgen, 2001). For each primer set, PCR reactions were run in triplicate and normalized to the average of triplicate reactions run with glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

STATISTICS

Data were analyzed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Mean values, standard errors, independent *t*-test, ANOVAs, and Pearson Correlations were calculated to compare the WT mice with the p75−/− mice.

RESULTS

IDENTIFICATION OF PRIMARY GUSTATORY CORTEX IN THE MOUSE

We first investigated whether the p75−/− mouse was an appropriate model in which to explore the impact of long-term, developmentally based cholinergic hyper-innervation on taste learning by examining whether there was increased innervation of CTA-relevant regions. Researchers have reported an increased number of cholinergic neurons in the BFs of p75−/− mice (Van der Zee et al., 1996; Naumann et al., 2002; Lin et al., 2007). This expansion of the number of cholinergic neurons appears to lead to increased cholinergic innervation in some, but not all sub-regions of forebrain structures such as the hippocampus (Yeo et al., 1997), but the regions projecting to insular cortex (where GC resides) have not been examined, nor has it been conclusively shown that increases in BF neurons result in cortical hyper-innervation. We therefore first set out to examine GC in the p75−/− mouse.

While gustatory sub-regions within insular cortex have been described using both structural and functional techniques in the rat (Yamamoto, 2006), the equivalent areas in the mouse have not been conclusively identified (Tokita et al., 2009). To isolate the appropriate region, we made use of the fact that GC is known to be vital for CTA learning (Yamamoto et al., 1995; Naor and Dudai, 1996; Yasoshima and Yamamoto, 1997; Berman and Dudai, 2001; Grossman et al., 2008; Bertrand et al., 2009), while the regions surrounding GC are not known to have any involvement in CTA. We were able to use a functional assay to pinpoint the taste-relevant part of insular cortex, identifying it as that part in which CTA was inhibited by localized infusions of the GABA-a agonist muscimol.

Intra-cranial cannulae were inserted bilaterally into presumptive GC (identified on the basis of anatomical landmarks) of WT mice in stereotaxic survival surgeries (see Materials and Methods; **Figure 1**). After at least 7 days of recovery, the mice were introduced to the behavioral protocol, wherein we tested their ability to develop conditioned aversions to taste stimuli when spiking of neurons in putative GC was silenced by infusions of muscimol (Krupa et al., 1998). First, a pilot group was used to ascertain a safe dose of muscimol – one that only minimally interfered with normal feeding behavior. Mice received bilateral infusions of 0, 0.25, 0.5, 0.75, or 1.0 μg/μl muscimol in 0.3 μl saline vehicle 15 min before being given access to a water-filled lick-spout for 30 min. The highest of these concentrations strongly inhibited water consumption, consistent with non-specific effects of the blocker on gustatory behaviors (data not shown). Lower concentrations had minimal effects on water consumption, however, and so we carried out CTA experiments using 0.50 μg/μl muscimol infusions. This, the lowest concentration that had any effect on water consumption in our pilot experiments [$t(7) = 2.644$, $p = 0.033$], has been shown to effectively block action potential generation *in vivo* in the region surrounding the infusion cannula (Krupa et al., 1998).

We tested the acquisition of CTA in a naïve group of cannulated mice. These mice received 0.3 μl of 0.5 μg/μl muscimol while control mice received infusions of vehicle. Mice then consumed 100 mM NaCl out of a lick-spout *ad lib* for 30 min, and their consumption was measured in grams. The drinking session was immediately followed by an ip injection of the emetic LiCl, after which mice were returned to their home cages for the night. The following day the mice were offered 30 min of access to the same taste. **Figure 2A** shows the result of this experiment: control mice developed substantial aversions to the emesis-paired taste, measured as a decrease in consumption between the training and testing sessions. Muscimol infusions completely blocked this decrement in consumption [$t(12) = 2.24$, $p = 0.045$, two-tailed *t*-test]; in fact, consumption of NaCl increased between training and testing for this group – a result that is consistent with what

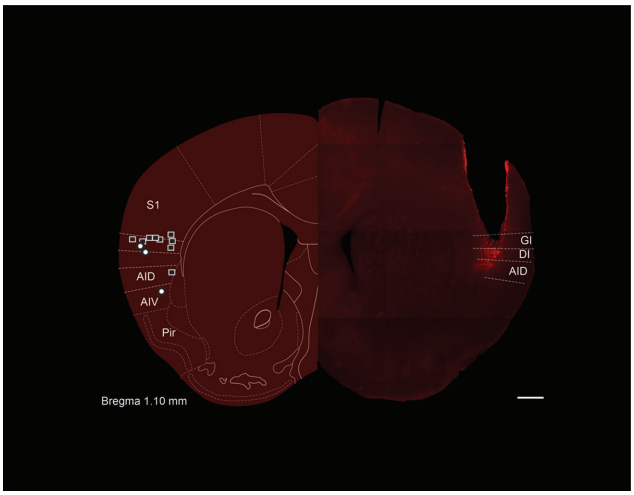

FIGURE 1 | Gustatory cortex in the mouse. The left hemisphere of this figure is a schematic diagram of the likely coronal plane of mouse (reprinted with permission from Paxinos and Franklin, 2001) gustatory cortex (GC), selected for its homology to the known location of rat GC (Katz et al., 2001). The super-imposed squares and circles show the location of cannula tips for muscimol and control mice, respectively, in the GC localization experiments – most tips are found in granular (GI) or dysgranular (DI) insular cortex; also noted are dorsal and ventral agranular insular (AID and AIV) as well as piriform cortex (Pir). The right hemisphere shows a photomicrograph from one mouse subject (with the approximate demarcations of granular, dysgranular, and agranular cortex noted). The cannula track is readily visible, as is the localized spread of fluorescent muscimol infused through that cannula just before perfusion. Scale bar = 500 μm.

is known about cessation of neophobia with experience (Domjan and Gillan, 1976). Given the anatomical (Fortis-Santiago et al., 2010) and functional evidence (Wang et al., 2006), as well as our direct imaging evidence (using fluorescent muscimol, see **Figure 1**), all of which suggests the spread of such muscimol infusions is highly circumscribed, we conclude that our implantation coordinates correctly localize mouse GC. This conclusion received further support from control experiments demonstrating that infusion of muscimol in mice in which the cannulae were placed outside of IC had no effect on acquisition of CTA ($p > 0.1$). **Figure 2B** shows a parasaggital view of the most effective muscimol infusion sites, along with estimations of muscimol spread based on fluorescence measurements (see Materials and Methods); our localization of mouse GC is essentially identical to that region identified on the basis of tracer injections into taste thalamus (Chen et al., 2011).

CHOLINERGIC HYPER-INNERVATION OF IC IN THE P75−/− MOUSE

We next examined cholinergic innervation of identified GC in p75−/− and WT mice. Cholinergic fiber analysis was carried out in 40 μm coronal slices made through GC. Choline acetyltransferase (ChAT) density was calculated as the fraction of the image covered with fibers. Data were averaged across slices and across mice. This analysis of ChAT density revealed a significantly heavier cholinergic innervation of GC in p75−/− mice compared to WT controls [$t(9) = 3.988$, $p = 0.003$; two-tailed; **Figure 3A**].

A concern with such analyzes is that they may fail to distinguish between true increases in number/length of fibers and increased intensity of staining of individual fibers. We therefore re-analyzed the slices to evaluate the length of ChAT-positive fibers on skeletonized images using ImageJ software (NIH, USA). Such pre-processing eliminates information relating to intensity. These fiber-length measurements confirmed the results of the previous analysis, showing a significant increase in cholinergic innervation in the GC of p75−/− mice compared to WT controls [$t(9) = 3.648$, $p = 0.005$; two-tailed; **Figure 3B**].

Loss of p75 expression did not have gross secondary effects on GC in these mice beyond cholinergic hyper-innervation itself. Despite the increase in both BF cholinergic neuron number and cholinergic innervation of the IC, we found no change in the total number of cortical neurons in the GC of adult p75−/− mice compared to WT control animals. Coronal slices used for the fiber-length analysis were co-labeled with the NeuN antibody to identify all neuronal cell bodies in the slice. The number of neurons per section was counted using the watershed algorithm and the analyze particles tools in ImageJ (**Figure 4A**). There was no difference in total cortical neuron number between regionally matched slices taken from p75−/− and WT animals [$t(8) < 1$].

We examined the specificity of p75-dependent cholinergic increases in GC by comparing these data to measurements of cholinergic fiber length in the gustatory thalamus. The parvicellular division of the ventroposterior medial thalamic nucleus conveys gustatory information and contains extensive cholinergic innervation. However, this innervation is predominantly derived from the brain stem (notably the pedunculopontine tegmental nuclei, Parent and Descarries, 2008) rather than from the BF. We therefore predicted that thalamic innervation would be normal in p75−/− mice. In line with this expectation, and in contrast to the cortical innervation pattern, we found no difference in ChAT staining in the gustatory thalamic nuclei of p75−/− mice [$t(2) = 1.39$, $p = 0.30$, **Figure 4B**].

Furthermore, we found only limited evidence that the increased cholinergic innervation of GC causes compensatory changes in muscarinic receptors. We examined mRNA expression of the major CNS muscarinic receptors – M1, M2, and M4 – in RNA isolated from the GC of WT and p75−/− mice using RT-PCR. We found no change in mRNA levels of the M1 and M4 receptors (**Figure 5**), but we did observe a significant decrease in signal for M2 receptor mRNA: M1, $t(11) = 0.11$, $p = 0.915$; M2 $t(11) = 2.598$, $p = 0.025$, M4 $t(11) = 0.074$, $p = 0.404$.

THE IMPACT OF CHOLINERGIC HYPER-INNERVATION ON TASTE LEARNING

Based on the above data, and the literature linking reductions of cholinergic function to learning impairments, we hypothesized that p75−/− mice should form stronger memories than WT mice on a standard taste learning task. Our evidence reveals that this is in fact the case.

To test this prediction, we returned to the CTA paradigm. Groups of p75−/− and WT mice were adapted to the testing chamber, given a single training session involving a pairing of 10 mM saccharin with ip injections of 0.15 M LiCl (2% body weight), and tested for their post-training consumption of the conditioned

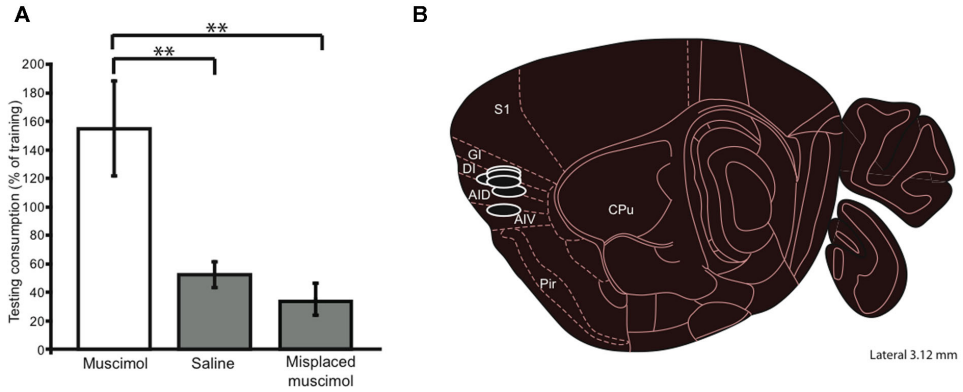

FIGURE 2 | Functional test of mouse GC localization. (A) A muscimol concentration that had relatively little impact on *ad lib* consumption (i.e., 0.50 μg/μl), infused into putative GC just before a CTA training session (a single pairing of orally administered NaCl and ip injected LiCl), inhibited taste learning – these mice did not reduce their NaCl consumption in the testing session (*y*-axis). Mice receiving control (saline) infusions into putative GC learned normally, consuming much less NaCl after training, as did controls receiving muscimol infusions into non-GC sites. Thus we can conclude that the infusion cannulae have correctly targeted mouse GC. **(B)** In a parasagittal view, the locations, and approximate spreads, of muscimol infusions that were most effective at blocking CTA. Error bars, here and in every figure, represent the standard error of the mean, $** = p < 0.01$; see text for further details. S1, somatosensory cortex; GI, DI, AID, AIV, gustatory insular cortex (granular, dysgranular, agranular dorsal and ventral); Pir, piriform cortex; CPU, caudate putamen.

taste. Consumption was followed across several post-training days, so that the speed with which behavior returned to baseline (i.e., extinction of learning) could also be assessed. **Figure 6A** shows the result of this experiment, with consumption normalized to pre-learning saccharine consumption. Strong initial learning (significant reduction of consumption in testing session compared to baseline), appeared to extinguish more quickly in WT mice than p75 mutants – by extinction session 5, WT mice drank 119% of their naïve (training day) consumption, reflecting the fact that consumption in that initial session was affected by mild neophobia for the novel taste (Domjan and Gillan, 1976), whereas p75−/− mice had re-attained 93% of naïve consumption. A two-way mixed ANOVA of these data revealed, as expected, a significant effect of day [F (5,195) = 44.16, $p < 0.001$] as well as a day × mouse interaction [F (5,195) = 2.83, $p < 0.02$] consistent with the appearance of **Figure 6A**, in which p75−/− and WT mice drank similarly little saccharin in the testing session, and WT mice drank more than p75−/− mice in each of the extinction sessions. Pairwise *post hoc* tests were somewhat equivocal and difficult to interpret, with significant strain differences appearing on extinction days 2 and 5.

It is tempting to conclude that p75 mice learned stronger aversions than WT mice, on the basis of the fact that their learning extinguished more slowly. The failure to observe differences in initial learning (i.e., in testing session 1) could well be explained as a function of a floor effect: CTA is powerful and evolutionarily important, and "normal-strength" learning consists of a near elimination of consumption; increases in that strength should therefore be difficult to detect. In fact, when the ANOVA was repeated without the initial testing session, the session × strain interaction vanished ($F < 1$), demonstrating that the interaction was wholly dependent on the initial testing session, and suggesting that the rate of extinction did not actually differ as a function of cholinergic innervation. The alternative possibility must also be considered, however – the possibility that the p75-deficient and WT mice developed equivalent CTAs, but that p75−/− mice show an "extinction deficit." Acquisition and extinction of learning are known to involve distinct network and sub-cellular mechanisms (Berman and Dudai, 2001), and while extinction has not been specifically linked to cholinergic function, it is possible that cholinergic hyper-innervation of GC targets precisely this process.

To distinguish between these possibilities, we performed a second learning experiment on an additional sample of p75−/− and WT mice. This experiment was identical to the first, but the volume of injected LiCl was cut in half – a change that reduces the intensity of the induced emesis, and thus reduces the strength of the learned aversion (Sakai and Yamamoto, 1997). It was assumed that this reduction would "lift" consumption off of the "floor," allowing any inter-strain differences in initial learning to be more easily observed.

Figure 6B shows the result of this experiment: at the smaller LiCl dose, consumption is now far from the "floor" in all sessions for both strains, and p75 mice can now be seen to learn stronger aversions in the first testing session and all sessions thereafter. While modest, this difference confirms that the effect observed in **Figure 6A** does not reflect a selective difference in extinction learning. A two-way mixed effect ANOVA was performed on the testing session data: a significant main effect of session [F (5,175) = 68.78, $p < 0.001$] revealed that extinction occurred for both types of mice; a significant main effect of strain [F (1,35) = 5.32, $p < 0.03$], meanwhile, reveals that p75−/− mice did indeed learn stronger aversions than WT mice, consuming less fluid across testing sessions. While we observed substantial variability in basal consumption in the p75−/− group, subsequent analysis revealed that these large differences were positively ($r = 0.21$) but insignificantly ($t_z < 1$) related to consumption in the testing session. This suggests that mice that consumed more saccharin during training did not learn stronger CTAs (and thus removes basal consumption differences as a possible confound to the results).

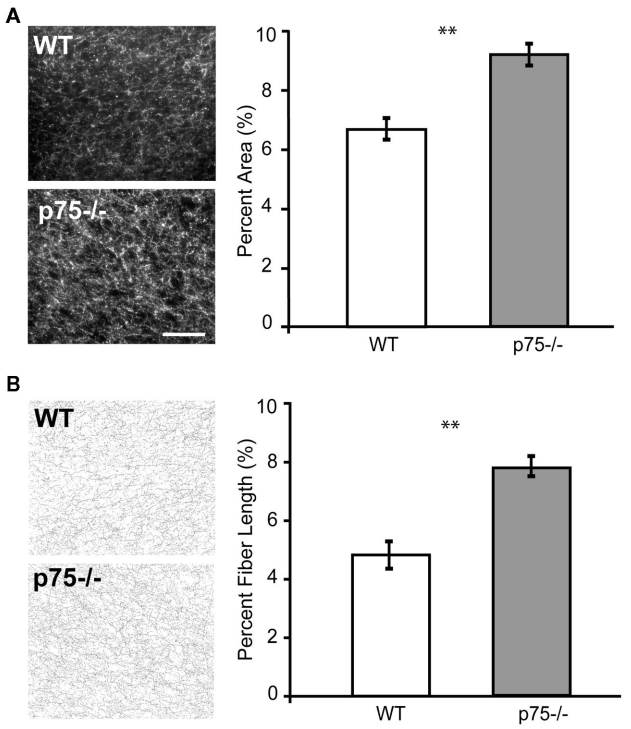

FIGURE 3 | p75−/− mice showed cholinergic hyper-innervation in GC compared to wild-type mice. (A) The two photomicrographs show ChAT-stained sections through GC. More fibers are visible in p75−/− mice than in wild-type. The group data are summarized in the panel to the right, which shows that in the p75−/− mouse, a higher percentage of the space in the images (*y*-axis) was taken up by ChAT-stained fibers than in the wild-type (WT) mouse. **(B)** The same analysis done on skeletonized images (see Materials and Methods for details), which allows us to rule out confounding explanations having to do with the possibility of brighter staining. The group data demonstrates that, when stained fibers are reduced to equi-luminant, equi-thick lines, these cholinergic fibers are longer in p75−/− mice than in wild-type mice. ** = $p < 0.01$, scale bar = 100 μm.

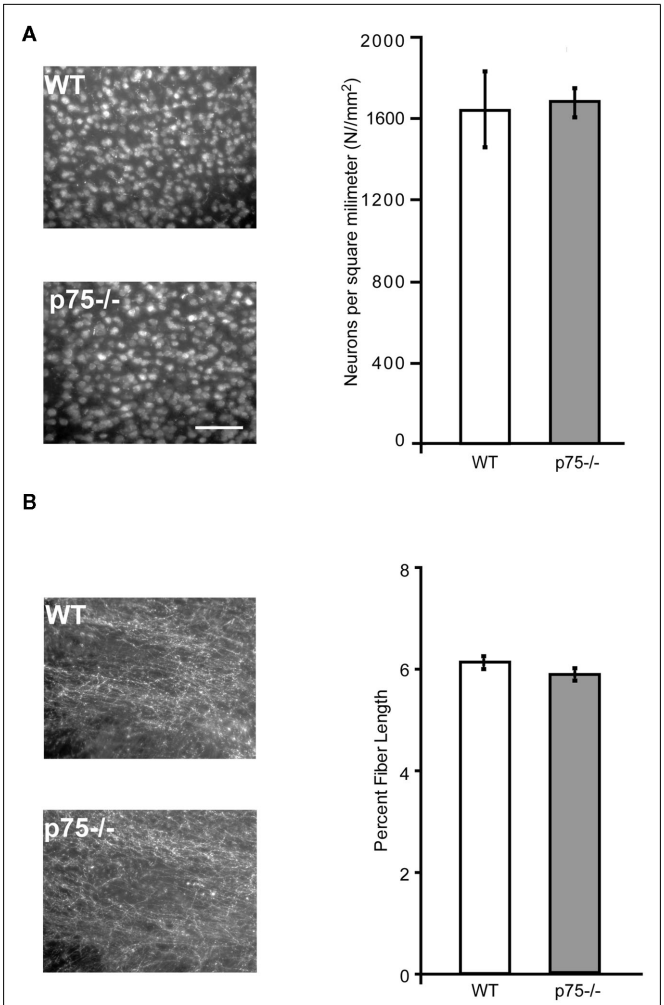

FIGURE 4 | p75−/− and wild-type mice are comparable in cortical neuron number and cholinergic innervation of gustatory thalamus. (A) The photomicrographs show NeuN staining, which labels all neurons in wild-type and p75−/− GC slices. The group data at right demonstrates that the two strains did not differ in the number of neurons in GC, indicating that cholinergic hyper-innervation did not change the number of cortical neurons. **(B)** Like **Figure 3B**, this figure shows ChAT staining of slices harvested from p75−/− and wild-type mice – this time from gustatory thalamus, which receives cholinergic innervation from brainstem rather than basal forebrain. The photomicrographs (left) and group data (right) reveal no major p75/wild-type differences in cholinergic innervation of this important part of the mouse gustatory system. Scale bar = 100 μm.

The utter lack of a strain × session interaction, both in this ANOVA ($F < 1$) and that comparing extinction days 1–5 in the high-LiCl experiment, reveals that the difference between p75−/− and WT mice did not vary significantly between session. While the difference between the groups was small, it was consistent from the first test until the last test in any session that was not confounded with a floor effect, which is to say that the extinction differences followed directly from differences in initial learning (statistical texts make it clear that it is inappropriate to probe for specific session-specific strain differences when a significant interaction is not present, see, e.g., Howell, 2007). Thus, we conclude that p75−/− mice learned stronger taste aversions that extinguished at normal rates.

DISCUSSION

Acute and long-term disruptions of the cholinergic system, including lesions of the BF and infusion of inhibitors into targets of BF including GC, are known to impair a rodent's ability to learn a CTA. Here, we used a genetic approach to confirm a key but largely untested implication of these findings: we show that cholinergic hyper-innervation of the taste system leads to stronger than normal taste aversion learning. ChAT immunostaining confirmed that GC of p75−/− mice, identified using a functional assay, contains a greater number of cholinergic fibers than that of WT mice. Subsequent analyses ruled out the possibility that the increase was due to increased staining intensity rather than fiber length, and revealed that p75 mutants were similar to WT mice with regard to numbers of neurons in GC, post-synaptic M1 and M4 ACh receptor expression in GC (but not M2, see below), and cholinergic innervation in

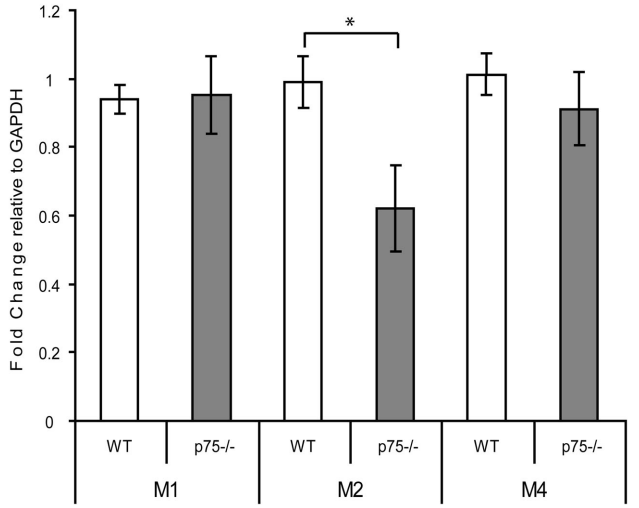

FIGURE 5 | p75−/− and wild-type GC differed in expression of the M2, but not M1 or M4, cholinergic receptor mRNAs. RNA was isolated from GC of wild-type and p75−/− mice and the levels of M1, M2, and M4 receptor mRNA were measured relative to expression of GAPDH using real-time PCR. Levels of mRNA are expressed relative to GAPDH mRNA expression levels, showing no change in M1 and M4 levels and a decrease in M2 mRNA. * = $p < 0.05$.

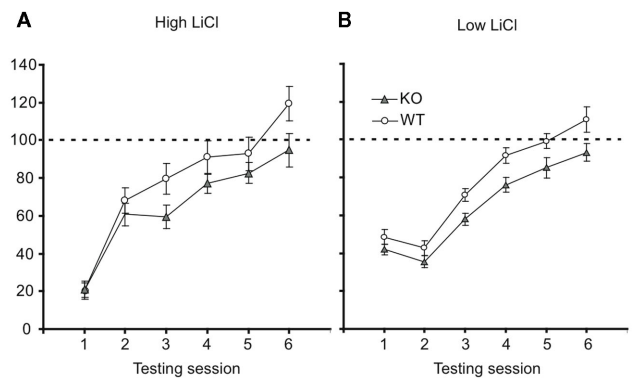

FIGURE 6 | p75−/− mice learn stronger CTAs than wild-type mice. (A) With the standard dose of LiCl (0.15 M, 2% of body weight), both wild-type (open ovals), and p75−/− mice (closed triangles) learn strong CTAs, reducing their consumption of saccharin in the first testing session to ∼20% of training-session consumption (*y*-axis). Across further testing sessions (*x*-axis) both groups re-learned to consume saccharin (i.e., the CTA underwent extinction), but this occurred faster for wild-type mice (see text for statistics). **(B)** When the LiCl dose was reduced to 1% of body weight, the learning was accordingly milder. By lifting consumption away from the floor, it became possible to observe a difference in initial learning: p75 mice learned stronger CTAs (i.e., consumed less saccharin) than wild-type mice. This difference was maintained through several extinction trials. See text for statistical details.

gustatory regions that receive their cholinergic input via the brainstem rather than BF (i.e., the gustatory thalamus). These mice thus show specific cholinergic hyper-innervation of the taste system, and thus would be predicted to show stronger than WT learning, a prediction that was borne out in our behavioral testing.

The assays that we used to determine cholinergic hyper-innervation of the taste system centered on GC, providing confirmation that increases in BF cholinergic neuron numbers (previously described for this mutant, Lin et al., 2007) resulted in an increase in the number of cholinergic fibers in the taste system. This does not imply that the actions of ACh, or the cholinergic changes observed in p75−/− mice, are restricted to GC. Cholinergic activity at the brain stem, amygdala, and cortical levels of the taste system has been implicated in CTA (Bermudez-Rattoni, 2004), and it is possible that all of these regions are hyper-innervated in the p75 behavioral phenotype. Most notably, the BLA – and amygdala–cortical connectivity – are known to be deeply involved in CTA (Bermudez-Rattoni, 2004; Grossman et al., 2008), and cholinergic hyper-innervation may well affect this circuitry although given the extremely high density of cholinergic innervation in the wild-type BLA it is hard to know if increases can be detected (see Muller et al., 2011). Cholinergic hyper-innervation has also been shown in the hippocampus of the p75-deficient mice (Yeo et al., 1997), but it is unlikely that this previously described effect underlies the results shown here, as hippocampal activity appears to exert an inhibitory influence on taste learning (Stone et al., 2005).

It may reasonably be asked whether altered cholinergic innervation – that is, the increased number and length of cholinergic fibers – is in fact the only reasonable explanation for our behavioral findings. For instance, we did observe a down-regulation of the M2 receptor, inhibition of which increases ACh release in the cortex through a presynaptic mechanism and enhances passive avoidance learning (Carey et al., 2001). It is therefore possible that down-regulation of the M2 receptor could function to further augment the effect of hyper-innervation, contributing partially or wholly to our observed enhancements in taste learning. This hypothesis fails to accord with experiments involving cortical application of M2 inhibitors, however, which report either no impact (Ramirez-Lugo et al., 2003) or impairments of CTA (Naor and Dudai, 1996). The interpretation of these latter studies is complicated by the possibility that the manipulations used to inhibit M2 also inhibited post-synaptic cortical neurons (Amar et al., 2010; Brown, 2010); regardless, while decreases in M2 receptor expression level in the p75−/− mice could potentially contribute to increased GC ACh levels and the enhancement of CTA seen in this study, either directly or indirectly, the current state of the field suggests that it is unlikely that loss of M2 receptors is a key mechanism for compensation of cholinergic hyper-innervation.

Finally, while the most prominent abnormality in p75−/− mice is in the BF, changes in this region involve both an increase in the number of cholinergic neurons and a decrease in GABAergic neurons (Lin et al., 2007). Furthermore, the cholinergic abnormality itself may well result in additional, secondary changes in neuronal structure and circuit function, implicating other neurotransmitter systems. Any (and all) of these effects could potentially have played a role in driving the observed behavioral phenomenon; most notably, cholinergic modulation

may ultimately impact dopaminergic and glutamatergic function, known to be involved in proper conditioning (Fenu et al., 2001; Jiménez and Tapia, 2004). All methods of perturbing ACh have such secondary impacts, however: the effect of lesions, and even of temporary inactivations, are inevitably non-local (Honey and Sporns, 2008; Alstott et al., 2009), and even the behavioral effects of selective BF cholinergic immuno-lesions may reflect the involvement of other BF cell groups (Gutierrez et al., 1999a,b); p75-Saporin, which cleanly targets cholinergic neurons, still causes non-specific damage to the distributed neuronal networks into which these neurons are connected.

Perhaps even more dramatic, cholinergic agonism can result in seizures, *via* profound disruption of extra-cholinergic pathways (Olney et al., 1983; Naor and Dudai, 1996), making it a particularly difficult preparation to work with. To our knowledge, one study has successfully examined taste learning using cholinergic agonism (specifically, carbachol); this study reported, consistent with our results, that boosting cortical cholinergic function allowed rats to acquire CTAs to normally ineffective familiar stimuli (Clark and Bernstein, 2009). Thus, while no single method can provide direct proof of cholinergic function in this system in the absence of potentially confounding indirect effects, the use of the p75−/− mice provides important independent evidence that increased cholinergic function results in enhanced taste learning.

In fact, the genetic approach may offer certain advantages over other methods. For one thing, it largely eliminates issues of spatial variability: the hyper-innervation observed here, at least as far as GC was concerned, appeared to be uniformly and broadly distributed; thus, our results are less subject to variability borne of regional differences in targeting dependent on the precise placement of infusion cannulae. In addition, developmental compensation of critical functions in knockout mice may stabilize the ancillary circuits that are acutely perturbed under more acute manipulations of ACh. p75−/− mice become cholinergically hyper-innervated through slow, non-traumatic developmental processes and, thus, do not experience acute perturbations of cholinergic and other systems; they retain fundamental abilities to feed, are not seizure-prone, and show normal behaviors including odorant recognition responses (Barrett et al., 2010). In many regards they appear largely normal – while there are reports of behavioral impairments in one type of spatial learning task in the p75-deficient mice (Wright et al., 2004), other researchers report enhancement of spatial learning (Greferath et al., 2000) and, at the cellular level, of hippocampal long-term potentiation (Barrett et al., 2010).

One question that remains to be answered has to do with the psychological effect of the knockout phenotype. Our behavioral results clearly show that p75-deficient mice condition more strongly than WT mice – while the results using a high dose of LiCl could be interpreted to suggest a p75-WT difference in extinction rather than learning, our data make it clear that saccharin was observably more aversive to trained p75−/− mice in every session that was not contaminated by a floor effect for consumption, including the first testing session in the low-LiCl experiment. Thus, the inter-strain extinction differences largely follow from differences in initial conditioning. Our conditioning data do not, however, enable us to say whether the observed behavioral effects represent a simple associative learning abnormality as opposed to a more permanent change. Since our knockout mice failed to achieve the same level of extinction as WT mice even across six testing sessions, we cannot say for sure that CTA in mice with cholinergic hyper-innervation did not permanently elevate a "fear-related component" of the response to saccharin. Future work will address this question.

More centrally with regard to the theory of cholinergic involvement in learning, our data do not speak to the issue of whether the p75−/− mice truly found the tastes to be more novel (i.e., less familiar) as opposed to more salient (i.e., more potent and intense). Either of these effects would result in stronger learning (Schmajuk et al., 1996; Berridge and Robinson, 1998) without reflecting a direct change to general learning circuitry. Several lines of evidence link cholinergic function to novelty, however. Familiarizing a rodent with a taste causes a down-regulation of taste-related ACh release (Miranda et al., 2000), whereas acute inhibition of cholinergic function inhibits the normal behavioral effects of such familiarization (Naor and Dudai, 1996; Berman et al., 2000; Miranda et al., 2000). Conversely, increasing cholinergic activity enhances both taste novelty and salience (Clark and Bernstein, 2009). It is therefore reasonable to speculate that p75−/− mice treat the taste as somehow "even less familiar than a novel taste" and thus are more likely to associate this taste with malaise than WT mice. Future work assessing the effect of familiarization on the inter-strain differences should shed light on this issue.

With few exceptions (one being mentioned above), the current understanding of the role of the cholinergic system in an animal's response to novel tastes is based upon experimental perturbations that destroy or inhibit key cells or signaling in the neural circuit, resulting in attenuation of gustatory behaviors such as CTA. Less common, but of great value in assessing the role of this system, are findings of enhanced functions following manipulations that increase cholinergic signaling. Our use of a knockout mouse strain that links cholinergic hyper-innervation to stronger CTA demonstrates that a developmental increase in the BF cholinergic projection to GC, brought about through genetic manipulation, is effective in regulating the strength of conditioning. In addition to providing independent evidence for cholinergic regulation of CTA, this study confirms the power of genetic approaches for studying taste behaviors (Masugi et al., 1999; Jacobson et al., 2006), and suggests new areas for investigation that include the developmental role of cholinergic signaling during the maturation of gustatory circuitry.

ACKNOWLEDGMENTS

This work was funded by NIH NS057305 to Susan J. Birren, CD006666 to Donald B. Katz, and by 2P30NS045713 for Core Facilities for Neurobiology. The authors wish to thank Jeanine Hinterneder and Suzanne Paradis for critical readings of the manuscript, and our editor and reviewers for a truly enjoyable submission and revision process.

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

Received: 11 August 2011; accepted: 09 November 2011; published online: 01 December 2011.

Citation: Neseliler S, Narayanan D, Fortis-Santiago Y, Katz DB and Birren SJ (2011) Genetically induced cholinergic hyper-innervation enhances taste learning. Front. Syst. Neurosci. ***5****:97. doi: 10.3389/fnsys.2011.00097*

frontiers in
SYSTEMS NEUROSCIENCE

MINI REVIEW ARTICLE
published: 08 November 2011
doi: 10.3389/fnsys.2011.00091

Taste learning and memory: a window on the study of brain aging

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Taste aversion learning exhibits advantages for research on memory brain systems and its reorganization throughout life. A review of the effects of aging on taste memory abilities offers a complex picture showing preserved, impaired, and enhanced functions. Some of the age-related changes in taste memory seem to be associated with an altered temporal processing. Longer taste–illness delays can be introduced for acquisition of conditioned taste aversions and the modulation of taste learning by the temporal context is absent in naïve old rats. It is suggested that an altered hippocampal function is involved in the peculiar performance of these rats. Evidence is also presented which suggests that hippocampal-dependent taste memory can be reactivated by previous learning experiences in old rats. Results obtained after reversible inactivation of the dorsal Hippocampus by tetrodotoxin (TTX) in old rats support such a view. Therefore, the interaction between the previous experience and acute brain interventions should be taken into account when studying the effect of aging on taste memory.

Keywords: aging, context, hippocampus, learning, memory, rat, taste, time-of-day

Aging offers a privileged opportunity to study the reorganization of brain memory systems throughout life. At advanced ages the learning and memory abilities have been shaped by several decades of previous learning experiences and face new adaptive challenges due to the modifications of biological conditions. In rodent studies the usual age groups range from young (3–6 months), middle-aged (14–18 months), and aged (24–27 months). Even though the effect of age in learning and memory is progressive and age-related changes in performance have been described as early as at 8-months of age (Gallo et al., 1997) the most pronounced changes occur in middle-aged and aged rats. Thus, 18- to 22-month-old animals are often defined as aged even though it has been claimed that they should be considered late middle-aged (Coleman et al., 2004). Contrary to the idea of a global memory decay, normal aging seems to selectively deteriorate some memory functions whilst others remain relatively unimpaired or even enhanced. This is the case in taste aversion learning. Aged rats readily acquire strong, long-lasting aversions to the taste of ingested food leading to visceral distress. The behavioral procedure to induce conditioned taste aversions (CTA) in the laboratory typically involves applying an illness-inducing i.p. injection of lithium chloride (LiCl) after ingestion of a flavored solution. The aversive memory formed after association of taste cues conditioned stimulus (CS) with the aversive visceral signals unconditioned stimulus (US) prevents later ingestion of poisons thus playing a critical role for survival.

Conditioned taste aversions in the rat relies on a brain circuit including areas located from the lower brain stem (nucleus of the solitary tract, parabrachial area) to the higher (amygdala, insular cortex) brain levels. The brain circuit of CTA is described elsewhere (Bermudez-Rattoni, 2004; Lundy and Norgren, 2004; see in this issue Scott, 2011; Yamamoto and Ueji, 2011). CTA also exhibits hippocampal-dependent complex learning phenomena that are selectively impaired by aging. It can be envisaged that the relationship between the hippocampal system and the CTA basic circuit might have been modified throughout life. Thus, the peculiar memory performance of healthy subjects at advanced ages reflects the altered organization of the neural systems involved. A widely accepted view to explain such reorganization is based on compensatory changes to the selective decay of the hippocampal function. Surprisingly, little attention has been paid to the effect of the accumulation of previous learning and memory experiences throughout a long life. Given the plasticity of the brain memory systems, it can be envisaged that changes of the brain systems connectivity have been the obvious outcome of previous learning episodes in order to enhance adaptation to the environmental conditions. Thus, the temporal parameters of learning experiences might become increasingly important throughout life.

This review focuses on the potential time processing changes for understanding the peculiar features of CTA at advanced ages. Special emphasis is given to the effect of previous learning experiences.

TASTE PROCESSING AND NORMAL AGING

A systematic approach to explore potential explanations of the superior CTA ability related with normal aging should take into account potential modifications of taste processing induced by life events at several steps.

Firstly, aging could alter sensory processing, thus modifying the salience of the taste stimuli to be used in the learning procedures. However no taste sensitivity changes have been reported in aged rodents that could significantly affect the outcome of conventional CTA protocols. Accordingly neuro-physiological responses to various tastes, such as KCl, sucrose, quinine-hydrochloride, HCl,

monosodium glutamate, and glutamic acid do not change with age in animals (Osada et al., 2003). Even though a decreased olfactory sensitivity has been associated with aging, old rats have been reported to discriminate between odors as readily as younger adult rats (Brushfield et al., 2008) and flavors conventionally used in flavor preference tasks, such as grape and cherry (Renteria et al., 2008).

Secondly, aging could alter the unlearned neophobic response to non-familiar tastes. Taste neophobia is evidenced in decreased consumption of novel taste solutions compared with later exposures as long as the taste becomes familiar. It is well known that stronger aversions are acquired to novel taste solutions rather than to familiar solutions, a well known phenomenon called latent inhibition (Lubow and Moore, 1959). Thus, age-related increases in taste neophobia could account for the higher CTA abilities in old rats.

However, research on this issue has yielded controversial results regarding the impact of aging on taste neophobia. Whilst some studies have found enhanced neophobic responses in aged rats (Collier et al., 2004), no effect of aging has been reported in the amount drunk of a novel taste when using either a grape juice solution (Gallagher and Burwell, 1989; Koh et al., 2009), 0.1% sodium saccharin (Moron and Gallo, 2007), 1% sodium chloride (Manrique et al., 2009), 0.5% sodium chloride, or 3% cider vinegar solutions (Moron et al., 2002a).

Moreover, the potential impact of aging on taste neophobia and attenuation of the neophobic response might be confounded. This is due to the fact that the full demonstration of the neophobic response to a novel taste requires taking into account not only decreased consumption during the first encounter but also later increases upon subsequent exposures. In fact, a lower rate of neophobia attenuation has been reported in old rats (Pelleymounter and Cullen, 1993) although no age-related differences have been found using a low NaCl concentration (Manrique et al., 2009).

Regarding the effect of previous experience, early studies showed the relevance of previous aversive taste learning on neophobia to later encountered taste solutions in adult rats (Domjan, 1975; Best and Batson, 1977; Kristal et al., 1980; Franchina and Dyer, 1985). Moreover, there are data supporting an even higher impact of previous aversive experiences on taste neophobia during aging. Thus, previous exposure without consequences leading to habituation of the neophobic response to a sodium saccharin solution disrupted subsequent neophobia to a NaCl solution both in young and old adult rats. However, a previous saccharin–lithium chloride (LiCl) pairing induced in aged rats a larger increase on later neophobic responses to the salty solution than in young adult rats (Moron and Gallo, 2007). Given that the strength of the previous aversive experience was equated across the age groups, the results could be attributed to a greater impact of aversive memories at advanced ages. Different explanations could account for it being the most suitable a superior learning ability to develop stronger taste aversions at advanced ages. Whatever the explanation, studying the effect of previous taste experiences on the aged rat's willingness to accept novel tastes may contribute to the understanding of controversial results.

To sum up, whilst no aged-related changes in sensory processing seem to be responsible for the superior ability of aged rats to acquire CTA, previous aversive learning experiences could induce increased taste neophobic reactions and/or decreased rates of neophobia attenuation. This might contribute to the formation of stronger learned aversions. This widely unexplored issue is especially relevant because even though many aging studies use naïve animals, it is not unusual to apply previous aversive learning tasks either for dissociating pathological and normal aging or to follow the recommendations for reusing the subjects.

TASTE AVERSION LEARNING AND MEMORY IN AGED RATS

As mentioned above the acquisition of learned taste aversions seems to be facilitated at advanced ages. As has been reviewed elsewhere (Manrique et al., 2007), stronger aversions are evident in old rats in comparison with young–adult rats during the first extinction test, provided that floor effects are avoided. Although the possibility that this enhancement arises because of impaired extinction cannot be discarded (see below), there are other features of CTA acquisition that point to a superior ability for associating taste and visceral distress in aged rats than in young adult rats. One of the most intriguing features of taste aversion facilitation in aged rats is the possibility of introducing longer intervals between the taste and the LiCl injections than in younger adult rats. Using a relatively low dose of LiCl (1% b.w., 0.15 M) and a 24 h two-bottle test, saccharin aversions have been found in aged rats but not in young–adult rats with taste-LiCl intervals ranging from 180 (Misanin and Hinderliter, 1989) to 360 min (Misanin et al., 2002b). This ability to associate a taste with an illness over long intervals develops gradually as rats get older. Thus, rats older than 18 months exhibit taste aversion at the 180-min interval, whilst only 24- and 30-month-old rats acquire learned taste aversions at 360-min delays (Misanin et al., 2002b).

Different explanations for the age-related facilitation of long-trace taste illness associations have been proposed. Previous results suggest that they cannot be attributed to age differences either in taste sensitivity or increased efficacy of LiCl injection (Misanin and Hinderliter, 1994). Other explanations based on deficits of learned irrelevance (Misanin and Hinderliter, 1995), age differences in the use of interval context cues (Hinderliter and Misanin, 1995b), context–illness associations (Hinderliter and Misanin, 1995a), relative taste novelty (Hinderliter and Misanin, 1993), or memory for specific taste attributes (Misanin et al., 1997) have also been ruled out.

Misanin et al. (2002b) have proposed a longer availability of the taste memory trace in aged rats, because increasing the illness intensity extends the interval over which trace conditioning is evident in old but not in young–adult rats. In order to explain how a memory trace can be available to old rats at a time when it is not longer available to young adult rats, the authors have proposed the slowing down of a metabolic pacemaker. The hypothesized pacemaker is compared to a countdown timer that regulates trace decay after taste processing. The timer would stop at a given duration. Thus, aging can slow the pace at which the clock counts down, thus extending the memory trace decay delay. The effect of aging on this metabolic pacemaker would be independent to that of other circadian clocks or brief interval timers (Misanin et al., 2002b,c). Support for the metabolic pacemaker has been obtained from studies with adult rats showing correlations between decreased metabolic

rate and the ability to establish long-trace taste aversions. Firstly, at low body temperatures rats displayed learned taste aversions with delays of up to 225 min, which was attributed to a cold-induced slowing of the biochemical clock (Misanin et al., 2002a). Secondly, increasing the metabolic rate by chronic water deprivation reduced the interval that can be introduced in a taste aversion learning protocol (Anderson et al., 2006). Explanation based on an altered sense of time may be related with other reports in animals (Walton, 2010) and humans (Fitzgibbons and Gordon-Salant, 2004; Gooch et al., 2010) pointing to age-related differences in temporal processing using other tasks. No attempts have been made to explore the potential role of age-related anatomical changes in different brain areas in the facilitation of CTA acquisition at advanced ages.

A similar explanation could account for the higher resistance to extinction of learned taste aversions in old rather than in young–adult animals even if no significant differences in acquisition are detected. Thus, Ingram and Peacock (1980) reported that old rats showed delayed extinction of a LiCl-induced saccharin aversion monitored over a period of 32 days. Similarly, resistance to the extinction of a saccharin aversion induced by a low dose of LiCl has been reported in old rats (Moron and Gallo, 2007). These results contrast with the impaired retention that has been reported in old rats using other learning tasks, such as fear conditioning (Kaczorowski et al., 2011), passive avoidance and learned helplessness (Martinez and Rigter, 1983), among others (Bevilaqua et al., 2008). Given the proposals considering the relevance of a time-induced context differentiation process during extinction, it is conceivable that an altered sense of time could also contribute to slower extinction during aging. An alternative explanation of the slower extinction rate found in older subjects can be related with the greater robustness of the aversion. Nevertheless, even though the age-related superiority in taste aversion learning might rest on the associative mechanisms acting during the acquisition session, enhanced taste memory abilities cannot be excluded given the long intervals supported at advanced ages.

Thus, whatever the explanation, a neural reorganization of the taste memory systems favoring the acquisition and retention of taste aversions at advanced ages seems to be evident. In addition a potential role of changes in temporal processing induced by such reorganization merits attention.

HIPPOCAMPAL FUNCTION AND TASTE MEMORY DURING AGING

While the hippocampus does not seem to be necessary for acquisition of basic CTA using conventional protocols, the effect of hippocampal damage is evident in adult rats with modified protocols. Firstly, both dorsal and ventral hippocampal neurotoxic lesions have been reported to selectively impair taste aversion learning when 3-h intervals were introduced between taste and illness (Koh et al., 2009). Secondly, temporary inactivation of the dorsal hippocampus by muscimol infusions during acquisition has been shown to enhance learned aversions in a procedure that involved no delay, two different taste solutions, and two conditioning trials (Stone et al., 2005). The authors pointed out to the potential relevance of avoiding ceiling effects due to the relative complex two-taste protocol used. Thirdly, permanent and reversible hippocampal inactivation selectively interferes with a variety of taste complex learning phenomena depending on previous experience (Gallo et al., 1999) as well as on temporal context cues (Molero et al., 2005). Both taste memory enhancement and impairment after hippocampal damage might reflect the interaction between multiple memory systems working in parallel that might induce competitive interference between them. Thus, the hippocampal functions supporting long-delay CTA and complex learning phenomena could be interfering with the acquisition of learned taste aversions (Schoenbaum and Stalnaker, 2005).

It is conceivable that the aging process might modify the potential interaction between the hippocampus and the basic taste memory system. Whilst the evidence from permanent lesion studies does not support an explanation based on hippocampal damage of the age-related changes in taste learning abilities (Manrique et al., 2007), a contribution of an altered functioning of the aged hippocampus cannot be excluded. If this were the case, acute hippocampal inactivation in the behavior of adult animals could be a better model than permanent lesions to study the potential hippocampal involvement in the age-induced facilitation of taste aversion learning (Stone et al., 2005).

PREVIOUS LEARNING EXPERIENCES AND THE TEMPORAL CONTEXT MODULATION OF CTA IN AGED RATS

Previous results in our lab have shown that a time-of-day shift between taste pre-exposure and conditioning interferes either with learned taste aversions retrieval (Moron et al., 2002b) or with the latent inhibition effect (Manrique et al., 2004). This depends on the extent of the previous habituation to water deprivation procedure (**Figure 1**). Thus, the comparison between the groups receiving the taste–illness pairings at the same (SAME) and at a different (DIFF) time-of-day than pre-exposure and testing yields an opposite pattern of results in a short-habituation (2 days) versus a long-habituation (5 days) protocol. In the former, DIFF groups exhibit weaker aversions than SAME groups whilst in the latter DIFF groups show stronger taste aversions than SAME groups. These patterns reflect the temporal context dependency of CTA (Moron et al., 2002b) and latent inhibition (Manrique et al., 2004), respectively. Both of them demonstrate the ability of the time-of-day to act as a context. The hippocampal integrity plays a crucial role in the temporal modulation of latent inhibition. Thus, neurotoxic lesions of the dorsal hippocampus in adult rats selectively disrupt the effect of the temporal context shift in the long-habituation procedure (Molero et al., 2005). Similarly intact aged rats have been reported to exhibit deficits in the long-habituation protocol. No differences between the aversions shown by SAME and DIFF groups were found (Manrique et al., 2009). This finding does not seem to be explained by a disruptive effect of aging on either latent inhibition (Moron et al., 2002a) or the ability to use the time-of-day as a context. In fact, modulation by the time-of-day was evident in hippocampal aged rats. The pattern of results induced in adult rats by the short-habituation protocol appeared in lesioned aged rats subjected to the long-habituation procedure (Manrique et al., 2009).

Additional data have shown that the temporal context modulation seen in the long-habituation protocol absent in old rats can be reinstated by previous learning experience. Previous training

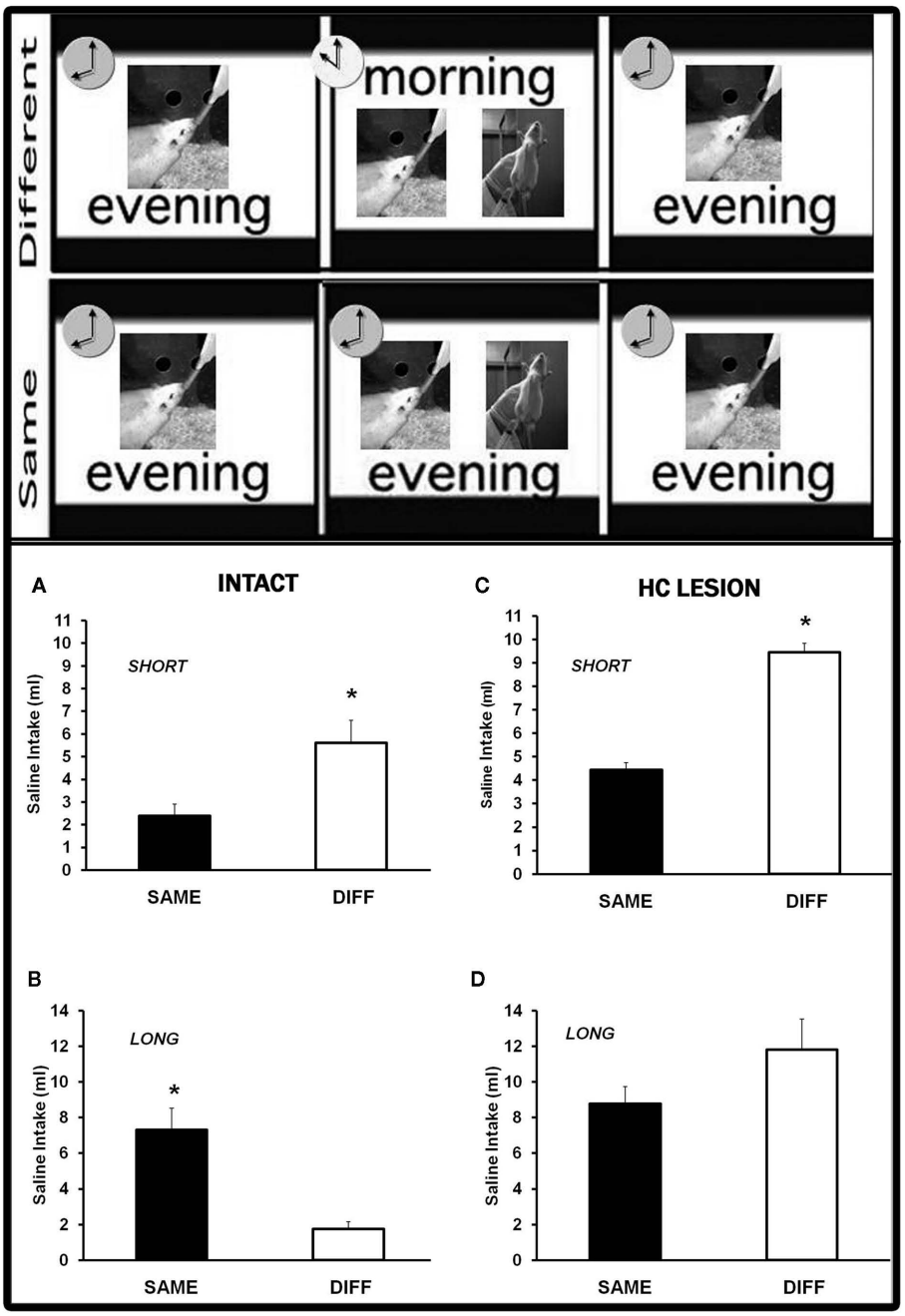

FIGURE 1 | Temporal context-dependent taste learning in adult intact and hippocampal-lesioned rats. Upper panel – the behavioral procedure consisted of four phases: habituation to drink water twice a day, taste solution pre-exposure, taste-LiCl pairing, and testing. In DIFF groups conditioning took place at a different time-of-day to pre-exposure and testing. Groups SAME received all the experimental phases at the same time-of-day. Sessions were performed either at 10 or 20 h. Lower panel – two different protocols were applied depending on the extent of the habituation phase: a short-habituation (2 days) or a long-habituation (5 days) protocol. The time-of-day shift induced opposite patterns of results in intact rats subjected either to the short **(A)** or the long **(B)** protocol. Hippocampal lesions impaired the effect of a temporal context shift in the long **(D)** but not the short **(C)** behavioral protocol. (For further details see Moron et al., 2002b; Manrique et al., 2004, 2009; Molero et al., 2005).

in our experiment included several tasks: (a) exposure to a first novel taste solution and subsequent attenuation of taste neophobia, (b) a latent inhibition protocol using a second novel taste solution, and (c) a novel object recognition task. Unexpectedly, trained aged rats exhibited a pattern of differences similar to that seen in adults. The DIFF group showed stronger aversions than the SAME group (**Figure 2A**). The reinstatement of this adult pattern known to require an intact hippocampus (Molero et al.,

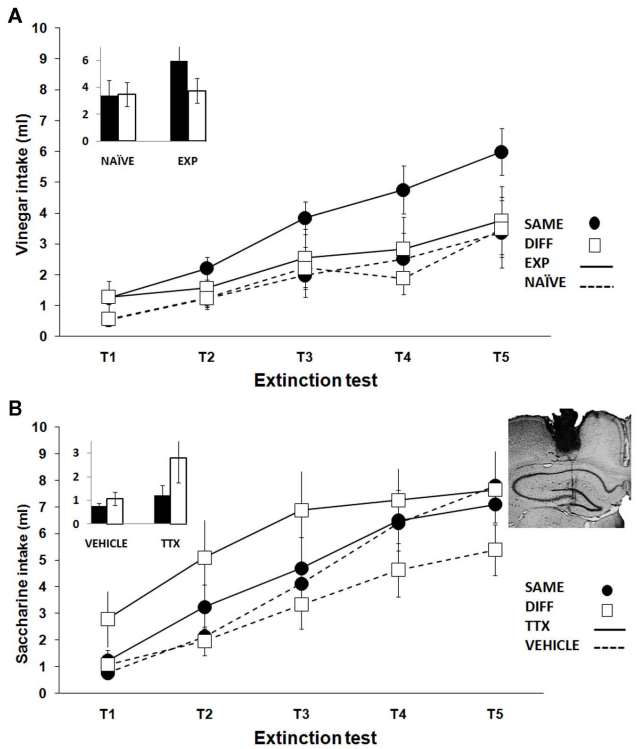

FIGURE 2 | Mean (±SEM) taste solution intake by the aged groups SAME and DIFF using a long-habituation protocol for exploring the hippocampal-dependent time-of-day modulation of taste learning. (A) Reinstatement of the hippocampal adult pattern of results by previous training (EXP groups). Naïve animals (NAÏVE groups) did not exhibit modulation of learning by time-of-day. **(B)** Emergence of the opposite non-hippocampal pattern of results in trained groups receiving tetrodotoxin (TTX groups) injection in the dorsal hippocampus. Vehicle injected groups (VEHICLE) did not differ between them. The inset highlights the key comparisons on the retention test. A cresyl violet-stained coronal section shows a representative injection needle trace indicating the infusion target area in the dorsal hippocampus.

2005) suggests a potential reactivation of the aged hippocampus function. In our experiments only the above mentioned discrete learning experience was effective. However, a similar 2-month-long exposure to unspecific environmental enrichment had no effect. Considering that either longer exposure or increased complexity of the enriched environment conditions may be needed, further research is required on this issue.

Moreover, previous learning experience seems to have a critical role in determining the effects of temporary hippocampal interventions during aging. Whilst permanent neurotoxic lesions of the dorsal hippocampus in naïve aged rats enhanced the non-hippocampal temporal modulation of taste aversion (Manrique et al., 2009), temporary inactivation by TTX during conditioning induced a similar effect only in trained old rats (**Figure 2B**). The results cannot be attributed to changes in the attenuation of neophobia during conditioning since hippocampal inactivation during exposure to a 3% cider vinegar solution had no effect either in the neophobic response or its habituation. It is, therefore, conceivable that reversible temporary inactivation may release functions modulated by the aged hippocampus that were previously reactivated by learning experience. However permanent damage would be required for the reorganization of neural circuits in naïve animals.

The fact that previous discrete learning experiences determine the outcome of hippocampal inactivation in taste learning at advanced ages shows up a complex interaction between parallel memory systems. It is conceivable that the aging process modifies the interaction between hippocampus and the taste memory systems. Therefore, the study of the interaction between the hippocampus and other taste memory systems at advanced ages should take into account the nature of the learning experiences throughout the life.

CONCLUSION

Temporal processing deficits may be at the root of the peculiar features of older subjects' performance in taste learning tasks. A compromised sense of time in aged animals is supported by both enhanced long-delay taste aversion learning and absence of temporal context modulation.

An altered interaction between the hippocampal system and CTA brain circuits could be responsible for the peculiar temporal attributes relevant for taste memory during aging. Thus, permanent hippocampal lesions facilitate basic non-hippocampal forms of CTA modulation by the time-of-day, thus indicating competition between systems. However transient, hippocampal inactivation produces similar effects only in trained aged rats.

Therefore, memory abilities which have been shaped by several decades of learning experiences throughout life should be taken into account in taste research on aging.

ACKNOWLEDGMENTS

Supported by the research projects HUM 02763 (Junta de Andalucía. Spain), PSIC2008-03933 (MICINN. Spain), PSI2011-23702 (MICINN. Spain), and FPU fellowship to Fernando Gámiz (MICINN, Spain). The authors are grateful to Angela L. Tate for her help in editing the English text.

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

Received: 15 July 2011; paper pending published: 20 August 2011; accepted: 23 October 2011; published online: 08 November 2011.

Citation: Gámiz F and Gallo M (2011) Taste learning and memory: a window on the study of brain aging. Front. Syst. Neurosci. ***5****:91. doi: 10.3389/fnsys.2011.00091*

frontiers in
SYSTEMS NEUROSCIENCE

MINI REVIEW ARTICLE
published: 29 November 2011
doi: 10.3389/fnsys.2011.00099

Sweet taste signaling and the formation of memories of energy sources

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The last decade witnessed remarkable advances in our knowledge of the gustatory system. Application of molecular biology techniques not only determined the identity of the membrane receptors and downstream effectors that mediate sweetness, but also uncovered the overall logic of gustatory coding in the periphery. However, while the ability to taste sweet may offer the obvious advantage of eliciting rapid and robust intake of sugars, a number of recent studies demonstrate that sweetness is neither necessary nor sufficient for the formation of long-lasting preferences for stimuli associated with sugar intake. Furthermore, uncoupling sweet taste from ensuing energy utilization may disrupt body weight control. This minireview examines recent experiments performed in both rodents and *Drosophila* revealing the taste-independent rewarding properties of metabolizable sugars. Taken together, these experiments demonstrate the reinforcing actions of sugars in the absence of sweet taste signaling and point to a critical role played by dopamine systems in translating metabolic sensing into behavioral action. From a mechanistic viewpoint, current evidence favors the concept that gastrointestinal and post-absorptive signals contribute in parallel to sweet-independent sugar acceptance and dopamine release.

Keywords: carbohydrates, dopamine, glucose oxidation, nutrient selection, reward, striatum, sweet taste, TRPM5

INTRODUCTION

Glucose-containing carbohydrates, the ingestion of which are critical for most forms of animal life, reliably elicit the highly pleasurable sensation of sweetness. Such mechanism allows the brain to rapidly trigger acceptance responses upon recognizing the presence of nutritive carbohydrates in the oral cavity. Given the consistency of this behavioral response, one would hypothesize that sweet taste is both necessary and sufficient for the appropriate control of sugar intake. However, mounting evidence from both mammals and insects now favors the possibility that long-term food choices depend primarily on the detection of the energy content of the food sources, without requiring the stimulation of sweet taste effectors. This minireview concentrates on describing recent studies in both rodents and *Drosophila* revealing that the formation of long-term sugar acceptance and preference requires the activation of energy-sensing pathways.

THE GUSTATORY SYSTEM

Recent reviews have covered in depth the anatomy of both the peripheral and central gustatory pathways (Carleton et al., 2010; Kinnamon, 2011), which we will mention only briefly. The peripheral gustatory system consists of the neural-epithelial machinery linking the sensory epithelial cells in the oral cavity to the first gustatory relay center in the brain. G-protein-coupled receptors (GPCRs) expressed on the apical end of taste receptor cells (TRCs) function as the receptors for sweet, some L-amino acids, and bitter tastants while ion channels of the transient receptor potential are thought to mediate sour and salty tastes (Chandrashekar et al., 2006; Roper, 2007). It is noticeable that taste receptor expression has been detected at other organs including the gastrointestinal tract (Margolskee et al., 2007; Hass et al., 2010), pancreas (Nakagawa et al., 2009), and brain (Ren et al., 2009), although extra-oral physiological functions remain to be determined. On the tongue, sweet taste is specifically mediated by the taste genes *Tas1r2* and *Tas1r3*, whose T1R2 and T1R3 products assemble to form the heterodimeric sweet receptor T1R2/T1R3 (Chandrashekar et al., 2006). T1R2/T1R3 activation is effected via the downstream signals phospholipase PLCβ2 and TRPM5, a non-selective ionic taste channel, the deletion of either inducing severe impairment in—if not taste-blindness for—sweet, umami, and bitter transduction (Zhang et al., 2003). Sweet tastant-induced TRC depolarization—mediated by TRPM5—produces the release of chemical messages onto cranial nerve afferents innervating the basolateral aspect of TRCs. The cell bodies of taste-responsive cranial nerve ganglia synapse into the rostral division of the nucleus tractus solitarius (rNTS) in the medulla (Hamilton and Norgren, 1984), which in turn projects, in rodents, ipsilaterally to the parabrachial nucleus (PBN, Norgren and Leonard, 1971). From PBN two pathways ascend in parallel to the forebrain: a "dorsal" pathway projecting to parvicellular areas of the ventroposterior medial nucleus of the thalamus (whose afferents define anatomically the primary gustatory cortex in the insula), and one ventral pathway projecting to the amygdala, lateral hypothalamus, and the bed nucleus of the stria terminalis (Norgren, 1976). Noticeably NTS taste projections seem to ascend directly to thalamus, therefore bypassing the PBN (Small and Scott, 2009).

SWEET TASTE AND BRAIN DOPAMINE RELEASE

Robust attraction toward sweet tastants is pervasive across most species. Animals will avidly consume sweet solutions even when required to learn complex operant behaviors (Kare, 1971). Innate attraction to sweetness is presumed in humans given the stereotyped oral/facial reactions observed in children upon their first exposure to sugary solutions (Ganchrow et al., 1983). However, recent evidence reveals that early childhood flavor exposures rather than innateness *per se* mold long-term food preferences (Beauchamp and Mennella, 2009; Ventura and Mennella, 2011). It does, therefore, seem logical to presume that neural pathways must exist that link peripheral sweet receptors to brain reward-related circuits (McCaughey, 2008). Among such circuits we must stress the brain cells producing the reward-related monoaminergic transmitter dopamine. Dopamine is a major regulator of sweet sugar intake. In fact, dopamine receptor antagonism decreases the attraction toward sweet-tasting nutrients given that animals pretreated with either D1- or D2-type dopamine receptor antagonists approach high concentrations of sucrose solutions as if they had been diluted (Xenakis and Sclafani, 1981; Wise, 2006). Conversely, palatable foods elevate extracellular dopamine concentrations in the nucleus accumbens (NAcc) of the ventral striatum (Hernandez and Hoebel, 1988), a brain region critical for the expression of normal feeding behaviors (Kelley et al., 2005). In humans, striatal dopamine release reflects the perceived pleasantness of a meal (Small et al., 2003). Sweet-elicited stimulation of the central dopamine systems occurs upon intra-oral stimulation but does not require intestinal sugar absorption (as demonstrated in "sham-feeding" preparations combined with microdialysis measurements, Hajnal et al., 2004), as effect found to depend on the integrity of the "ventral" taste pathway (Norgren and Hajnal, 2005). It does, therefore, appear that sensing sweetness *per se* would account for the high acceptance associated with sugar intake.

SWEETNESS-INDEPENDENT ATTRACTION TO SUGARS

While sweet sensation is a powerful drive of feeding behavior, it remains to be proven that animals rely entirely on the orosensory properties of sugars to evaluate energy sources. In fact, it has been long established that approach or satiation responses to a given flavor can be conditioned by postingestive consequences (Sclafani and Xenakis, 1984; Booth, 1985; Rolls, 2005). For instance, in flavor-nutrient conditioning experiments, gut infusions of a given nutrient or control solution are conditionally linked to the oral intake of a distinct flavor, usually represented by odorant solutions that had been artificially sweetened (Sclafani and Xenakis, 1984; Booth, 1985). These experiments show very clearly that rodents (Booth, 1985; Sclafani, 2001) and humans (Hellstrom et al., 2004) will develop strong preferences for flavors that had been paired to infusions of nutrients compared to control infusions, with a bias toward glucose-containing sugars over other isocaloric nutrients (Ackroff and Sclafani, 2006).

Would animals develop preferences for sugars even when not paired to distinct sweet flavors? To assess this possibility the author designed a conditioning protocol where mice are allowed to form memories of sipper locations that had been previously associated with the oral delivery of sugars, water, or non-caloric sweeteners (de Araujo et al., 2008). The study involved employing wild-type as well as knockout mice lacking functional TRPM5 channels (Zhang et al., 2003). As was mentioned above, the TRPM5 ion channel is expressed in TRCs (Perez et al., 2002) and is required for sweet taste signaling (Zhang et al., 2003). Accordingly, it was hypothesized that sweet-blind *Trpm*5 knockout mice would be able to form robust preferences for those spouts previously associated with the oral presentation of sucrose solutions as long as these animals were allowed sufficient time to detect the solutions' postingestive effects. This was accomplished by first determining the initial side-preferences using a series of pre-conditioning two-bottle water tests, followed by exposing animals to 30 min-long conditioning sessions where either water (assigned to the same side of initial bias) or sucrose (assigned to the opposite side) were consumed freely while access to the other sipper was blocked.

Results from this experiment demonstrated that, unlike during short-term exposure, during the 30 min conditioning sessions both wild-type and knockout animals consumed significantly larger amounts of sucrose compared to water. In addition, during post-conditioning two-bottle tests, both wild-type and knockout animals reversed their initial side-preference biases by consuming significantly more water from those sippers that during conditioning sessions had been associated with sucrose. Therefore, oral stimulation with sweetness or otherwise distinct flavors was not required to induce strong biases toward consuming nutritive sucrose. These effects were in fact dependent on the energy content, rather than sweetness *per se*, associated with sucrose since when the same experiments were performed using the non-caloric sucrose-derived sweetener sucralose instead of sucrose, only wild-type animals consumed more sucralose than water during the conditioning sessions.

However, and critical to our argument in this review, during the two-bottle post-conditioning water sessions, neither knockout nor wild-type mice showed preferences for sippers associated with the delivery of sucralose. Overall, these results provide evidence in favor of the hypothesis that sweetness is neither necessary nor sufficient to induce long-term sugar preferences if unaccompanied by detectable physiological effects.

POSTINGESTIVE SIGNALS AND BRAIN DOPAMINE RELEASE

Given the above, it would be natural to conclude that sweetness may not be required for dopamine to be released during sucrose intake. In fact, microdialysis measurements revealed on one hand that the non-caloric sweetener sucralose produced significantly higher increases in NAcc dopamine levels in wild-type compared to TRPM5 knockout animals (de Araujo et al., 2008). These results are consistent with the ability of sweetness *per se* to stimulate dopamine release in NAcc (Hajnal et al., 2004). Now, when the same comparison was performed using sucrose, no differences were found between NAcc dopamine levels in wild-type and TRPM5 knockout mice. In conclusion, while sweet taste stimulation without caloric content only produced significant increases in accumbal dopamine levels in wild-type, caloric sucrose evoked

the same levels of dopamine increase in both wild-type and sweet-blind mice.

The above is also consistent with the fact that rats treated with local infusions in NAcc with a D1-receptor antagonist display dose-dependent reductions in intake of a flavor paired with intra-gastric infusions of glucose (Touzani et al., 2008). Interestingly, the effect of dopamine signaling antagonism on post-conditioning preferences tests was less compelling (Touzani et al., 2008). In any event, these results demonstrate that D1-like receptors in the NAcc are required for the acquisition of glucose-conditioned flavor preferences. Finally it must be noted that in addition to striatum other brain regions including the amygdala, lateral hypothalamus, and medial prefrontal cortex mediate postingestive influences on behavior (Sclafani et al., 2011), although it is intriguing to note that all those are densely targeted by dopaminergic afferents.

SWEETNESS-INDEPENDENT ATTRACTION FOR SUGARS IN *DROSOPHILA*

The attraction to sugars in the absence of sweetness or distinct flavors does not seem to be limited to vertebrates. Two very interesting recent studies independently report that flies not only survive by feeding on a tasteless metabolizable sugar, but will form odor-sugar memories only when sugar cues provide metabolic benefit. Their strategy was based on the elegant idea of comparing the results obtained from attempting to condition behavioral approach to a sweet, non-metabolizable sugar against those obtained from conditioning approach to a non-sweet, nutritional sugar (reviewed in Wright 2011). Burke and Waddell (2011) have shown that flies will not form lasting memories for odors that had been previously associated with non-metabolizable sugars such as arabinose. In fact, when these authors added the non-sweet (to flies) alcohol sugar D-sorbitol to arabinose, memory retrieval was as efficient as when odors were paired to sucrose.

The ability of flies to recognize the nutritional value of sugars independently of taste was also shown by Fujita and Tanimura (2011). These authors have also shown that flies can form associations between odors and arabinose only if D-sorbitol is added. In addition, flies were able to maintain normal physiological functions when given D-sorbitol as the only nutrient available (Fujita and Tanimura, 2011). These authors have also shown that neural mechanisms must be involved in the learning processes described above, since null mutants of the synapsin gene syn^{97}, which encodes a protein necessary for synaptic function, showed significant reductions in the ability to associate arbitrary odors with tasteless nutritive sugars (Fujita and Tanimura, 2011).

It is intriguing to note that in *Drosophila*, as in mammals, dopaminergic pathways play a role in regulating behavioral responses to rewarding stimuli such as cocaine, nicotine, and ethanol (Bainton et al., 2000). In addition, blocking transmission in dopaminergic neurons abolishes the expression of conditioned preferences for ethanol-associated cues (Kaun et al., 2011). It is therefore plausible to hypothesize that dopamine may mediate the ability to sense the nutritional value of sugars, including tasteless sorbitol, in flies as it does in mammals.

UNCOUPLING SWEET TASTE FROM ENERGY UTILIZATION

Another interesting aspect associated with the relationship between sweet taste and sugar metabolism relates to the fact that the usage of non-caloric sweeteners may disrupt the predictive relationship between sweetness and energy intake. Swithers, Davidson, and colleagues developed an experimental rodent model to study the role of sweet taste as a predictor of energy intake (see e.g., Swithers and Davidson, 2008; Swithers et al., 2009). Overall, the results reveal that intake of foods (or fluids) containing non-nutritive sweeteners, when compared to the intake of glucose, leads to significant weight gain, increased fat deposition, and impaired ability to caloric compensation. Overall, these results suggest that consumption of saccharin or other non-caloric sweeteners may decrease the ability of the organism to upregulate energy utilization, a physiological response that usually follows sugar ingestion (Swithers et al., 2009).

ON THE IDENTITY OF THE POSTINGESTIVE REINFORCING SIGNAL

A critical question that remains to be resolved regards the identity of the taste-independent reinforcement signal. Generally speaking, candidate signals could be classified into two major groups, according to whether they are generated during either pre- or post-absorptive phases of food intake. The former group includes, broadly speaking, those signals occurring previous to nutrient delivery into the bloodstream but simultaneous to the arrival of nutrients to the gut. The latter group on the other hand refers to those events occurring after nutrients reach the bloodstream, and non-exclusively includes a variety of signals such as fuel utilization metabolites and changes in plasma hormonal levels.

Experiments based on flavor-nutrient conditioning paradigms indicate that pre-absorptive signals may mediate the ability to form associations between orally delivered flavors and intragastrically delivered sugars. In fact, no flavor preference learning was obtained when flavor intake was paired with portal infusions of glucose (Ackroff et al., 2010). It is interesting to note that flavor-nutrient conditioning is robustly achieved even when infusions bypass the stomach and are delivered directly to the small intestine (Ackroff et al., 2010). These results are supported by the fact that abdominal vagotomy does not interfere with flavor preferences conditioned by glucose-containing sugars (Sclafani and Lucas, 1996). Altogether, the above allowed Sclafani and colleagues to infer that a currently unknown glucose sensor expressed in the intestine mediates flavor-nutrient conditioning to glucose-containing sugars (Ackroff et al., 2010).

Physiological signals generated post-absorption also seem to regulate sweet-independent attraction to sugars. We have recently assessed in our laboratory the potential role of metabolic signals in taste-independent nutrient selection by comparing the behavioral responses to glucose and weakly gluconeogenic L-amino acids in wild-type and *Trpm5* knockout mice. Briefly, *Trpm5* knockout mice, despite displaying insensitivity to the tastes of both glucose and L-serine during short-term (10 min) tests, did form strong preferences for glucose-associated sippers during conditioning sessions, as well as ingested significant larger amounts of glucose during longer-term sessions (Ren et al., 2010). These results were confirmed by indirect calorimetry

measurements, which demonstrated that higher intake levels of glucose were closely associated with glucose oxidation levels, in such a way that respiratory quotient measures functioned as highly efficient predictors of intake, even more so than blood glucose levels. This finding points to a role for post-absorptive nutrient utilization in postingestive reinforcement.

Consistent with the above, glucose utilization rates were also found to act as one powerful regulator of dopamine release. In fact, we have shown in the same study that an intravenous infusion of the anti-metabolic glucose analog, 2-deoxy-D-glucose (henceforth "2-DG") resulted in significant decreases in extracellular dopamine levels. In addition, such inhibitory effects of

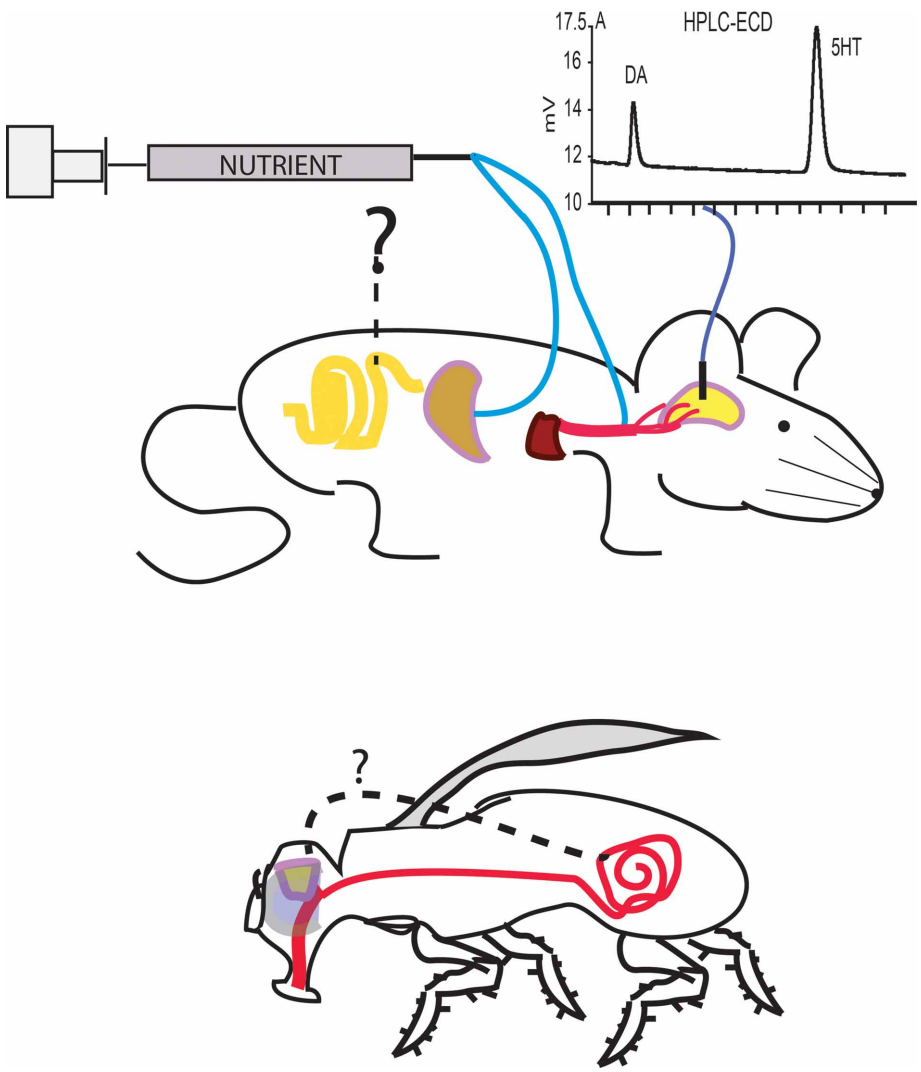

FIGURE 1 | Post-oral pathways modulating dopamine release. Infusions of nutrients into post-oral peripheral sites exert controlling actions over dopamine release, as measured from microdialysates collected from either the ventral or dorsal striatum. The figure illustrates the concept that different peripheral sites may produce stimulatory effects on dopamine release. *Upper panel* gastric injections of either non-gluconeogenic amino acids or sugars produce marked changes (reductions or increases, respectively) in extracellular dopamine levels in ventral striatum while isocaloric injections of glucose do increase dopamine levels in dorsal striatum (Ren et al., 2010). This finding establishes that stimulating the oral cavity is not required for stimulating dopamine release during nutrient intake. However, the relative contributions of gastric vs. intestinal sensing remain to be dissected because the effects if infusing nutrients directly into the intestine on dopamine release remain to be assessed (interrogation mark). Furthermore, jugular infusions of a glucose antimetabolite, 2-DG, suppress dopamine release in dorsal striatum, an effect that can be attenuated by subsequent infusions of glucose (Ren et al., 2010). This finding establishes that stimulating the gastrointestinal system is not required for stimulating dopamine release during nutrient intake. Therefore, a network of pre- and post-absorptive physiological signals converges onto dopamine circuits to regulate ingestive behavior. Chromatogram represents the use of liquid chromatography coupled to electrochemical detection (HPLC-ECD) methods to separate and quantify dopamine (DA) and serotonin (5HT) content in brain dialysates. *Lower panel* invertebrates, such as *Drosophila melanogaster*, figure as a promising model for investigating the molecular bases of how post-oral nutrient sensing exerts influence over the central nervous system. However, a number of important questions remain to be addressed, in particular whether postingestive reinforcement in these insects require gut stimulation or, likewise rodents, also involves post-absorptive pathways (see also Wright, 2011).

2-DG on dopamine release were attenuated, or almost reversed, by a subsequent intravenous glucose infusion that counteracted 2-DG effects and contributed to restore normal glucose oxidation rates (Ren et al., 2010). Finally, but always consistent with a role for glucose utilization in mediating sweet-independent sugar reinforcement, we have also found that after 2-DG injections *Trpm5* knockout mice produced significantly higher numbers of licks to glucose compared to after saline injections. Thus, within 30 min of 2-DG administration, glucose becomes more attractive if contributing to reinstate glucose oxidation levels.

Altogether, the results above indicate that both intestinal and metabolic factors simultaneously convey physiological signals that affect the central nervous system. It is important to note that these two pathways are not necessarily mutually exclusive. In fact, they are more likely to cooperate to control both acceptance and preference for environmental stimuli associated with sugars. For example, it is possible that metabolic signals are important for regulating overall sugar intake levels (as demonstrated by the 2-DG experiments performed by Ren et al., 2010) whereas preferences for flavors associated with intra-gastric glucose depend on vagus-independent intestinal signals (Ackroff et al., 2010). Alternatively, the intestine may trigger the release of incretin factors that ultimately may enhance insulin release, and, therefore, glucose uptake and utilization. Future research must determine the signaling pathways that allow brain dopamine systems to sense the energy of sugars without requiring inputs form the oral cavity. One interesting hypothesis consists in the possibility that dopamine neurons have the ability to sense the internal energy levels of the cell, modulating transmitter release accordingly, possibly via cellular sensors such as AMPK. This feature would place dopamine neurons among the brain's glucosensors, as hypothesized previously (Levin, 2000).

CONCLUSION

We have reviewed evidence that favors the conclusion that sweet taste signaling is neither necessary nor sufficient to allow for the formation of lasting memories or preferences for sugar-associated stimuli. Furthermore, brain circuits, particularly dopaminergic systems, show marked sensitivity to the energy content of sugars independently of oral stimulation. Current evidence points to the possibility that the brain monitors both gastrointestinal signals and energy utilization rates to control sugar intake independently of the sense of sweet. Future research must determine the physiological pathways allowing the gastrointestinal system and intracellular energy sensors to control dopamine release during sugar intake (See **Figure 1**).

ACKNOWLEDGMENT

This work was supported by NIH grant DC009997 to IEA.

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

Received: 10 September 2011; accepted: 18 November 2011; published online: 29 November 2011.

Citation: de Araujo IE (2011) Sweet taste signaling and the formation of memories of energy sources. Front. Syst. Neurosci. 5:99. doi: 10.3389/fnsys.2011.00099

frontiers in
SYSTEMS NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 01 March 2012
doi: 10.3389/fnsys.2012.00005

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	One-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 ∼1 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 × 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 ± 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, ∼39%) relative to the pre-conditioning sessions (14 out of 61 neurons, ∼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

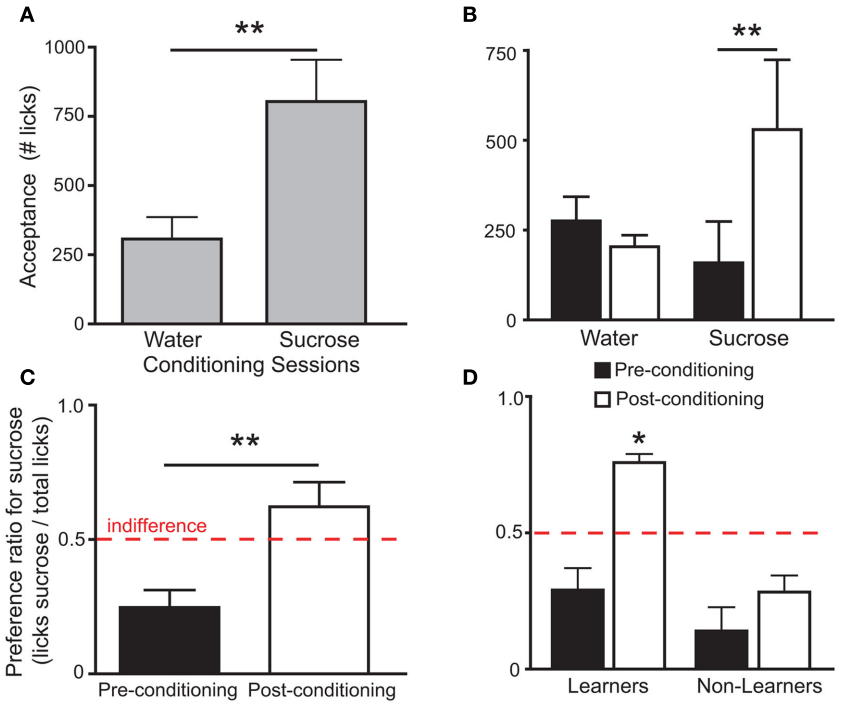

FIGURE 1 | Behavioral responses to sucrose and water in *Trpm5*$^{-/-}$ mice before and after conditioning. In all figures, error bars indicate mean ± SEM. **(A)** In the *Trpm5*$^{-/-}$ animals where IC activity was recorded, average acceptance of sucrose during conditioning sessions (see **Table 1**) 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 ± 3.4%) and Non-Learners (−6.1 ± 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

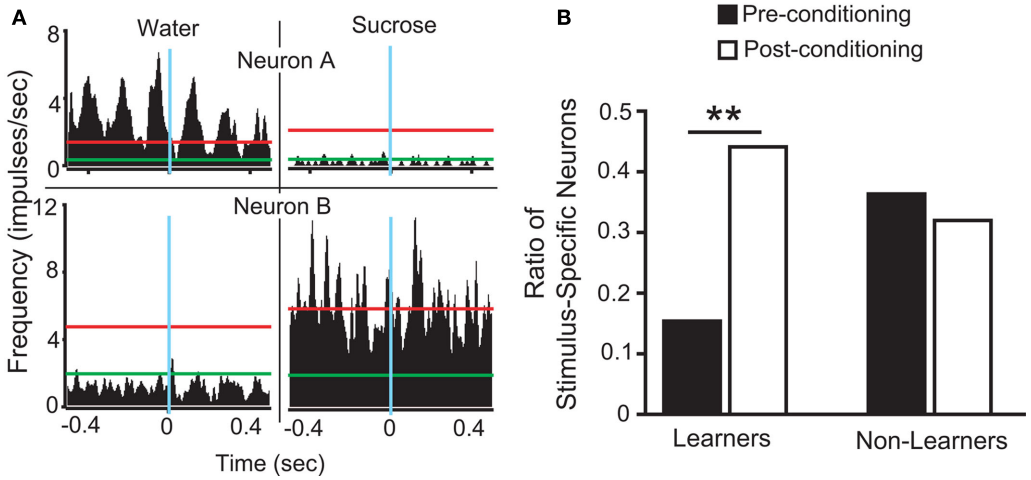

FIGURE 2 | Stimulus selective IC responses to sucrose and water in *Trpm5*$^{-/-}$ mice before and after conditioning. (A) Each panel corresponds to a peri-event histogram centered on licks (blue line) for water (left panels) and sucrose (right panels). The green lines indicate baseline level of activity, and red lines define the 95% confidence interval, as defined in Section "Materials and Methods." Neurons "A" and "B" are examples of stimulus-specific neurons, i.e., those neurons that responded only to water (neuron "A") or to sucrose (neuron "B"). **(B)** The proportion of stimulus-specific neurons increased when post-conditioning sessions were compared to pre-conditioning sessions in Learners (15/34 vs. 6/39 neurons in post- vs. pre-conditioning respectively; $^{**}p < 0.01$), but not in Non-Learners (8/25 vs. 8/22 in post- vs. pre-conditioning respectively; $p > 0.7$). This proportion did not differ significantly between the two groups during pre-conditioning sessions ($p > 0.1$; Fisher's exact test).

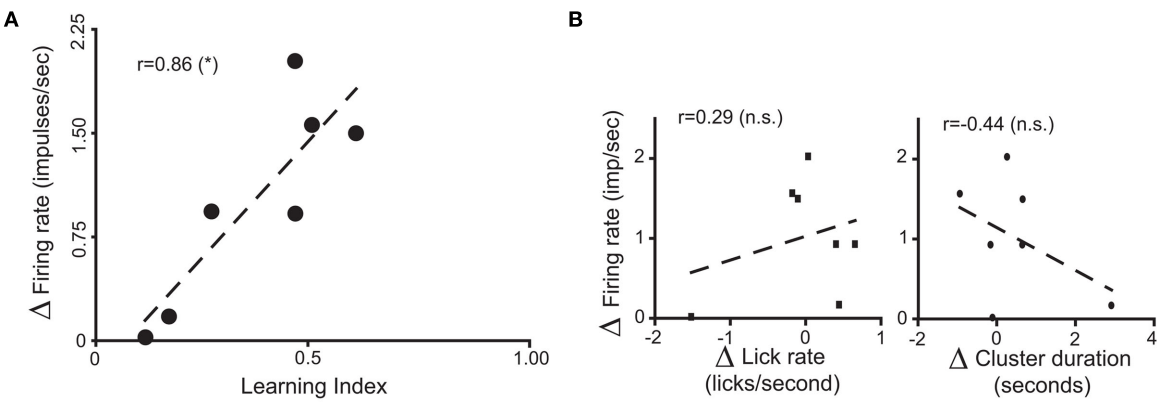

FIGURE 3 | Population responses of IC neural ensembles before and after postingestive learning. (A) "Δ Firing Rate" or "Δ FR," where $\Delta FR = |(FR^{SUC} - FR^{H_2O})_{POST} - (FR^{SUC} - FR^{H_2O})_{PRE}|$, represents the changes in the differential neural population responses to water and sucrose as a function of conditioning (POST and PRE refer to post- and pre-conditioning test sessions respectively). "learning index" (LI) was defined as $LI = (P_{Sucrose})_{POST} - (P_{Sucrose})_{PRE}$, and is a measure of the extent to which *Trpm5*$^{-/-}$ animals increased their preference (*P*) for the sipper associated with sucrose during conditioning (see Materials and Methods for further details). These values were calculated for each animal (each circle represents data from one animal) and a significant positive correlation ($r = 0.86$, $^{*}p < 0.02$) was found between Δ FR and LI. **(B)** To show that the correlation between ΔFR and LI seen in (A) does not arise from learning-induced licking-related, oromotor behaviors, we tested if ΔFR would also correlate with changes in measures of oromotor activity before and after conditioning. To that point, after conditioning, neither Δ of lick rate (left panel) nor Δ of cluster duration (right panel) were correlated with ΔFR ($r = 0.29$ and $r = -0.44$ respectively; $p > 0.3$ for both), showing that they were not responsible for the learning-related changes in IC neural population responses seen in (A).

modifications in licking-related oromotor behaviors. To investigate this possibility, we tested if ΔFR 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 ΔFR (**Figure 3B**), thereby showing that these parameters cannot account for the relationship between ΔFR 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

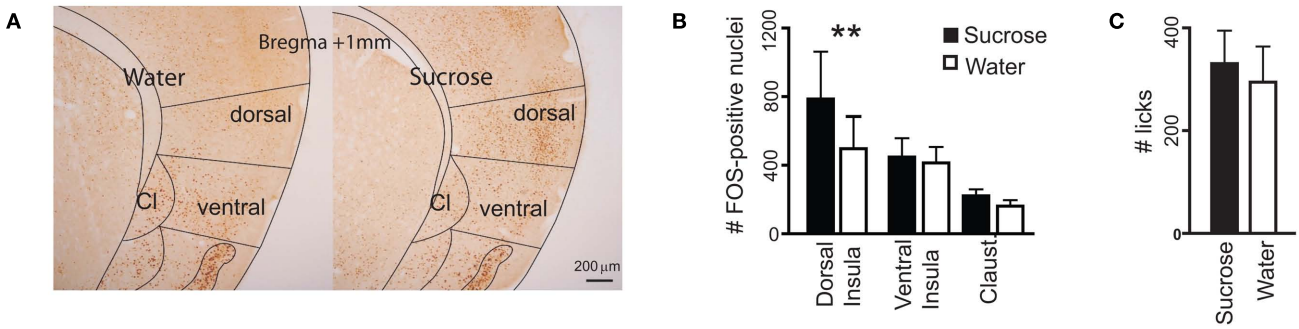

FIGURE 4 | Consumption of sucrose-induced FOS protein expression in the dorsal insula of *Trpm5*$^{-/-}$ mice. (A) Coronal sections of the IC showing FOS expression. The number of FOS-positive nuclei, averaged across brain sections in each animal, was compared between animals following consumption of water or sucrose (examples in left and right panels respectively), in the dorsal and ventral insula and the claustrum (Cl), divided as shown. **(B)** The number of FOS-positive nuclei was not significantly different between the three brain areas ($F_{2,9} = 2.3$, $p > 0.15$), but was impacted by the consumption of sucrose ($F_{1,9} = 5.9$, $p < 0.005$) and the two factors interacted significantly ($F_{2,9} = 5.9$, $p < 0.03$, two-way repeated measures ANOVA). The effect of sucrose ingestion was significant in the dorsal insula (water – 497 ± 188, sucrose – 787 ± 276; $t = 5$, $^{**}p < 0.01$) but not the ventral insula (water – 413 ± 93, sucrose – 447 ± 110; $t = 0.6$, $p > 0.05$) or the claustrum (water – 162 ± 35, sucrose – 221 ± 38; $t = 1$, $p > 0.05$; *Post hoc* Bonferroni). **(C)** Consumption of the two tastants was yoked (water: 295 ± 69 licks, sucrose: 331 ± 64; $t_6 = 0.4$, $p > 0.7$, unpaired two-sample *t*-test).

be found. To further investigate insular sites where postingestive 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 postingestive 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 µg 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 postingestive 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 postingestive 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).

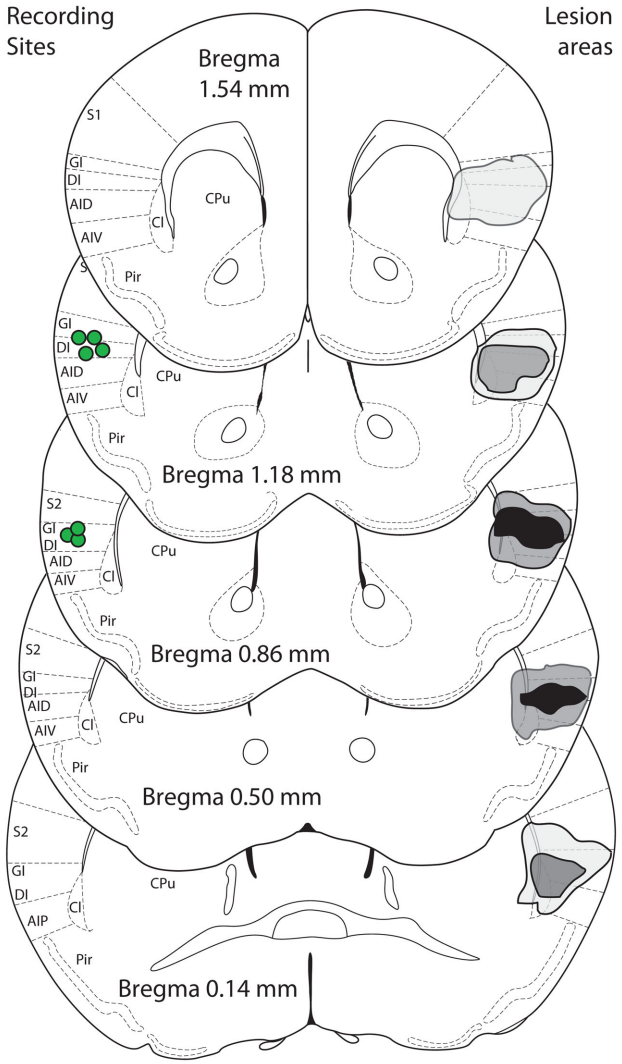

FIGURE 5 | Bilateral IC lesions. Sucrose-induced changes of FOS expression were found in the same area of the insula (dorsal) from which neural activity was recorded. A bilateral lesion study was performed to target that same area. Schematic figures (modified from Paxinos and Franklin, 2001) show composite lesion areas on the right side of image, as assessed by microscopic examination for cannula tract and areas of gliosis. Black areas are where all animals were lesioned bilaterally, light gray when only one animal was lesioned unilaterally or bilaterally and dark gray for intermediate situations. The center of recording sites in animals with multielectrode arrays implanted is shown on the left side (green circles). S1/S2, somatosensory cortices; GI, granular insula; DI, dysgranular insula; AID/AIV/AIP, agranular insula; CI, claustrum; Pir, piriform cortex; CPu, caudate/putamen.

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

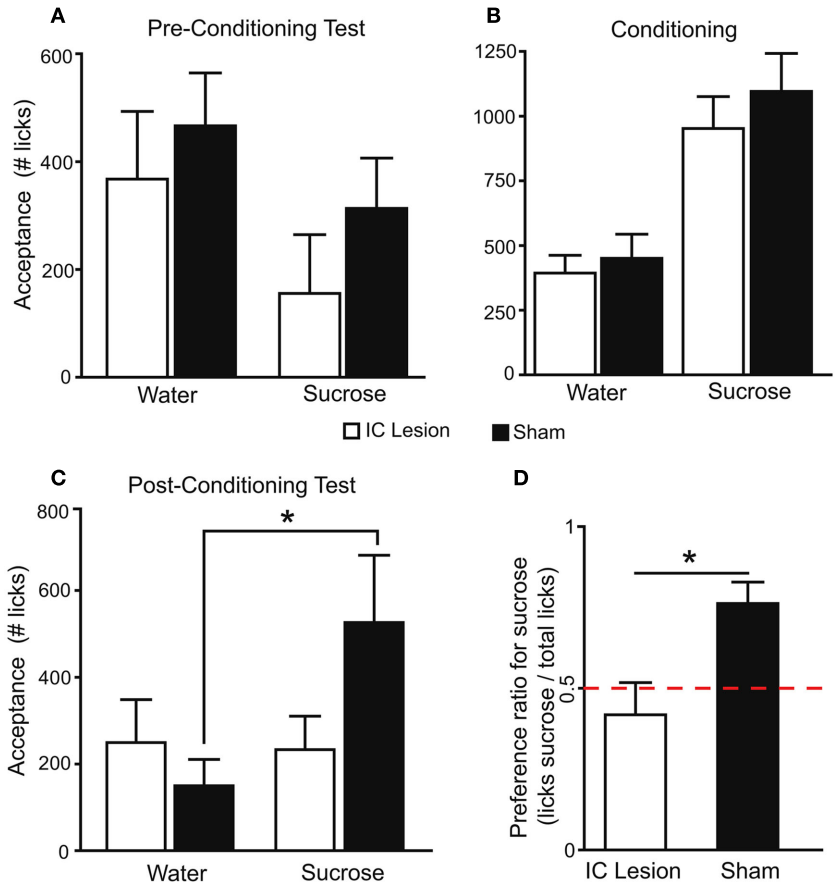

FIGURE 6 | The IC is necessary for the expression of taste-independent preferences conditioned by postingestive reward.(A) In pre-conditioning sucrose vs. water two-bottle preference tests, since sucrose was presented on the side opposite to bias, mice consumed more water (lesion: 368 ± 125 licks; sham: 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.

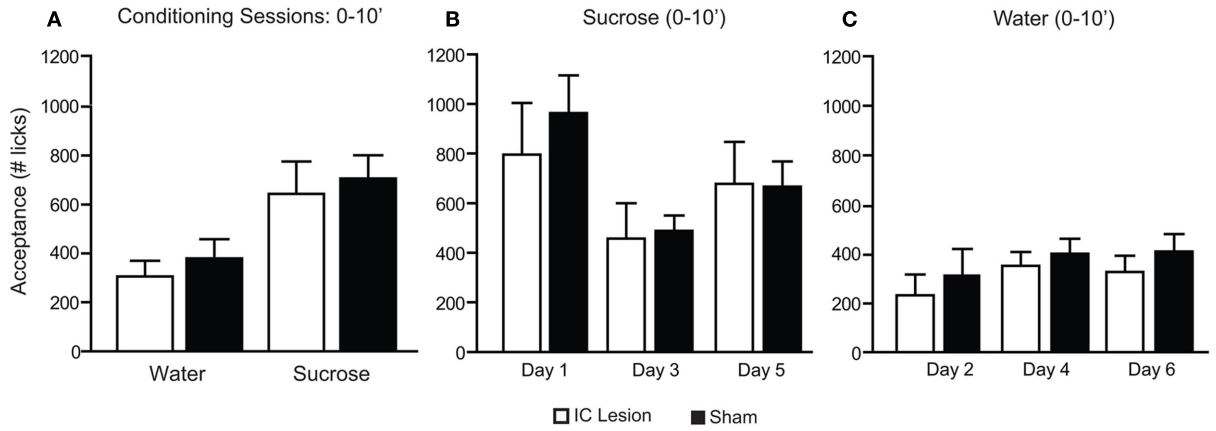

FIGURE 7 | Consumption patterns of sucrose and water during the first 10 min of conditioning sessions in animals with bilateral IC or sham lesions. (A) During the initial 10 min (10′) of conditioning sessions, animals consumed more sucrose (sham: 706 ± 95; lesion: 644 ± 132) than water (379 ± 79 and 306 ± 65 licks respectively; $F_{1,11} = 39.4$, $p < 0.0001$) and no lesion-dependent effects were found ($F_{1,11} = 0.3$, $p > 0.59$ and $F_{1,11} = 0.01$, $p > 0.91$, respectively for lesion and interaction; two-way, repeated measures ANOVA). **(B)** When the initial 10′ of sucrose consumption was analyzed across sessions a significant effect was found for the comparison of conditioning day 1 (sham: 963 ± 152; lesion: 796 ± 208), day 3 (sham: 488 ± 62; lesion: 457 ± 144) and day 5 (sham: 666 ± 102; lesion: 678 ± 169; $F_{2,11} = 7.2$. $p < 0.005$) but no lesion-dependent effects were found ($F_{1,11} = 0.2$, $p > 0.7$ and $F_{2,11} = 0.4$, $p > 0.68$, respectively for lesion and interaction; two-way, repeated measures ANOVA). Reduced sucrose consumption across days could be potentially ascribed to learned satiety. **(C)** For water sessions a significant effect was also found for the comparison of conditioning day 2 (sham: 320 ± 108; lesion: 234 ± 85), day 4 (sham: 405 ± 62; lesion: 355 ± 57) and day 6 (sham: 414 ± 72; lesion: 330 ± 67; $F_{2,11} = 5.6$, $p < 0.02$) but no lesion-dependent effects were found ($F_{1,11} = 0.5$, $p > 0.49$ and $F_{2,11} = 0.2$, $p > 0.83$, respectively for lesion and interaction; two-way, repeated measures ANOVA).

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 oromotor 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**).

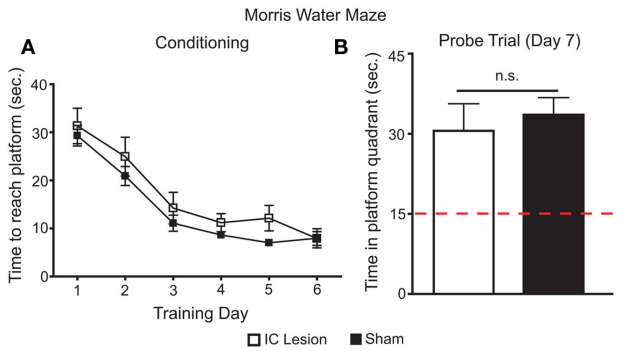

FIGURE 8 | Effects of bilateral IC lesion on spatial orientation learning. **(A)** Sham-operated and IC-lesioned animals were trained, for 6 days, to find a hidden and submerged platform in a Morris water maze (MWM). During training, there was an overall main effect for lesioning (lesioned vs. control subjects, $F_{1,55} = 5.2$, $p < 0.045$), but both groups improved their performance across the six training days ($F_{5,55} = 29.4$, $p < 0.0001$) in a similar fashion (interaction – $F_{5,55} = 0.3$, $p > 0.91$; two-way repeated measures ANOVA) and reached equal levels of performance in the last day (lesion: 7.9 ± 1.4 s to reach platform; sham: 7.9 ± 2; $t = 0.02$, $p > 0.05$; Bonferroni *Post hoc* test). Thus, during training there were only small differences between sham and real lesioned animals. **(B)** On the seventh day, animals were tested for 60 s in a probe trial where the platform was removed from the maze. Here, both groups spent similar times searching for the platform in the correct quadrant of the maze (30.6 ± 5.1 and 33.6 ± 3.2 s respectively; $t_{11} = 0.5$, $p > 0.6$; unpaired two-sample *t*-test) and, in both cases, this search period was significantly higher than what would be expected if they were moving randomly (lesion – $t_5 = 3.1$, $p < 0.03$; sham – $t_6 = 5.8$, $p < 0.003$; one sample *t*-tests vs. 15 s with Bonferroni–Holm's correction for multiple comparisons). Thus, the minor differences found in MWM during training do not seem to explain the deficits found in two-bottle tests (see **Figures 6C,D**) since both groups learned the spatial location of the submerged platform and IC-lesioned animals have seemingly normal spatial orientation memory in the MWM.

In fact, the post-sucrose consumption increase in IC FOS immunostaining in *Trpm5*$^{-/-}$ mice was *restricted to the dorsal subdivision* 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, were 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 postingestive 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 postingestive sensory information. First, we showed that the IC can represent positive postingestive 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 postingestive outcomes, even in the absence of orosensory taste input (**Figure 6**).

While our results support the concept that postingestive 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 postingestive 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 postingestive dependent conditioning seem to reflect a deficit in the capacity to associate between postingestive 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 postingestive 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 postingestive 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).

ACKNOWLEDGMENTS

We thank Teresa Maia, Pedro Nicolelis, Jim Meloy and Gary Lehew for technical support; Drs. Sidney Simon, Ranier Gutierrez, and Shih-Chieh Lin for invaluable discussions and suggestions, and Susan Halkiotis for review of the manuscript. This work was supported in part by funding from NIH R01DC001065 to Dr. Sidney Simon, the Duke School of Medicine Professorship in Neurosciences and NIH R01DE011451 to MALN, Philip Morris USA Inc and Philip Morris International to Dr. Sidney Simon and IEdeA, a GABBA fellowship from FCT and a post-doctoral grant from the AXA Research Fund to Albino J. Oliveira-Maia.

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

Received: 15 December 2011; accepted: 30 January 2012; published online: 01 March 2012.

Citation: Oliveira-Maia AJ, de Araujo IE, Monteiro C, Workman V, Galhardo V and Nicolelis MAL (2012) The insular cortex controls food preferences independently of taste receptor signaling. Front. Syst. Neurosci. ***6****:5. doi: 10.3389/fnsys.2012.00005*

frontiers in
SYSTEMS NEUROSCIENCE

REVIEW ARTICLE
published: 08 December 2011
doi: 10.3389/fnsys.2011.00098

Behavioral and neurophysiological study of olfactory perception and learning in honeybees

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The honeybee *Apis mellifera* has been a central insect model in the study of olfactory perception and learning for more than a century, starting with pioneer work by Karl von Frisch. Research on olfaction in honeybees has greatly benefited from the advent of a range of behavioral and neurophysiological paradigms in the Lab. Here I review major findings about how the honeybee brain detects, processes, and learns odors, based on behavioral, neuroanatomical, and neurophysiological approaches. I first address the behavioral study of olfactory learning, from experiments on free-flying workers visiting artificial flowers to laboratory-based conditioning protocols on restrained individuals. I explain how the study of olfactory learning has allowed understanding the discrimination and generalization ability of the honeybee olfactory system, its capacity to grant special properties to olfactory mixtures as well as to retain individual component information. Next, based on the impressive amount of anatomical and immunochemical studies of the bee brain, I detail our knowledge of olfactory pathways. I then show how functional recordings of odor-evoked activity in the brain allow following the transformation of the olfactory message from the periphery until higher-order central structures. Data from extra- and intracellular electrophysiological approaches as well as from the most recent optical imaging developments are described. Lastly, I discuss results addressing how odor representation changes as a result of experience. This impressive ensemble of behavioral, neuroanatomical, and neurophysiological data available in the bee make it an attractive model for future research aiming to understand olfactory perception and learning in an integrative fashion.

Keywords: olfaction, neural processing, perception, appetitive learning, optical imaging, electrophysiology, brain circuits, insect

INTRODUCTION

Chemical molecules, especially volatile ones, are the vessel of crucial information that may determine an animal's eventual survival and reproductive success. Perhaps for this reason, the sense of chemoreception is ubiquitously represented in the animal kingdom (Ache and Young, 2005). The role of the olfactory system is to decode the complex eddies of molecules in the environment and shape them into pieces of relevant information that will allow the animal to make decisions and engage in adapted behaviors. Major tasks of the olfactory system are for instance the identification of food sources, the detection of possible dangers (such as fire or predators), the recognition of potential mates as well as allowing social interactions. How the nervous system operates this transformation from the detection of chemical molecules via the formation of neural representations until the creation of percepts has been the focus of intense research especially in vertebrates (Lledo et al., 2005; Mori et al., 2006; Leon and Johnson, 2009; Mandairon and Linster, 2009) and in insects (Galizia and Menzel, 2001; Laurent, 2002; Galizia, 2008; Masse et al., 2009). A general finding of these studies is that the basic rules underlying olfactory processing in these different classes of animals are highly similar (Hildebrand and Shepherd, 1997; Ache and Young, 2005). For the most part, this resemblance is thought to result from evolutionary convergence due to similar constraints (Eisthen, 2002).

Olfaction consists in a series of transformations from the chemical world of odor molecules into spatiotemporal patterns of neural activity in the animal's brain, eventually giving rise to a perceptual odor representation. Odor molecules exist in a myriad of chemical compositions, three-dimensional shapes, and vibration properties, to name but a few of their characteristics. They cannot be easily described based on simple dimensions like the wavelength and intensity of stimulus light when studying color vision. Therefore, only multiple descriptors can adequately describe an odorant molecule. In olfaction, the first transformation is thus the detection of particular features of the molecules by dedicated receptor (and associated) proteins, leading through a transduction of the signal to the activation of a subset of receptor cells (Touhara and Vosshall, 2009). This combinatorial code will then be conveyed to a series of structures in the brain and will undergo intense processing leading to a reformatting of the odor representation that will allow the extraction of the most relevant information for the system (Laurent, 2002; Kay and Stopfer, 2006). This processing will then give rise to a perceptual representation used for behavioral decision, and may link odor quality with hedonic value and learned relationships between odor and probable outcomes.

For a century now, the honeybee *Apis mellifera* L. has been a key insect model in which behavioral, neuroanatomical, and neurophysiological approaches have been performed to unravel the basis of olfaction and olfactory learning. Honeybees are social insects which present a wide range of behaviors relying on olfaction both within and outside of the colony (Winston, 1987; Seeley, 1995). Moreover, the study of olfaction is easily amenable to the laboratory, since dedicated protocols have been developed in which bees show rapid and robust odor learning abilities (Menzel, 1999; Giurfa, 2007). In addition, the olfactory pathway of the honeybee brain has been extensively described (Kenyon, 1896; Mobbs, 1982; Strausfeld, 2002; Kirschner et al., 2006) and the bee brain is easily accessible to neurophysiological experiments like electrophysiological or optical imaging recordings (Galizia and Menzel, 2001; Sandoz et al., 2007). We will discuss in turn these different aspects.

OLFACTORY BEHAVIOR IN THE HONEYBEE

ROLE OF PHEROMONES IN SOCIAL LIFE

Honeybees employ a rich repertoire of pheromones to ensure intraspecific communication in many behavioral contexts (Free, 1987; Slessor et al., 2005; Sandoz et al., 2007). The social organization of a honeybee colony is determined by chemical signals produced by the queen, but also by workers. Most honeybee pheromones are complex blends of many substances which are most effective when all components are present in appropriate ratios in the blend. The most important pheromonal components, which were sometimes used in olfactory learning experiments, are detailed below.

The queen, the only fertile female in the colony, communicates her presence mostly by means of a mixture of substances released from her mandibular glands. The queen mandibular pheromone (QMP) was originally considered to be a unique substance, 9-oxo-(E)-2-decenoic acid (9-ODA) (Barbier and Lederer, 1960; Butler et al., 1962), but later studies revealed that the actual pheromone contains several additional components (Slessor et al., 1988; Keeling et al., 2003). The queen pheromone reinforces social cohesion, by attracting workers and enticing them to groom the queen. It also has a physiological effect on workers, inhibiting their ovarian development (Hoover et al., 2003) and modifying gene expression (Grozinger et al., 2003). An interesting aspect of this pheromone is that it acts on different receivers. The queen component 9-ODA thus also acts on males (drones) and plays a crucial role for in-flight mating, attracting them from as far as 60 m (Free, 1987).

The second major source of pheromones is workers, who perform different tasks depending on their age (Winston, 1987). Aggregation pheromones are used by workers to mark and elicit attraction of other workers to important locations (profitable food source, potential nest site, etc.). This pheromone is a complex blend comprising many volatiles among which geraniol and citral are principal components (Pickett et al., 1980). On the other hand, alarm pheromones are released when confronting potential enemies (Breed et al., 2004). The main alarm pheromone is released near the sting and consists of more than 40 highly volatile compounds, among which the major component isopentyl acetate (IPA; Boch et al., 1962; Collins and Blum, 1982; Pickett et al., 1982). Release of this pheromone attracts other bees and causes them to sting and attack. Another alarm pheromone, 2-heptanone, is released by workers' mandibular glands (Shearer and Boch, 1965) and exerts a repellent action on potential intruders and robbers from other hives. Additionally, it is used by foragers to mark recently depleted flowers to avoid immediate revisit (Giurfa and Núñez, 1992).

ROLE OF FLORAL ODORS IN FOOD SEARCH

When reaching 2–3 weeks of age, workers engage in foraging for nectar or pollen outside the hive (Seeley, 1982). Honeybees are generalist pollinators and are not bound to a limited number of plants for gathering food. However, at the individual level, they are "flower constant," memorizing the features of a given floral species, and exploiting it as long as profitable (Grant, 1950; Chittka et al., 1999). Floral cues include color, odor, shape, and texture, but among those, odors play the most prominent role, being most readily associated with nectar or pollen reward (von Frisch, 1967; Menzel et al., 1993). The scent of a flower is a mixture of many volatile compounds that varies with respect to genotype, stage of development, and local environmental conditions (Pham-Delègue et al., 1989; Dobson, 1994; Dudareva and Pichersky, 2000). Flowers of the same plant may show differences in volatile compounds according to the time of day and with respect to their pollination status (Tollsten and Bergström, 1989; Schiestl et al., 1997). To maximize their profit from foraging, honeybees have to show good *olfactory discrimination* capacity. In other words, they have to be able to distinguish between fine differences in the volatile emissions of the visited flowers, to choose flowers whose volatile blend indicates good forage (Menzel, 1985). Indeed, honeybees are able to differentiate between very subtle differences in odor blends, as for instance between two genotypes of the same species or between flowering stages (Pham-Delègue et al., 1989; Wright et al., 2002). On the other hand, many of the variations in volatile emissions displayed by flowers are not indicative of any difference in reward quality, and therefore, another key ability is *olfactory generalization*. This ability corresponds to extending a behavior learned for a given stimulus to other, novel, stimuli, which are perceived as different, but sufficiently similar, to the learned one (Shepard, 1987). As for many lines of work about honeybee behavior and sensory capacities, both of these abilities were first recognized experimentally by Karl von Frisch. In a pioneering investigation, von Frisch (1919) trained free-flying bees to visit an artificial feeder presenting several essential oils (odor mixtures). Using a set of 32 such odors, von Frisch observed that after learning that one odor was associated with sucrose solution, bees tended to prefer this odor over others, clearly discriminating among odors, although they also sometimes visited other odors that were, to the human nose, similar to the rewarded one, thus displaying clear generalization behavior. This work laid the ground to a plethora of experimental studies on the olfactory detection, perception, and learning capacity of honeybees with odors.

OLFACTORY LEARNING PROTOCOLS IN FREELY FLYING AND RESTRAINED BEES

Many experiments have been performed with free-flying bees visiting scented feeders (e.g., Kriston, 1971, 1973; Pham-Delègue et al.,

1993; Laska et al., 1999). These experiments have the advantage of providing an ecologically relevant context, but many variables of the experiment, like bees' physiological status, or the time intervals between learning trials, cannot be precisely controlled. Moreover, the search for the neural basis of olfaction needs the use of neurophysiological methods to monitor the bee brain while it processes and learns odors (Menzel, 1999; Giurfa, 2007). For these reasons, an experimental protocol allowing the study of olfactory learning on restrained individuals was developed, the conditioning of the proboscis extension reflex (PER; **Figure 1A**). The PER was initially described by Minnich (1932) in flies and Frings (1944) in bees. When the antennae, mouthparts, or tarsi of a hungry bee are touched with sucrose solution, the animal reflexively extends its proboscis to suck the sucrose. This response was later conditioned by Kuwabara (1957) and Takeda (1961), by associating visual and olfactory stimuli respectively with a sucrose reward. Perfecting the olfactory version of this protocol, Bitterman et al. (1983) also showed that it corresponds to a case of associative Pavlovian conditioning. Odors to the antennae do not usually release a PER in naive animals. If an odor is presented immediately before the sucrose solution (forward pairing), an association is formed and the odor will subsequently trigger the PER in a following test (**Figures 1A,B**). Thus, the odor can be viewed as the conditioned stimulus (CS) and sucrose solution as the reinforcing unconditioned stimulus (US). This association is thought to recapitulate the final phase of the foraging behavior, when bees drink nectar from an odorous flower.

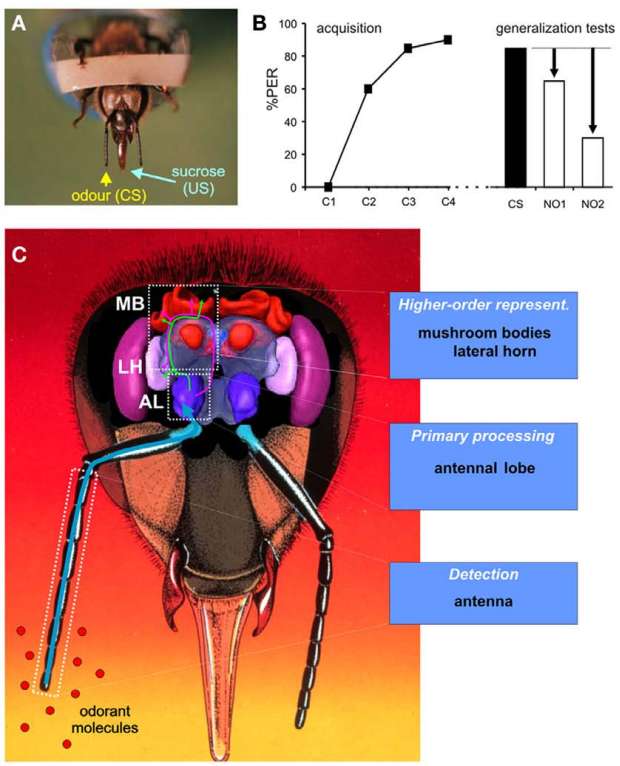

FIGURE 1 | The study of olfactory learning and generalization in bees. **(A)** Conditioning of the proboscis extension reflex (PER) on restrained bees. The PER is a reflex shown by bees when their antennae, tarsi, or mouthparts are contacted with sucrose solution. During conditioning, an odor (conditioned stimulus, CS) is presented in temporal association with sucrose solution to the antennae and to the proboscis (unconditioned stimulus, US), so that the odor progressively gains control over the PER [see acquisition in **(B)**]. After conditioning, presentation of the odor alone triggers the PER. **(B)** Generalization experiment. During acquisition, bees learn to associate the CS with the sucrose reinforcement and respond with a PER to the CS. In a test phase, bees are presented in a random order with the CS and novel odorants 1 and 2 (respectively NO1 and NO2). The perceptual similarity between the CS and each novel odor can be measured as the response level to this novel odor relative to responses to the CS. In this example, NO1 would be considered as more perceptually similar to the CS for bees, than NO2. **(C)** Frontal view of the bee head showing a three-dimensional model (from Brandt et al., 2005) of the brain. Olfactory processing follows three main steps. First, odors are detected at the level of the antenna. Information is conveyed to the antennal lobe (AL) for primary processing. Processed information is then relayed by different pathways to higher-order centers, the mushroom bodies (MB), and the lateral horn (LH), creating multiple olfactory representations in the bee brain (see **Figure 4C**).

More recently, another type of Pavlovian conditioning protocol on restrained individuals was developed, which is based on aversive associations. The sting extension reflex (SER) is a defensive response of bees to potentially noxious stimuli (Breed et al., 2004), which can be elicited experimentally by delivering a mild electric shock to the thorax (Núñez et al., 1983, 1998; Balderrama et al., 2002). During conditioning, harnessed bees learn to associate an initially neutral odor (CS) with the electric shock (US; Vergoz et al., 2007; Giurfa et al., 2009; Roussel et al., 2009). While PER conditioning is appetitive and induces attraction toward the CS in a choice test (Sandoz et al., 2000; Chaffiol et al., 2005; Carcaud et al., 2009), SER conditioning is aversive and bees will accordingly avoid the CS (Carcaud et al., 2009). Hence, olfactory processing, detection and learning capacities of honeybees can now be studied and compared with respect to different reinforcement modalities.

LEARNING OF ODORS WITH DIFFERENT BIOLOGICAL MEANINGS

The olfactory abilities and behavior of honeybees are the fruit of millions of years of co-evolution between hymenoptera and angiosperms. One could imagine that it would be beneficial to bees to not only be able to learn the features of rewarding flowers, but also to "know" in advance the sensory characteristics of a potential food source (Menzel, 1985). Do bees have an "innate search image"? It was initially expected that bees would only be able to learn floral odorants in an appetitive context, but the extreme plasticity of their olfactory learning behavior was soon recognized (von Frisch, 1919). While certain odorants are clearly attractive to bees prior to foraging (essential oils, aggregation pheromones), others are also clearly repulsive (propanol, 3-methyl indole). However, bees can still learn to associate these stimuli with sucrose reward, both in free-flying (Kriston, 1971, 1973) and in restrained conditions (Vareschi, 1971). Nevertheless, some odorants will be learned more quickly than others (above studies), and after learning, may produce stronger or longer responses (Smith and Menzel, 1989). However, it is difficult to interpret such differences as truly "innate," since bees already learn odors within the hive (Farina et al., 2005) and may not be truly naïve when used in conditioning experiments.

Interestingly, bees can even learn to associate pheromonal odors with sucrose reinforcement. This has been proven with aggregation pheromones (citral, geraniol, Getz and Smith, 1987; Smith, 1991; Laska et al., 1999), and even more surprisingly with alarm pheromones (IPA and 2-heptanone, Smith and Menzel, 1989; Laska et al., 1999; Sandoz et al., 2001). However, even though learning does take place, social pheromones do not seem to be treated like general odors (Getz and Smith, 1991; Sandoz et al., 2001). For instance alarm pheromones (IPA and 2-heptanone) produce very high generalization to other odors (Sandoz et al., 2001). Most puzzling, although these two molecules do not have a similar structure, very high generalization was observed between them, suggesting that bees may have also associated their biological value (here alarm) with the appetitive reward and used this information to generalize.

ODOR DISCRIMINATION AND GENERALIZATION

As explained above, stimulus discrimination and generalization are two crucial abilities for bees. To study discrimination, researchers use *differential conditioning* procedures: bees are repeatedly presented with two odors, one (CS+) that is associated with reinforcement, while the other (CS−) is presented without reinforcement. If bees respond significantly more to the CS+ than to the CS−, it can then be concluded that they can discriminate between them. To study generalization, bees are simply conditioned to one odorant (CS) and are then presented with novel odorants without reinforcement (**Figure 1B**). The perceived similarity between the CS and each novel odorant is measured as the level of response to this odorant relative to the CS (amount of generalization, **Figure 1B**).

An important question in sensory neuroscience is along which dimensions animals measure similarity among stimuli (Shepard, 1987). Vareschi (1971) was the first to use PER conditioning to study the discrimination capacities of honeybees with a wide range of odors. He used a kind of differential conditioning, with one rewarded odor (CS) and 27 non-rewarded odors presented in-between CS trials. Bees were found to differentiate the odors from >95% of the 1816 tested odor pairs. The same high discrimination ability is also found in free-flying bees (97% of 1848 tested odor pairs, Laska et al., 1999).

In the bee, as in vertebrates (Mori et al., 2006; Johnson and Leon, 2007), aliphatic odor molecules have attracted the interest of researchers because they can be described by two main characteristics: their chemical group and the length of their carbon chain. Bees generalize more often between odors with similar carbon chain lengths or belonging to the same functional group, as found with restrained (Smith and Menzel, 1989) and with free-flying bees (Getz and Smith, 1990; Laska et al., 1999). Recently, Guerrieri et al. (2005b) systematically studied the generalization behavior of bees with 16 odorants presenting all combinations of four possible functional groups (primary and secondary alcohols, aldehydes, ketones) and chain lengths (six to nine carbons). These authors found that generalization is not always symmetrical, so that generalization from odor A to odor B is not always the same as from B to A. Strikingly, learning an aldehyde induced low generalization to other odors, while bees often responded to aldehydes after learning other odorants. In this study, the first factor determining honeybees' generalization behavior was a molecule's chain length, followed by the chemical group. This was the demonstration on a simple set of odor molecules that chemical dimensions are somehow encoded in the brain of honeybees and determine their behavior (Guerrieri et al., 2005b). However the bees' natural environment provides an incredible wealth of possible odor molecules and we are still far from knowing the encoding dimensions for all these molecules.

ODOR CONCENTRATION

The fact that honeybees are able to learn absolute odor concentrations was recognized by Kramer (1976), who trained individual workers in simulated odor gradients using a locomotion compensator with feedback control of odor concentration. The bees were reinforced with sucrose solution at a particular concentration of an odor, and were then placed at different concentrations. They showed a typical upwind walk when placed in a range of concentrations relatively close to the learned one (20–180%), but walked downwind when placed outside of these boundaries. Moreover, bees showed a particular alerting behavior at about 85–90% of the learned concentration (Kramer, 1976). Similarly, free-flying bees visiting a vertical odor array choose the right odor at the right concentration and reject higher or lower concentrations (Ditzen et al., 2003). In contrast to the freely moving situation, differential conditioning with two concentrations of the same odor is difficult in harnessed bees (Bhagavan and Smith, 1997; Pelz et al., 1997). Honeybees' sensory capacity and motivation may be different in these two situations. In the visual modality, for instance, honeybees easily associate colors or patterns with sucrose reward when flying freely, but show much lower performance when restrained (especially when the antennae are not cut, Hori et al., 2006; Mota et al., 2011).

Concentration strongly influences the salience of olfactory stimuli. Generally, odors are learned more quickly at higher concentration (Bhagavan and Smith, 1997; Wright et al., 2009), and support better memory consolidation (Pelz et al., 1997). Moreover, conditioned responses to a high concentration are produced more quickly, suggesting that the olfactory system needs less time to determine odor quality at high than at low concentration (Wright et al., 2009). The discrimination power between different odorants also increases with their concentration (Getz and Smith, 1991; Wright and Smith, 2004). Lastly, bees generalize more from low to high concentrations, than from high to low concentrations (Marfaing et al., 1989; Getz and Smith, 1991; Bhagavan and Smith, 1997; Pelz et al., 1997). However, in some instances bees generalize more between different odors at the same concentration, than between different concentrations of the same odor (Wright et al., 2005). To summarize, odor identity is not totally invariant as a function of concentration, so that it is both possible for bees to differentiate between concentrations of an odorant, but also to show high generalization between different concentrations of this odorant. Such versatile capacities may be crucial when foraging for identifying and locating floral sources.

THE CASE OF OLFACTORY MIXTURES

Natural floral odors encountered by foraging bees are not single molecules but complex mixtures (Knudsen et al., 1993). Honeybees are thus confronted to the problem of discriminating among complex blends but also of recognizing the same floral source although its blend composition varies. Some authors have attempted to understand complex mixture processing in learning experiments with whole floral extracts (Pham-Delègue et al., 1986; Le Métayer et al., 1997) or with synthetic mixtures of six to 14 components (Pham-Delègue et al., 1993; Wadhams et al., 1994; Blight et al., 1997; Reinhard et al., 2010). A general finding of these experiments is that when bees learn a mixture and are afterward tested with the individual components, they usually respond to some components more strongly than to others. Such components have been termed key-compounds (or key-components, Wadhams et al., 1994; Laloi et al., 2000; Reinhard et al., 2010). What determines that a component is a key-component? Neither relative quantity nor volatility are predictive (Wadhams et al., 1994; Le Métayer et al., 1997; Reinhard et al., 2010). Rather, the perceptual salience of a component appears to be important, as measured by the conditioning success with this odor presented alone (Laloi et al., 2000). Additionally, whether a component will be learned in a mixture depends on the identity of the other components (Laloi et al., 2000; Reinhard et al., 2010). Thus, the processing of different odorants simultaneously produces unpredictable outcomes, a phenomenon termed "mixture interaction." Due to the apparent complexity of mixture processing, research on mixture interactions has focused on binary mixtures (Getz and Smith, 1987, 1990, 1991; Chandra and Smith, 1998; Smith, 1998; Deisig et al., 2001). Generally an odor is better learned when presented alone, than when together with a second odorant (Smith, 1998). Usually, when learning a mixture AB, bees can recognize the components (Getz and Smith, 1987, 1990). However, one component is often learned better than the other, a phenomenon called "overshadowing." Using three odors presented in the form of binary mixtures, Smith (1998) found that overshadowing depended on which odors were in a pair, so that overshadowing was difficult to predict. Mixture interactions may also depend on the sequence of experiences the bee has had with the stimuli. In the phenomenon of "blocking," initial learning of an odorant A blocks learning of odorant B when the mixture AB is subsequently trained. Although this effect has been observed in different studies (Smith and Cobey, 1994; Linster and Smith, 1997; Hosler and Smith, 2000), it remains controversial, as it only rarely appeared when possible confounding variables were controlled (Gerber and Ullrich, 1999; Guerrieri et al., 2005a).

On a theoretical level, concepts from psychophysical theories have been used to attempt to understand how a mixture is represented in the bee brain (Chandra and Smith, 1998; Deisig et al., 2001; Lachnit et al., 2004). Two widely differing theories have been put to the test. First, the elemental approach assumes that a compound AB will be represented in the brain as two elements, A and B, each of which can be associated with the US (Rescorla and Wagner, 1972). In other terms, "the whole equals the sum of its parts." On the other hand, configural approaches propose a radically different view by assuming that the representation of AB is a different entity from those of A and B ("the whole is different from the sum of its parts," Pearce, 1987, 1994). In fact both accounts were shown to be wrong using the so-called patterning experiments (Chandra and Smith, 1998; Deisig et al., 2001). In such experiments, bees have to differentiate between two single odorants A and B and the mixture AB. In negative patterning, the single elements are both reinforced when presented alone (A+, B+), while the mixture is non-reinforced (AB−). Conversely, in positive patterning, the two elements are non-reinforced when presented alone (A−, B−), while the mixture is reinforced (AB+). Honeybees can be trained to solve both tasks with odors (Deisig et al., 2001, 2002). The elemental approach can explain positive patterning but not negative patterning, because when each component is reinforced, a mixture would elicit, through elemental summation, twice as much responding as each component. On the other hand, in its principle, the configural approach could cope with both patterning tasks, as compound and elements are associated with reinforcement independently (Pearce, 1994). However, it ran into problems when analyzing response summation between elements and mixtures at the beginning of conditioning (Deisig et al., 2003). Experiments in bees thus suggested that the best model for explaining mixture learning was an expansion of elemental models, called the unique cue hypothesis (Rescorla, 1972, 1973; Whitlow and Wagner, 1972). In addition to the representations of the elements, the compound would give rise to a supplementary (internal) representation, the *unique cue*. During the negative patterning problem, the unique cue U would build inhibitory associations with the US, while the elements A and B would build excitatory associations. When A and B are presented alone, the excitatory association would thus trigger behavioral responses, but during AB presentations, the added inhibitory strength of the unique cue would hamper the response. Using different types of patterning tasks, it was possible to show that a version of the unique cue hypothesis best coped with all the experimental results (Deisig et al., 2001, 2002, 2003; Lachnit et al., 2004).

For simplicity, all experiments described above considered that mixture composition is stable in time, which is not the case in nature, as floral aroma changes throughout the day and the plant's state. Honeybees seem to focus on those components which remain relatively constant in their concentration (Wright and Smith, 2004). Such ability may be beneficial for bees in order to recognize the same floral species in spite of fluctuations in the composition of its odor blend.

NEUROANATOMY OF THE HONEYBEE OLFACTORY SYSTEM

An advantage of the bee model for understanding olfaction and olfactory learning is that the neuroanatomy of its olfactory pathway is known in great detail (e.g., Kenyon, 1896; Pareto, 1972; Suzuki, 1975; Mobbs, 1982; Abel et al., 2001; Strausfeld, 2002; Kirschner et al., 2006). Olfactory processing follows different steps, from the detection of molecules at the periphery, via primary processing by antennal lobe (AL) networks, until the establishment of olfactory representations in higher-order brain centers (**Figure 1C**). A simplified model of the different neuron types involved in olfactory processing is provided in **Figure 2**.

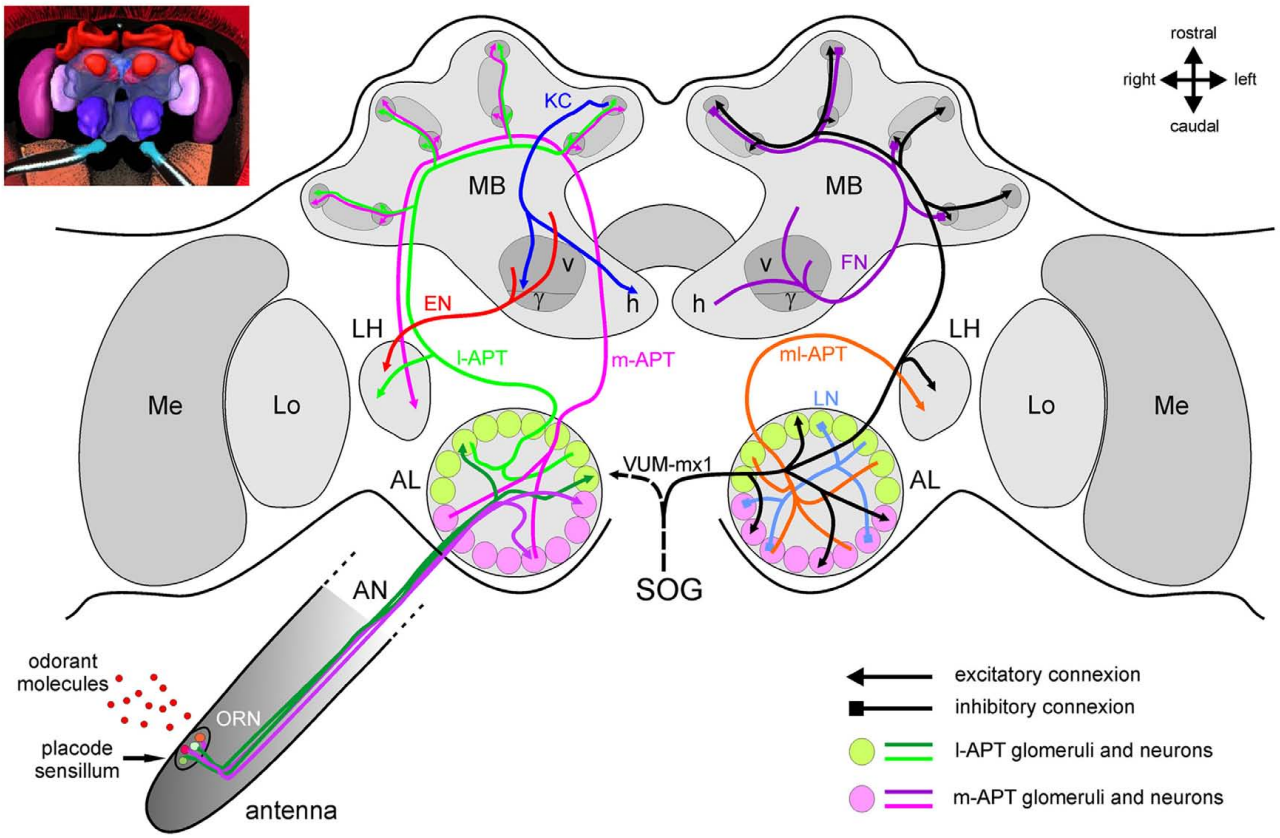

FIGURE 2 | The honeybee brain and the olfactory pathway. For clarity, different neuron types have been presented separately in the two brain hemispheres. On the left, major excitatory pathways involved in the transmission of olfactory information in the brain are shown. On the right, mostly inhibitory connections and modulatory neurons are presented. The antennal lobe, first-order olfactory neuropil, receives input from ∼60,000 olfactory receptor neurons (ORNs) which detect odorants within placode sensilla on the antenna. Within the AL's anatomical and functional units, the 165 glomeruli, ORNs contact ∼4000 inhibitory local neurons (LNs) which carry out local computations, and ∼800 projection neurons which further convey processed information via different tracts. The lateral antenno-cerebralis tract (l-APT) projects first to the lateral horn (LH) and then to the mushroom body (MB) calyces (lips and basal ring), while the medial tract (m-APT) projects to the same structures, but in the reverse order. Both tracts are uniglomerular, each neuron taking information within a single glomerulus. They form two parallel, mostly independent olfactory subsystems (in green and in magenta), from the periphery until higher-order centers, where they project in non-overlapping regions. Multiglomerular projection neurons form a medio-lateral tract (ml-APT) which conveys information directly to the medial protocerebrum and to the LH. The dendrites of the Kenyon cells (KCs), the mushroom bodies' 170,000 intrinsic neurons, form the calyces, while their axons form the pedunculus. The output regions of the MB are the vertical and horizontal lobes, formed by two collaterals of each KC axon. Within the MBs, feedback neurons (FN) project from the pedunculus and lobes back to the calyces, providing inhibitory feedback to the MB input regions. Extrinsic neurons (ENs) take information from the pedunculus and the lobes and project to different parts of the protocerebrum and most conspicuously to the LH. It is thought that descending neurons from these areas are then involved in the control of olfactory behavior. The figure also presents a single identified octopaminergic neuron, VUM-mx1, which was shown to represent reinforcement during appetitive conditioning. This neuron projects from the suboesophageal ganglion (SOG), where it gets gustatory input from sucrose receptors, to the brain and converges with the olfactory pathway in three areas, the AL, the MB calyces, and the LH.

PERIPHERAL ODOR DETECTION: THE ANTENNA

Peripheral odor detection starts at the level of olfactory receptor neurons (ORNs), which are located below cuticular structures on the antennae, called sensilla (Kaissling, 1987). Different morphological types of sensilla exist on the insect antenna, but sensilla placodea (pore plate sensilla) are the main olfactory sensilla in the honeybee (Esslen and Kaissling, 1976). A sensillum placodeum is formed by an oval-shaped (9 μm × 6 μm) thin cuticular plate with numerous minute pores and is innervated by five to 35 ORNs (Schneider and Steinbrecht, 1968; Esslen and Kaissling, 1976; Kelber et al., 2006). Odorant molecules reach the dendrites of ORNs by diffusing through an extracellular fluid, called the receptor or sensillum lymph, filling the sensillum cavity (Kaissling, 1987; Masson and Mustaparta, 1990). In this fluid, odorant binding proteins (OBPs) may help transporting odorants to the ORNs but very little is known about them in bees.

When reaching the ORN membrane, the odorant molecule interacts with the olfactory receptor protein (OR). Insect ORs

belong to a family of highly divergent proteins with seven-transmembrane domains, which are different from the vertebrate OR family (Benton, 2006; Touhara and Vosshall, 2009). The functional receptor is a heteromeric complex of an OR and a broadly expressed co-receptor AmOr2, which is the honeybee ortholog to the co-receptor Or83b of *Drosophila* (Benton et al., 2006; Robertson and Wanner, 2006). Honeybees present a remarkable expansion of the insect odorant receptor family relative to the repertoires of the fly *Drosophila melanogaster* and the mosquito *Anopheles gambiae*, which respectively possess 62 and 79 ORs, with a total of 170 OR genes including seven pseudogenes (Robertson and Wanner, 2006).

THE PRIMARY OLFACTORY CENTER: THE ANTENNAL LOBE

ORN axons form the antennal nerve and project to a primary olfactory center in the brain, the AL (**Figure 2**). The bee AL is compartmentalized in 165 spheroidal neuropile units called glomeruli. Glomeruli are the anatomical and functional units of the AL and constitute the first site of synaptic interaction between ORNs and other neuron types. Glomeruli can be recognized based on their relative position, size, and shape, using an anatomical atlas of the AL (Flanagan and Mercer, 1989a; Galizia et al., 1999a). In *Drosophila* axons of ORNs expressing the same odorant receptor converge onto the same glomerulus (Vosshall et al., 2000; Dahanukar et al., 2005). Thus, the array of AL glomeruli corresponds to an array of OR types. Noticeably, the number of 163 potentially functional ORs in bees coincides with the number of glomeruli in the AL (∼165). This would thus support the one-receptor/one-ORN/one-glomerulus hypothesis in bees.

Within each glomerulus, ORNs release acetylcholine (ACh), the primary excitatory transmitter of the insect brain (Bicker, 1999). Thus doing, they activate local neurons (LNs) connecting different glomeruli and projection neurons (PNs), which relay the olfactory message processed at the level of the AL to higher-order centers such as the lateral horn (LH) and the mushroom bodies (MBs).

Local neurons are neurons whose branching patterns are restricted to the AL (**Figure 2**). The ∼4000 LNs can be classified in two main types. One type innervates most if not all glomeruli in a uniform manner, and are therefore called homogeneous LNs (homo-LNs; Flanagan and Mercer, 1989b; Fonta et al., 1993). Neurons of the second type innervate only a small subset of glomeruli and are called heterogeneous LNs (hetero-LNs). They have one dominant glomerulus with very dense innervation and a few other glomeruli with very sparse processes (Flanagan and Mercer, 1989b; Fonta et al., 1993). Hetero-LNs branch in the core of the sparsely innervated glomeruli but branch in the whole (core and cortex) of their densely innervated glomerulus (Fonta et al., 1993; Abel et al., 2001). LNs are thought to carry out the first processing of olfactory information, with two different functions of Homo-LNs and Hetero-LNs, respectively global inhibition for gain control and asymmetrical lateral inhibition between glomeruli for refining odor representation and allowing better discrimination among olfactory representations (Sachse and Galizia, 2002).

Local neurons in bees are mostly inhibitory and may use many different neurotransmitters. About 750 LNs are GABAergic (Schäfer and Bicker, 1986) and functional data indicate that GABA is indeed inhibitory in the AL (Stopfer et al., 1997; Sachse and Galizia, 2002; Dupuis et al., 2010). In addition, glutamate (for review see Bicker, 1999) and histamine (only about 35 neurons, Bornhauser and Meyer, 1997) have been identified in the AL. Several lines of evidence indicate that glutamate (Barbara et al., 2005; El Hassani et al., 2008) and histamine (Sachse et al., 2006) also act as inhibitory neurotransmitters in the bee brain. Lastly, the AL houses many, often small, subpopulations of LNs which each express characteristic peptides, including allatostatins, allatotropin, tachykinins, FMRFamide, and other RFamide peptides (Galizia, 2008; Kreissl et al., 2010).

Projection neurons connect the AL with higher-order brain areas (**Figure 2**), following five different pathways, called antennoprotocerebral tracts (APTs; Mobbs, 1982; Abel et al., 2001). From their morphology, PNs can also be classified in two types. Uniglomerular projection neurons (uPNs) branch in a single glomerulus within the AL and have axons that project to the MBs and to the LH using the two major APT tracts (see below). On the other hand, multiglomerular projection neurons (mPNs) branch in most glomeruli and are therefore potentially capable, in contrast to uPNs, to extract combinatorial information across glomeruli. Their axons follow the three lesser tracts, the medio-lateral (ml) APTs, leading not to the MBs, but to other areas of the protocerebrum, surrounding the α-lobe of the MB or extending toward the LH (Abel et al., 2001; Kirschner et al., 2006).

The more numerous uniglomerular PNs (∼800) form two roughly equal tracts toward higher-order brain centers, the lateral (l-APT), and medial (m-APT) tract. The l-APT leaves the AL dorsally and then runs on the lateral side of the protocerebrum, forming collaterals in the LH and then continuing on to the MB calyces. The m-APT runs along the brain midline first toward the MBs where collaterals enter into the calyces, and then travels laterally to end in the LH (Abel et al., 2001; Kirschner et al., 2006). The current understanding of this anatomical organization is that the honeybee brain may arbor two parallel olfactory subsystems, as excitatory transmission of the olfactory message follows essentially two independent pathways in each brain hemisphere toward higher-order brain centers. L- and m-APT neurons take their information from two non-overlapping groups of 84 and 77 glomeruli respectively (Abel et al., 2001; Kirschner et al., 2006). Following the current hypothesis that each glomerulus is the projection center for all ORNs expressing a given OR, one may thus say that PNs from the l- and m-APT each transmit information about two independent portions of the honeybee odor detection repertoire. Moreover, the central projection areas of the two PN tracts are segregated in the MB calyces and in the LH with only partial overlap (Abel et al., 2001; Kirschner et al., 2006). How are the two subsystems connected through local networks? Several hypotheses are possible, from almost total segregation of the subsystems with hetero-LNs providing lateral inhibition only within each system, to an equal and symmetrical weight of lateral inhibition between both subsystems (Galizia and Rössler, 2010). However, no data are available yet concerning this question.

SECOND-ORDER OLFACTORY CENTERS: THE MUSHROOM BODIES AND THE LATERAL HORN

Olfactory information leaving the AL takes several routes to the MBs and LH (**Figure 2**). While the function of the LH is still unclear, the MBs are thought to be involved in a sparsening of olfactory representation, in olfactory learning and memory, as well as the integration of olfactory information with other sensory modalities (Menzel et al., 1994; Menzel, 1999; Giurfa, 2003, 2007).

MB-intrinsic neurons are the Kenyon cells (KCs; Kenyon, 1896), which form two cup-shaped regions called calyces in each hemisphere. MB calyces are anatomically and functionally subdivided into the lip, the collar, and the basal ring (Mobbs, 1982, 1984; Gronenberg, 2001; Strausfeld, 2002). The lip region and the inner half of the basal ring receive olfactory input, whereas the collar and outer half of the basal ring receive visual input (Gronenberg, 2001), in addition to input from mechanosensory and gustatory pathways (Strausfeld, 2002; Schröter and Menzel, 2003). The projections of individual PNs extend in most parts of each calyx (Müller et al., 2002). PN boutons form multisynaptic microcircuits in the MB lips, with GABAergic input and KC output connections arranged to form particular structures termed microglomeruli (Ganeshina and Menzel, 2001). KC axons project in bundles into the midbrain, forming the peduncle and the vertical and horizontal lobes (also called α and β lobes). The calyx is topologically represented in the lobes (Mobbs, 1982; Strausfeld et al., 2000; Strausfeld, 2002). About 55 GABAergic feedback neurons from the MB output lobes project back to the calyces (Bicker et al., 1985; **Figure 2**). Due to the parallel arrangement of intrinsic KCs, most subcompartments in the calyx receive feedback from their corresponding layer in the α lobe (Grünewald, 1999). Most KCs provide bifurcating axons to both α and β lobes. In the bee, about 800 PNs diverge onto a major proportion of the 170,000 KCs of each MB (i.e., onto olfactory KCs). Each PN contacts many KCs and each KC receives input from many PNs. If the figures calculated for the locust *Schistocerca americana* (Jortner et al., 2007) were to apply to the honeybee, each KC would contact about 400 PNs (i.e., 50% of the total PN count). This organization appears ideal for a combinatorial readout across PNs (Laurent, 2002).

The second major target area of both the m- and l-APT uPNs is the LH. In addition to the uPN innervation, the LH receives input from mPNs via the ml-APTs (Fonta et al., 1993). Similarly to the olfactory input of the MB calyx, the LH shows a PN tract-specific compartmentalization, with at least four subcompartments: one receives exclusively projections of m-APT uPNs, while others receive mixed input from m- and l-APT PNs, from l-APT and ml-APT PNs, or from the latter type alone (Kirschner et al., 2006). Possible local computations within this structure as well as the connectivity between PNs and other neurons are still mostly unknown.

MUSHROOM BODY OUTPUT

A number of neuron populations project from the MBs toward other brain centers (**Figure 2**), with two major output regions being the α and β lobes (Mobbs, 1982). About 400 extrinsic neurons (ENs) from the α lobe have been studied in details (Rybak and Menzel, 1993). Some are unilateral neurons with projection fields restricted to the ipsilateral protocerebrum, while others are bilateral neurons connecting both α lobes, or projecting from one lobe to the contralateral protocerebrum around the α lobe (Rybak and Menzel, 1993). A single conspicuous neuron in each MB, called Pe-1, forms a major output pathway from the peduncle of the MBs (Mauelshagen, 1993). This neuron arborizes extensively in the peduncle and projects to the lateral protocerebral lobe, and more specifically to the LH where it synapses directly or via interneurons onto descending neurons involved in behavior.

Although the anatomical description of projections to the LH is good, knowledge of the neurons leaving the LH and of descending pathways involved in behavioral output is still scarce in bees. Some anatomical descriptions of descending neurons in other insects, like cockroaches, suggest that their dendrites are distributed mainly in the lateral and medial protocerebrum, which are major termination areas of MB output neurons, but not in the AL, MBs, or regions of the LH receiving PN input (Okada et al., 2003). Thus it is possible that both MB output neurons and yet unknown LH output neurons contact descending neurons and can therefore modulate behavior. Investigation of descending neurons and the neural pathways involved in behavioral control in bees may help bridge this gap (Ibbotson and Goodman, 1990; Ibbotson, 2001; Schröter et al., 2007).

AVERSIVE AND APPETITIVE REINFORCEMENT INFORMATION

The olfactory pathway also receives input from different modulatory systems. Of special importance are the reinforcement systems necessary for the formation of neural associations between odors and particular outcomes. Such associations rely on the coactivation of two neural pathways, the olfactory pathway and a pathway representing the specific reinforcement. As in other insects, appetitive reinforcement in bees depends on octopamine (Hammer and Menzel, 1998; Farooqui et al., 2003) and aversive reinforcement on dopamine (Vergoz et al., 2007). A single, putatively octopaminergic, neuron in the bee brain, VUM-mx1 (**Figure 2**), was shown to represent a neural substrate of the sucrose US pathway (Hammer, 1993), because the forward (but not backward) pairing of an odor CS with an artificial depolarization of VUM-mx1 produces an associative memory trace. VUM-mx1, has its cell body in the suboesophageal ganglion (SOG), and converges with the olfactory pathway in both brain hemispheres at three sites, in the AL, in the MB calyces, and in the LH. Another neuron with a similar projection pattern has been found with its cell body in another neuromere of the SOG (VUM-md1, Schröter et al., 2007). On the other hand, many dopaminergic neurons are found in the bee brain (Schäfer and Rehder, 1989; Schürmann et al., 1989) but until now, none of them could be shown to provide aversive reinforcement.

NEUROPHYSIOLOGICAL STUDY OF OLFACTORY PROCESSING AND LEARNING

PERIPHERAL ODOR DETECTION: THE ANTENNA

The search for the neural correlates of olfactory detection and processing has started at the periphery, using extracellular recordings

of single placodes (e.g., Lacher and Schneider, 1963; Lacher, 1964; Vareschi, 1971; Akers and Getz, 1992, 1993). The first demonstration that placode sensilla were responsible for olfactory detection was provided by Lacher and Schneider (1963) with individual placode recordings showing responses to benzylacetate in workers, and caproic acid in drones, but no answers to light, sound, water vapor, or CO_2. ORNs show little spontaneous activity, but respond to odors mostly with a spike frequency increase in a phasic-tonic manner. Sometimes, they also show inhibitory responses or off-responses (response at the end of the stimulus). Because a single placode houses many ORNs it is difficult to segregate the responses of individual cells based on spike amplitude. Using statistical techniques to attempt such segregation, Akers and Getz (1992) found that units with similar odor-response spectra were more likely to be found in different placodes than within the same placode. This observation fits with anatomical data showing that ORNs from a placode innervate different glomeruli (Kelber et al., 2006). The complex organization of ORNs in the honeybee antenna has strongly hindered efforts to study peripheral odor detection in this species.

OLFACTORY PROCESSING IN THE ANTENNAL LOBE

Thanks to the technique of *in vivo* calcium imaging, it was however possible to record neural activity at the glomerular level (Joerges et al., 1997, **Figure 3**). This recording technique uses fluorescent dyes to measure the increase of intracellular calcium (coming from the extracellular medium and/or released from internal stores) following neuronal excitation (Joerges et al., 1997; Galizia and Vetter, 2004). In the most simple form of this technique, bees are fixed in a recording chamber, and the head capsule is carefully opened (**Figures 3A,B**). Membranes and tracheas covering the brain are removed, and a calcium-sensitive fluorescent dye (for instance, Calcium Green 2-AM) is bath-applied onto the brain (**Figures 3C,D**). After about 1 h incubation, during which the dye has penetrated AL cells, the brain is rinsed with saline solution and the bee is placed under an upright fluorescence microscope in front of an odor stimulation device (**Figure 3A**). This bath-application of Calcium Green 2-AM allows recording a composite calcium signal which could potentially come from all cell populations of the AL: ORNs, LNs, PNs, and glial cells (Joerges et al., 1997). Due to the numerical preponderance of ORNs and because odor-induced signals have a very stereotypical time course and do not show spontaneous activity or inhibitory responses (the hallmark of LNs and PNs, see below), these recordings are thought to emphasize presynaptic calcium variations from ORNs (Galizia et al., 1998; Sachse and Galizia, 2003), with a possibly significant contribution from glial cells surrounding each glomerulus (Galizia and Vetter, 2004). The compound signal has therefore been long interpreted as representative of sensory input (Sachse and Galizia, 2003; Deisig et al., 2006, 2010; Sachse et al., 2006).

Optical imaging experiments showed that odors elicit combinatorial activity patterns across glomeruli (Joerges et al., 1997; see **Figure 3D**). Combining imaging recordings with anatomical staining allowed assigning activity patterns to identified glomeruli using the published anatomical AL atlas (Galizia et al., 1999a). This showed that odor quality is represented in the AL according to a specific distributed code conserved between individuals (Galizia et al., 1999b; Sachse et al., 1999). Each glomerulus – representing an ORN type expressing a given OR – shows a rather broad molecular receptive range (Galizia et al., 1999b). Because the optical imaging technique allows recording activity only at the tissue surface, only a small part of the 165 glomeruli could be accessed (up to 38 glomeruli; Sachse et al., 1999). The question therefore arose whether the signals recorded in this subpart of the AL had any significance with regards to odor representation and olfactory behavior. To answer this question, Guerrieri et al. (2005b) studied the generalization behavior of honeybees among a panel of 16 odorants for which the activity patterns in these glomeruli were known (Sachse et al., 1999). As mentioned above, these authors built a complete generalization matrix among the 16 odorants differing according to their functional group and their carbon chain length (**Figure 4A**). Importantly, this work demonstrated for the first time a significant correlation between the similarity among odors in the behavior and in the neurophysiological recordings (**Figure 4B**). Thus, calcium signals in this subpart of the AL could to some extent allow predicting bees' generalization behavior. As shown on the figure, the data showed however some scatter and the question whether extending the neurophysiological recordings to more glomeruli, or to other parts of the brain may ameliorate this prediction remains unanswered. In theory, the bee brain contains different sets of olfactory representations in its different olfactory structures, which each can be characterized by an odor-similarity matrix based on combinatorial activity of its neuronal units (**Figure 4C**). It will be the goal of future research to compare the capacity of these different levels for predicting bees' olfactory behavior.

AL neurons are involved in the processing of incoming odor information provided by ORNs. Intracellular recordings of LN and PN responses provided some insights about this processing (Flanagan and Mercer, 1989b; Sun et al., 1993; Stopfer et al., 1997; Abel et al., 2001; Müller et al., 2002; Krofczik et al., 2009). LNs are odor-specific, responding in a differential manner to different odors. They can show excitatory responses to some odors and inhibitory responses (i.e., a reduction of spiking activity relative to background) to others (Sun et al., 1993). Staining of hetero-LNs allows identifying the glomerulus in which this LN most intensively branches (Galizia and Kimmerle, 2004). Generally, the response profile of the recorded LN corresponded to the known response profile of the innervated glomerulus, suggesting that hetero-LNs take their input in this glomerulus. LNs tend to show a shorter latency than PNs, which allows them to rapidly and efficiently inhibit the firing of PNs (Krofczik et al., 2009).

Projection neuron responses are the product of direct excitation from ORNs, direct inhibition from LNs and possibly disinhibition from LN–LN connections and can therefore be temporally complex (Sun et al., 1993; Müller et al., 2002). PNs are usually spontaneously active and can change their responses upon odor presentations in an either excitatory or inhibitory manner (respectively increasing or decreasing firing rate; Abel et al., 2001;

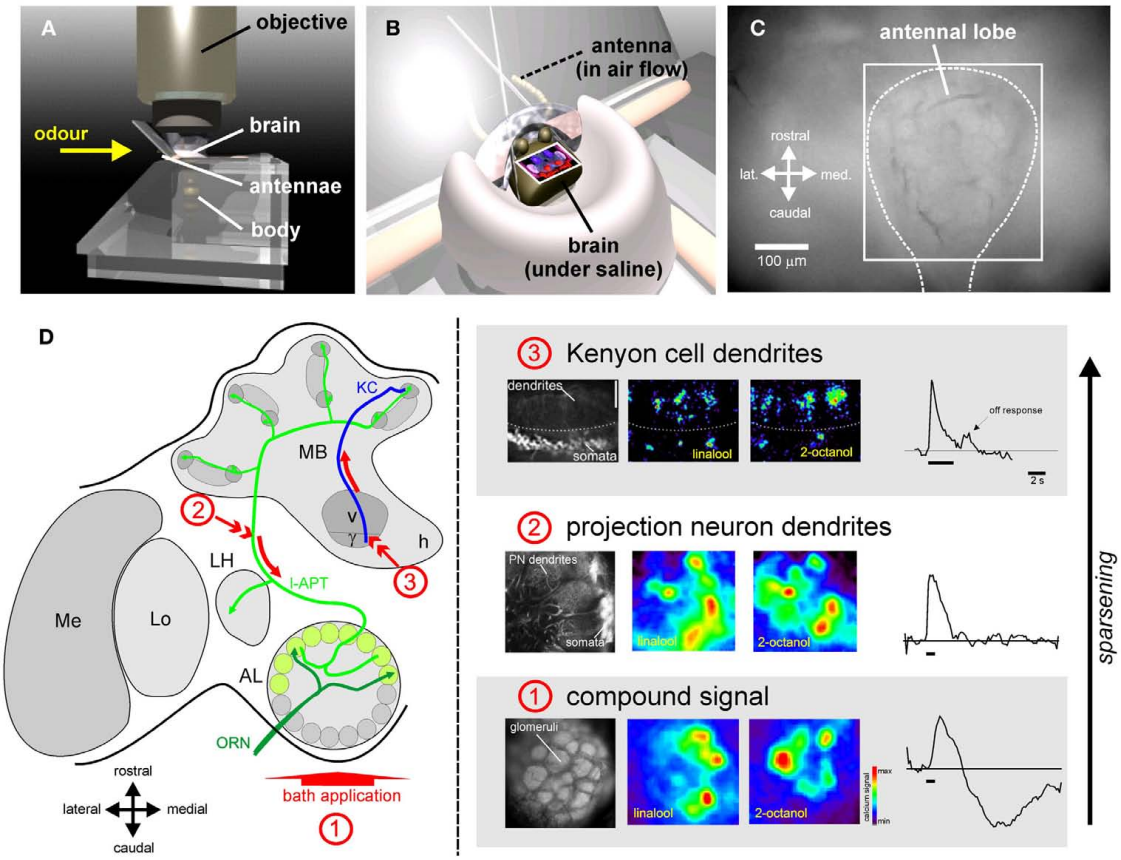

FIGURE 3 | Optical imaging of odor representations in the bee brain. Thanks to *in vivo* calcium imaging, odor representation can be recorded in the bee brain. **(A)** Bees are placed under an epifluorescence microscope in front of an odor-delivery device delivering a permanent airflow. **(B)** Their head capsule is opened revealing the brain, which is then kept under saline solution at all times, while the antennae are maintained in the airflow. **(C)** Example view of the brain surface after bath-application of a calcium dye (method 1, below). The recording can be restrained to the region corresponding to the antennal lobe (square). **(D)** Using different staining techniques, odor representation was recorded at different levels of olfactory processing. On the left the staining technique and the imaged neuronal population are shown, while on the right, activity maps evoked by two sample odorants (1-hexanol and linalool) as well as an exemplary time course are presented. (1) Using bath-application of a calcium-sensitive dye (Calcium-Green 2-AM), a compound signal can be recorded in the antennal lobe in response to odors (Joerges et al., 1997). This signal is thought to represent mostly olfactory input from the ORN population (see text). Different odors induce different, but overlapping, multiglomerular activity patterns. Bath application signals are temporally slow and biphasic. (2) Using retrograde staining with a migrating dye (Fura-2 dextran), projection neurons can be selectively stained (Sachse and Galizia, 2002). A dye-coated electrode is inserted into the PNs axon tract (arrow number 2). The dye is taken up by the neurons and migrates back to their dendrites in AL glomeruli. Such staining allows the selective recording of AL output information sent to higher-order centers. Odors also induce multiglomerular activity patterns, but these are scarcer (less glomeruli are activated) and more contrasted than the compound signals. The time course is mostly phasic-tonic, but also presents some complex temporal patterns and inhibitions. (3) Inserting the dye-coated electrode into the ventral part of the vertical lobe allowed recording activity from Kenyon cell dendrites and somata (Szyszka et al., 2005). Olfactory representation becomes even sparser in the MBs as few KCs respond to each odorant. Responses are phasic and often present off-responses at stimulus offset. (Recordings 1 and 2 from Deisig et al., 2006, 2010 – Recordings 3 from Szyszka et al., 2005).

Müller et al., 2002). However, PNs belonging to the two anatomical tracts conveying information to MBs and LH may have different response properties. In contrast to initially thought (Müller et al., 2002), there does not seem to be very clear-cut differences between PN pathways in their propensity to respond to odorants, all PNs responding rather non-specifically to many odors (Krofczik et al., 2009). On average, the dynamic response profiles of l- and m-APT neurons were found to be similar so that in both systems odor identity would be encoded both in the pattern of response latencies and in the subset of activated PNs. However, even though responses to single odors may be similar, this work and a recent imaging study (Yamagata et al., 2009) both showed that the two subsystems may treat odor mixtures differently. Moreover, the two systems seem to respond differently to odor concentration (Yamagata et al., 2009).

While intracellular recordings precisely describe the temporal response patterns of individual AL neurons to odors, imaging methods allow recording the combinatorial responses of many PNs simultaneously. This was possible using back-tracing of PN processes with the calcium dye Fura-dextran (**Figure 3D**). By placing a high concentration of dye into the axonal tract of l-APT PNs on their way to the MBs, the dye is taken up by the

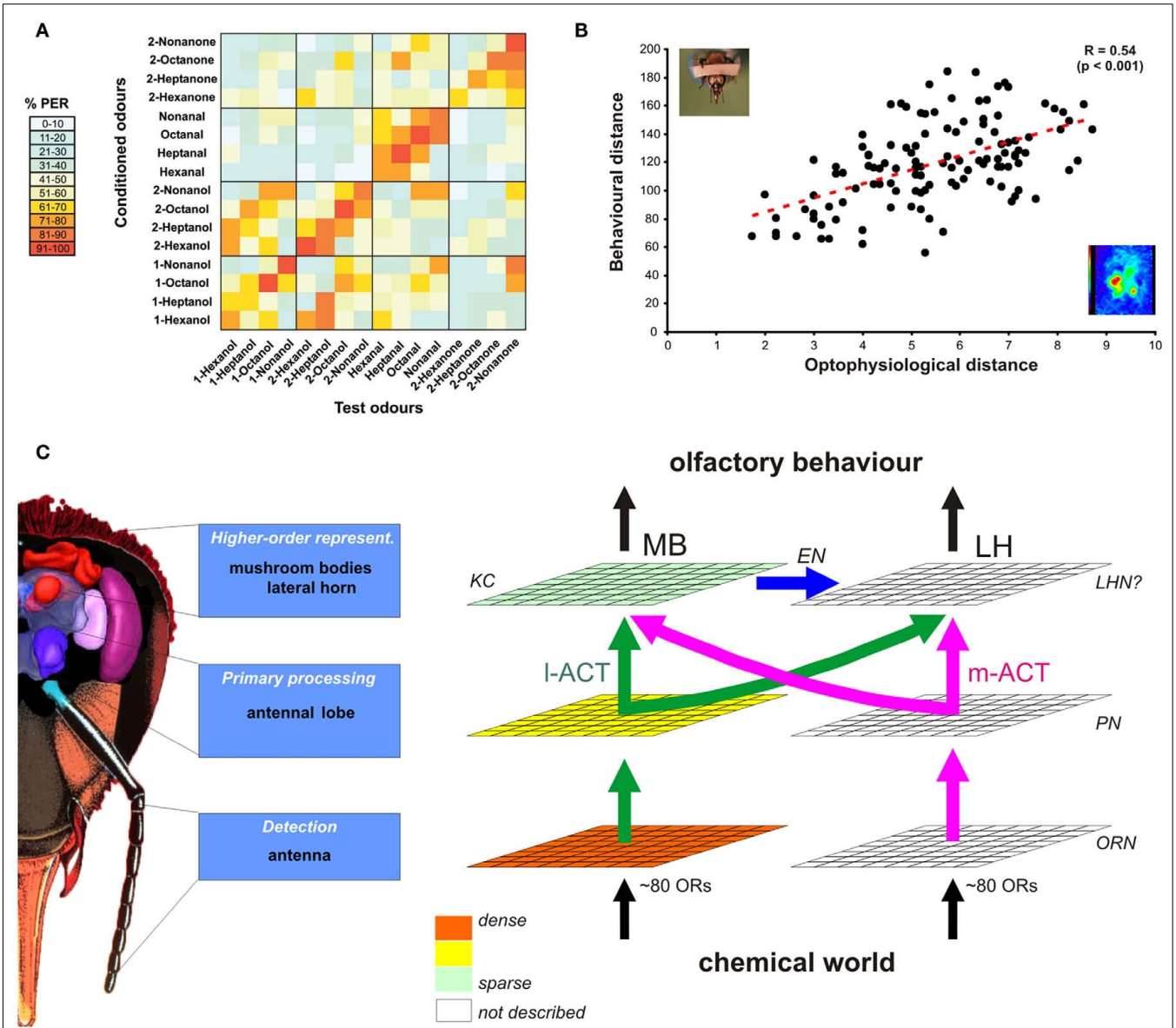

FIGURE 4 | Neural representations of odors and olfactory behavior. **(A)** Using a generalization experiment as shown in **Figure 1B**, it was possible to measure the perceptual similarity among all possible pairs of 16 aliphatic odorants. The table presents the amount of generalization between any two of these odors. Odors used for conditioning are presented vertically, while odors used in the generalization tests are presented horizontally. Bees respond preferentially to the learned odor (main diagonal), but also to other – perceptually similar – odors. For instance, they generalize between odors sharing the same carbon chain length (smaller diagonals) or the same functional group (boxes along diagonals, see for instance the high generalization among aldehydes). **(B)** Similarity among odors at the neural level (measured in the AL using bath-application of the calcium dye, method 1 in **Figure 3**) significantly correlates with similarity at the behavioral level, as measured in **(A)**. Thus, the AL contains a neural representation of odors which allows predicting to some extent the bees' olfactory behavior. **(C)** At its different processing levels, the bee brain is thought to contain multiple odor representations, which can be characterized by different odor-similarity matrices. Sequential and/or parallel transformation of olfactory information shapes odor representations in higher-order centers that would eventually determine olfactory behavior. Thus, higher-order representations should correlate more strongly with behavioral output than more peripheral representations [like AL input, see **(B)**]. As shown in **Figure 3**, we have access to representations at the level of ORNs, PNs, and KCs corresponding to the l-APT subsystem. There, a sparsening of olfactory representation is found, but its influence on predicting behavior is still unknown. In addition, representations in the m-APT subsystem, as well as in the LH are still unknown and should be studied in future work.

neurons and transported retrogradely to the soma near the AL, and to the dendrites within AL glomeruli (Sachse and Galizia, 2002). In agreement with electrophysiological recordings, the imaging recordings showed that PN odor-response patterns are temporally more complex than the input activity (bath application), and can show both excitation and inhibition phases. These calcium

responses seem to follow – although with a lower temporal resolution – the spiking activity of the neurons, as shown by consecutive intracellular electrophysiology and optical imaging of the same AL neurons (Galizia and Kimmerle, 2004). Imaging of PN population activity allowed comparing glomerular activity patterns between the input (Sachse et al., 1999) and PN output representation, even within the same animal (Sachse and Galizia, 2003). Such comparison showed that most glomeruli which are intermediately or weakly active in the compound signal, do not present any calcium increase in PNs (Sachse and Galizia, 2002). Thus, PN patterns are sparser than input patterns. Moreover, it was found that AL networks improve the separability of odor representations, both with single odors over a wide concentration range (Sachse and Galizia, 2003) and with mixtures (Deisig et al., 2010, see below).

From this, AL processing appears to perform mainly two operations: *gain control*, which quantitatively controls the overall amount of PN activity and *contrast enhancement* which qualitatively modifies the activity patterns. These two properties can be attributed to the action of inhibitory LN networks, in particular GABAergic ones. Indeed, application of GABA onto the brain blocks spontaneous activity and totally abolishes calcium response to odors (Sachse and Galizia, 2002). Conversely, application of a $GABA_A$-like receptor antagonist, picrotoxin, stimulates spontaneous activity, and increases the number of activated glomeruli upon odor presentation, also modifying the time course of the signals. Picrotoxin also abolishes network oscillation dynamics (Stopfer et al., 1997). Imaging recordings confirmed the existence of (at least) two different inhibitory networks, following the anatomical features of the bee AL (Sachse and Galizia, 2002). The first one would be a global inhibitory network driven by all glomeruli and affecting all glomeruli, corresponding to homo-LNs. Based on the above results, it would be sensitive to picrotoxin and have a gain control function. The second network would be an asymmetrical inhibitory network driven by one glomerulus and affecting mainly another glomerulus, corresponding to hetero-LNs. The neurotransmitter for the hetero-LN network involved in contrast enhancement is still unknown. Glutamate (Barbara et al., 2005) or histamine (Sachse et al., 2006) may play such a role, but this has not been demonstrated to date.

Which rule underlies the inhibition relationships of hetero-LNs between individual glomeruli? Comparison of the result of computational modeling with imaging experiments established that the transformation of odor representation between AL input and output is best achieved by an interglomerular inhibition based on functional similarity between glomeruli and less so by inhibition based on anatomical neighborhood relationships or random connections (Linster et al., 2005).

ODOR REPRESENTATION IN THE MUSHROOM BODIES

After AL processing, l-APT and m-APT PNs convey information to the MB calyces. In honeybees, KCs, the MB-intrinsic neurons, are too small to perform intracellular recordings. Data from locusts suggest that whereas PNs respond to odors with trains of spikes, KCs often respond with a single or very few spikes (Perez-Orive et al., 2002). KCs do not show any spontaneous activity, and respond to very few odorants, i.e., representation at the KC level is highly sparse. Optical imaging recordings used Fura-dextran forward- and back-fills of PNs and KCs respectively, to study this transformation of odor representation from the AL to the MB (Szyszka et al., 2005, **Figure 3D**). This study showed that the proportion of cells responding to only one odor out of a four-odor panel increased at each level, respectively 55, 70, and lastly 92%. Thus, olfactory representation would follow a series of transformations with a progressive sparsening of odor representation (**Figure 4C**). The last step of sparsening, which takes place at the level of the MB calyx involves several mechanisms. First, the low synaptic strength between PNs and KCs would imply that coherent input from many PNs at the same time is needed to excite a KC (Perez-Orive et al., 2002). Second, KCs would detect coincidence among many PNs thanks to odor-driven inhibition produced by LH inhibitory neurons locked in anti-phase to PN oscillations (Perez-Orive et al., 2002, 2004). Third, local microcircuits involving GABA processes in the MB microglomeruli would also shape KC responses (Ganeshina and Menzel, 2001; Szyszka et al., 2005).

Odor representation at the KC level is thus highly sparse, and each KC represents a particular pattern of PN inputs, possibly for a particular concentration of an odorant (Stopfer et al., 2003) or a particular composition of a mixture (Broome et al., 2006). Therefore, they are thought to be the ideal representation of a particular odorant for storing associative memories, i.e., storing the information that one particular odor has been associated with a sucrose reward or with a noxious stimulus (Heisenberg, 2003; Gerber et al., 2004).

MUSHROOM BODY OUTPUT AND THE LATERAL HORN

The most studied MB output neuron is the Pe-1 neuron, which is recognizable by a characteristic firing pattern in doublets or triplets of action potentials (Mauelshagen, 1993; Rybak and Menzel, 1998; Okada et al., 2007). This wide-field neuron does not only respond to odors, but also to other sensory modalities (visual, mechanosensory). Moreover, it changes its responses during conditioning (Okada et al., 2007). At this level of the olfactory pathway, odor information is thus integrated with other modalities, and the function of neurons such as Pe-1 might not be to represent specific information about the learned odor like its identity, concentration, or multimodal context, but rather that this particular stimulus combination has been learned.

Practically nothing is known about odor processing and representation in the honeybee LH. In *Drosophila*, recent neuroanatomical work could reconstruct putative maps of olfactory input to the LH (Jefferis et al., 2007). In this species, the response spectra of individual ORNs to odors are known (Hallem et al., 2004; Hallem and Carlson, 2006; Galizia et al., 2010) and glomeruli receiving input from ORNs carrying each receptor have been mapped (Couto et al., 2005; Fishilevich and Vosshall, 2005). Moreover, the projection patterns of uniglomerular PNs from identified glomeruli have been retraced to the higher-order centers. The putative olfactory maps at the level of the LH predict a clear segregation between candidate pheromone responsive PNs and fruit odor responsive PNs (Jefferis et al., 2007). Such functional segregation was not apparent in the MBs, although PNs from different

glomeruli also project there in at least 17 different areas (Jefferis et al., 2007). Thus, in *Drosophila*, particular subregions of the LH may code the biological nature of olfactory stimuli. If a similar organization of the olfactory circuit exists in the honeybee, one could expect the honeybee LH to exhibit pheromone processing regionalization. Anatomical and electrophysiological work in ants also confirms this idea (Yamagata and Mizunami, 2010). Moreover, because the LH receives input from associative neurons like Pe-1, it was proposed that it may represent a pre-motor center for both innate biological behavior (pheromones) and acquired behavior (associative learning). Future research should invest more efforts in anatomical and physiological experiments for addressing this question.

CONCENTRATION CODING

Odor concentration strongly affects the odor map in the AL as the number of activated glomeruli increases with increasing concentrations of the odor (Sachse and Galizia, 2003). Thus, neurons integrating the overall excitation over many glomeruli, like multiglomerular PNs, may be adequate for monitoring absolute stimulus concentration. But how can odor-specific concentration coding as well as concentration invariance be achieved given the changing nature of the odor representation with concentration? The identity of an odorant is combinatorial and resides more in the *relative* activation of different glomeruli (or PNs) than in the absolute activation of individual glomeruli (Galizia and Szyszka, 2008). Therefore, neurons recognizing a particular pattern of inputs, such as KCs, could perform both operations, as was shown in locusts (Stopfer et al., 2003): while some KCs were found to be tuned to a narrow concentration range of one particular odorant, other KCs recognized the same odorant on a wide concentration scale. Some concentration invariance can be achieved earlier in the olfactory pathway, mainly through gain control mechanisms. Imaging experiments showed that processing in the AL makes odor representation more reliable over a broader concentration range (Sachse and Galizia, 2003). Moreover, the two PN subsystems may provide differential information to higher-order centers. Imaging recordings of PN boutons in the MB lips showed that while l-APT neurons display low concentration dependency (i.e., concentration invariant representation), m-APT neurons show a clear concentration effect and change their response quickly with concentration (Yamagata et al., 2009). Thus, concentration coding and concentration invariance may be extracted by differential processing at the level of PNs, and/or differential readout by KCs.

MIXTURE PROCESSING

In vivo calcium imaging at the AL input showed that usually a glomerulus is activated by a mixture when at least one of its components activates this glomerulus (Joerges et al., 1997; Deisig et al., 2006). A putative presynaptic inhibition process induces a gain control at the system's input, so that complex mixtures do not saturate the capacity of the olfactory system. The more components a mixture contains, the more *suppression* phenomena were observed (Deisig et al., 2006), i.e., cases in which the response to a mixture was lower than to the components (Duchamp-Viret et al., 2003). Taking into account all measured glomeruli, the whole mixture representation follows essentially an elemental rule, because it can be predicted linearly from the responses to the components: the more a component activates the AL when presented alone (in number of activated glomeruli, for instance), the more present it is in the mixture representation (Deisig et al., 2006). The situation was slightly different at the PN level, as AL processing via LN networks increased the number of suppression cases, allowing the emergence of synthetic properties, i.e., the appearance of a representation that cannot be predicted based only on component information (Deisig et al., 2010). Indeed, similarity relationships between mixtures and their components were more homogeneous than at the input with a more equal representation of weak- and strong-components in the mixture. These recordings showed that reformatting by LNs in the AL increases separability among odor mixture representations, probably facilitating olfactory mixture discrimination by bees (Deisig et al., 2010).

How mixture representation further transforms along the olfactory pathway is mostly unknown. Recordings at the level of PN boutons in the MB lips confirmed an important proportion of suppression effects in l-APT PNs, but showed that such mixture non-linearities are mostly absent in m-APT PNs, providing an additional hypothesis for the functional role of this dichotomous system: one system would be involved in synthetic processing, while the other would conserve component information (Yamagata et al., 2009). The strong sparsening of odor representation from PNs to KCs and their coincidence detection properties could be the basis for mixture-specific units.

OLFACTORY PLASTICITY

In bees, olfactory processing is not a static phenomenon, but is subject to plasticity as a function of both age and experience. This plasticity is manifested by structural and functional changes of olfactory circuits.

DEVELOPMENTAL PLASTICITY

The olfactory system of bees goes through intensive remodeling during the pupal stage and metamorphosis. The compartmentations of AL and MB calyces first take place during the beginning of pupal development (Menzel et al., 1994; Hähnlein and Bicker, 1997). At pupal stage 1, the AL neuropil is still homogeneous without any trace of the first spherical neuropil regions, called "preglomeruli" which appear starting at pupal stage 3 (Masson and Arnold, 1984). During subsequent stages the number of preglomeruli progressively increases, so that at pupal stage 7, all glomeruli appear adult-like (Gascuel and Masson, 1991). At the MB level, a small, homogeneously structured neuropil that is not yet divided into subcompartments appears at the prepupal stage (Menzel et al., 1994). Then, starting at pupal stage 3, the calyces gradually become separated from each other, with the lip, collar, and basal ring regions being clearly developed at pupal stage 6 (Hähnlein and Bicker, 1997). Interestingly, PNs achieve their adult arborization pattern within their main output region (MB lip) earlier during development (pupal stage 1) than their dendritic processes within their input region, the AL (pupal stage 2; Schröter and Malun, 2000). The olfactory system remains highly plastic throughout adulthood: an age-dependent increase in neuropil volume is observed for most of the MB, but the lip (olfactory),

and collar (visual) regions show both age-related and experience-dependent volume increases (Withers et al., 1993; Durst et al., 1994; Fahrbach et al., 1998; Ismail et al., 2006). Moreover, the density of microglomerular complexes in the lips also undergoes changes with both age and experience (Groh et al., 2006; Krofczik et al., 2008).

The hive is a highly odorous environment, and bees at all ages are subject to constant olfactory stimulation from honey and pollen stores and from pheromones produced by the queen, workers, and brood (Winston, 1987). This olfactory environment can have a significant effect on the maturation of the olfactory system of young bees. A number of experiments attempted to understand the effect of a passive olfactory exposure on honeybees' behavior. Some studies showed an increase in orientation toward a prior passively exposed odor, both in bees walking in an olfactometer (Pham-Delègue et al., 1990) and in free-flying bees visiting an artificial feeder (Jakobsen et al., 1995). In contrast, in the PER conditioning procedure, no effect of passive exposure was found, or if it was found, exposed bees tended to learn the exposure odor less efficiently than naïve bees (Getz and Smith, 1991; Gerber et al., 1996; Sandoz et al., 2000). At the same time exposed bees were found to spend more time than controls in this odor in a four-armed olfactometer (Sandoz et al., 2000). As control bees tended to avoid the odor, this increased time spent in the exposure odor field was interpreted as a reduced sensitivity of bees after passive exposure. Several processes may explain this effect. For example, constant passive exposure could have induced sensory adaptation of the bees' olfactory system. This would decrease a spontaneous avoidance by bees of the pure compound in the olfactometer, and make it a less salient compound to be learnt in a PER conditioning procedure. As sensory neurons continue to mature until 8 days after emergence (Masson and Arnold, 1984; Allan et al., 1987), exposure at an early age may permanently alter bees' olfactory sensitivity.

In another series of experiments, odors were provided mixed with a sucrose solution for different periods during young adulthood (Arenas and Farina, 2008). Bees clearly associated the odor with the sucrose reward and showed long-term memory performance in a PER test at a later stage (17 days). Interestingly, the odor associated with sucrose reward when bees are 5–8 days old resulted in better olfactory retention at the adult stage than when the same exposure was performed before (1–4 days old) or – more surprising – after this critical period (9–12 days old; Arenas and Farina, 2008). *In vivo* calcium imaging showed that precocious olfactory experience increased general odor-induced activity as well as the number of glomeruli activated by the learned odor in the adult AL, but also affected qualitative odor representations (Arenas et al., 2009b). Thus early olfactory experiences inside the hive may have long-lasting effects, reflected in behavioral responses to odorants and concomitant neural activity in the adult olfactory system. Fitting with the idea of developmental plasticity, bees were found to memorize novel odor-sucrose associations more efficiently after such early experience than controls (Arenas et al., 2009a).

NEURAL CORRELATES OF OLFACTORY LEARNING

During the adult stage, honeybee foragers experience odors in the context of food search, and learn to associate floral odorants with sucrose reward (see above). A number of studies have searched for possible structural and functional plasticity of the olfactory pathway during or at different moments after the formation of an odor-sucrose association. Usually, in such experiments, differential conditioning is used so that changes in neural responses to a reinforced odorant (CS+) can be compared to changes observed to a non-reinforced odorant (CS−). Doing so, several studies found learning-correlated changes in odor-evoked patterns in the AL, taking place either shortly (10–30 min) after differential conditioning (Faber et al., 1999) or later (2–5 h, Rath et al., 2011; 24 h, Sandoz et al., 2003; Fernandez et al., 2009). At short-term, the amplitude of calcium responses to the CS+ were found to increase (Faber et al., 1999). Electrophysiologically, increases and decreases in PN spike rates were found in response to odors after conditioning, with a strongest effect for the CS+ (Denker et al., 2010). Later, between 2 and 6 h after training, differential increases and decreases in the responses of individual glomeruli were found (Rath et al., 2011), which was not the case at shorter-term (Peele et al., 2006). Lastly, at 24 h, PN calcium signals were found to increase to the CS+ (Fernandez et al., 2009). A general observation of these studies was that the similarity between the patterns of the CS+ and of the CS− was decreased after learning, suggesting that olfactory learning improves the discrimination of the learned odorant from other ones (Faber et al., 1999; Fernandez et al., 2009; Rath et al., 2011).

On a structural level, olfactory experience during foraging was shown to induce glomerular volume and structure changes (Sigg et al., 1997; Brown et al., 2002). It was long unclear whether such changes were actually due to olfactory experience *per se*. Recently, however, a specific glomerular volume increase was demonstrated in a subset of glomeruli as a result of the formation of a long-term appetitive olfactory memory after 72 h (**Figures 5A,B**; Hourcade et al., 2009). It thus seems that in the AL, learning-induced plasticity takes different forms at different moments after the associative event.

Likewise, modified odor-evoked responses to a learned odor were found in the MB calyces shortly after conditioning (10–30 min, Faber and Menzel, 2001; Szyszka et al., 2008). In particular, specific imaging of KC activity showed that repeated presentation of an odor induces a reduction of the evoked response (interpreted as habituation), while appetitive training induced a recovery from this decrease (Szyszka et al., 2008). On a structural level, a long-term olfactory memory trace 72 h after training was revealed as an increase in the density of microglomeruli in the MB lips (**Figures 5A,C**, Hourcade et al., 2010). MB output neurons are also subject to changes through associative learning, as exemplified by the Pe-1 neuron (Okada et al., 2007), by recurrent PCT neurons (Hähnel and Menzel, 2010) or by other ENs (Strube-Bloss et al., 2011). In some cases too, specific changes are found in responses to the CS+ and response differences between CS+ and CS− were increased (Strube-Bloss et al., 2011).

Thus many electrophysiological, functional imaging, or neuroanatomical studies find strong neural plasticity within olfactory circuits, especially after associative conditioning. However, it is often difficult to relate such neural plasticity to its exact function. Are the observed changes related to modifications of

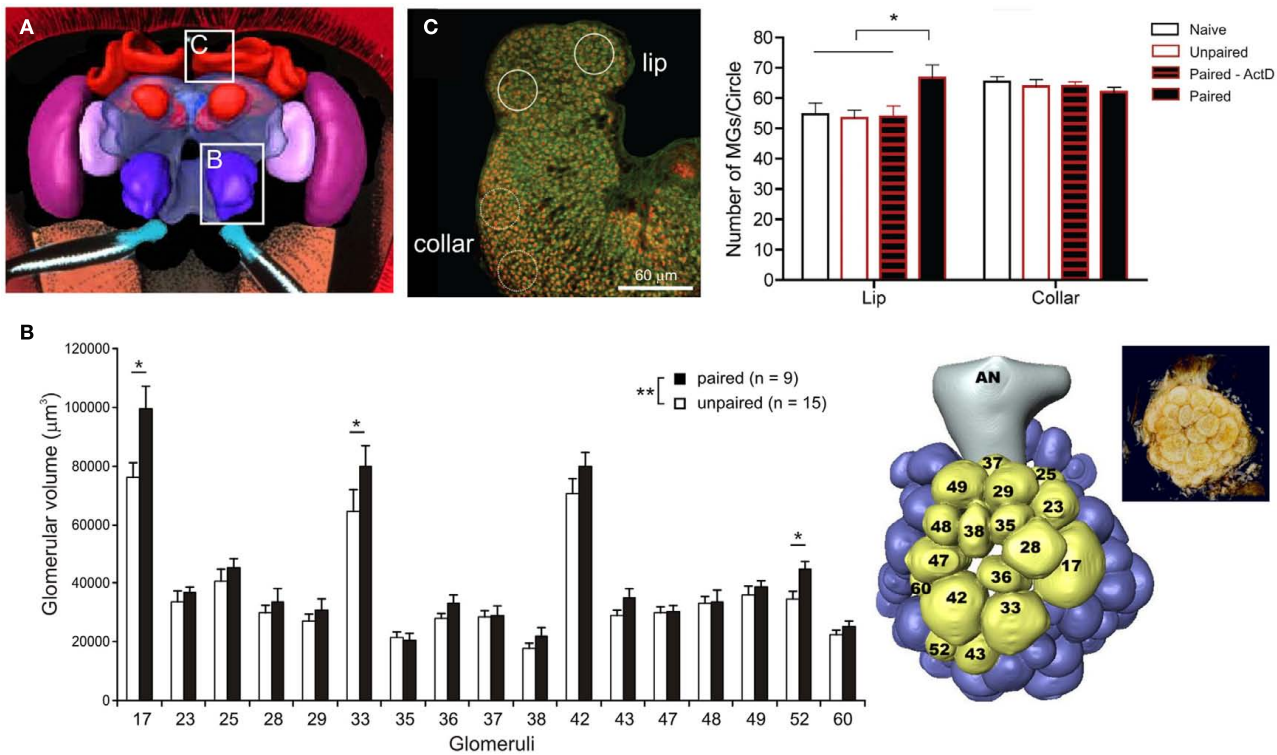

FIGURE 5 | Structural plasticity related to olfactory long-term memory. (A) Structural changes in olfactory circuits of the bee brain were evaluated as a result of the formation of a long-term appetitive olfactory memory. Bees conditioned to an odor CS (paired bees) are compared to bees subjected to pseudoconditioning in which CS and US are presented explicitly without temporal association (unpaired bees). After 72 h, paired but not unpaired bees show strong behavioral olfactory long-term memory. At that time, the brains were prepared and either the volume of olfactory glomeruli in the antennal lobe **(B)** or the number of microglomeruli in the mushroom body calyx **(C)** were measured. **(B)** Volumetric analysis of 17 identified glomeruli in the antennal lobe, based on neutral red staining and 3D reconstruction. A global increase in glomerular volume was found in paired bees relative to unpaired bees. For each of the learned odors (here 1-hexanol), three glomeruli showed a significant volume increase (data from Hourcade et al., 2009). **(C)** Counts of microglomeruli numbers in the MB calyx, based on synapsin/phalloidin double staining. Olfactory long-term memory induced an increase in microglomeruli numbers in the lip region (olfactory) compared to unpaired or naive bees. This long-term plasticity relies on transcription as injection of Actinomycin D blocked the effect. This structural plasticity related to olfactory long-term memory was logically found only in the calyx lip (olfactory input region) and not in the collar (visual input region). (Data from Hourcade et al., 2010).

odor processing, modulating the neural representation of the learned odors so that it can be better distinguished from environmental background? Or are they related to an "engram," revealing the storage of odor-reinforcement associations in the brain? Currently we think that the AL is mostly responsible for the former, while the MB would be crucial for the latter, but considerable work is still needed to confirm this hypothesis. Future neurobiological studies will need a combination of approaches, asking in particular whether the observed cells (and their plasticity) are necessary and/or sufficient for the expression of olfactory plasticity at the behavioral level (Gerber et al., 2004).

CONCLUSION

One century of experiments have provided extensive data on the olfactory behavior of honeybees, on the neuroanatomical organization of their olfactory pathway as well as on the neural representation of odors within these circuits. All these experiments concur to show that the honeybee olfactory system is tuned for performing a number of operations that are crucial for meeting the demands of social life, food search, and mating. This system thus allows to (1) detect and identify odor stimuli, allowing graded responses to increasingly similar odors; (2) measure stimulus concentration allowing both concentration invariant and concentration-specific odor recognition; (3) detect components within a mixture as well as extract mixture-unique properties; (4) constantly adapt to the odorous environment; and (5) learn relationships between almost any odor and appetitive or aversive outcomes. Although our understanding of odor representation at the different levels of the bee brain has greatly improved in the last years thanks to state-of-the-art recording techniques, entire brain regions have yet to be explored. The most prominent are the m-APT dependent parts of AL and MBs, as well as the utterly unstudied LH. Thanks to optical imaging, our understanding of the spatial representation of odors has greatly improved, but temporal aspects are still poorly understood. Even in such a simple system, as compared to vertebrates, olfactory coding involves complex interactions between different neuron types, so that only computational approaches feeding on comprehensive sets of experimental data may help understanding the

dynamics and processing rules of the olfactory system. Lastly, plasticity appears in multiple regions of the olfactory pathway, but their respective implications for tuning the olfactory system or for storing outcome-related memories is still unknown. It shall be the goal of future research to progress in these questions, so that a comprehensive model of olfactory detection, processing, and learning in the honeybee can be constructed, the ultimate goal of sensory neuroscience.

ACKNOWLEDGMENTS

I would like to thank M. H. Pham-Delègue, R. Menzel, M. Giurfa, and C. G. Galizia for their intellectual contribution to the ideas developed in this review. Tilman Franke produced the pictures in **Figures 2A,B**. This research is financed by the CNRS and by grants from the National Research Agency (ANR-NEURO-2007 and ANR-BLANC-2010) as well as from NERF and R2DS Ile-de-France Programs.

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

Received: 18 August 2011; accepted: 16 November 2011; published online: 08 December 2011.

Citation: Sandoz JC (2011) Behavioral and neurophysiological study of olfactory perception and learning in honeybees. Front. Syst. Neurosci. ***5****:98. doi: 10.3389/fnsys.2011.00098*

frontiers in
SYSTEMS NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 11 October 2011
doi: 10.3389/fnsys.2011.00084

Map formation in the olfactory bulb by axon guidance of olfactory neurons

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The organization of representations in the brain has been observed to locally reflect subspaces of inputs that are relevant to behavioral or perceptual feature combinations, such as in areas receptive to lower and higher-order features in the visual system. The early olfactory system developed highly plastic mechanisms and convergent evidence indicates that projections from primary neurons converge onto the glomerular level of the olfactory bulb (OB) to form a code composed of continuous spatial zones that are differentially active for particular physico-chemical feature combinations, some of which are known to trigger behavioral responses. In a model study of the early human olfactory system, we derive a glomerular organization based on a set of real-world, biologically relevant stimuli, a distribution of receptors that respond each to a set of odorants of similar ranges of molecular properties, and a mechanism of axon guidance based on activity. Apart from demonstrating activity-dependent glomeruli formation and reproducing the relationship of glomerular recruitment with concentration, it is shown that glomerular responses reflect similarities of human odor category perceptions and that further, a spatial code provides a better correlation than a distributed population code. These results are consistent with evidence of functional compartmentalization in the OB and could suggest a function for the bulb in encoding of perceptual dimensions.

Keywords: olfaction, plasticity, axonal guidance, olfactory coding, olfactory bulb, glomeruli, odor category, perception

1. INTRODUCTION

Complex repertoires of olfactory receptors (OR) evolved in different numbers over species. In humans, each ORN expresses only one of a possible 384 identified functional types (Aloni et al., 2006), and axons from ORNs that express the same type converge on the surface of the OB at stereotyped positions. The OB is a crucial processing station for olfactory signals (Buschhüter et al., 2008) and glomeruli are thought to be functional units and convergence target for axons from many ORNs of just one type of odorant receptor (OR) (Kauer and Cinelli, 1993; Mori, 1999; Bozza et al., 2002; Mombaerts, 2004), so that each odorant elicits a specific map of glomerular activation (e.g., Ressler et al., 1994). Olfactory bulb output neurons, mitral, and tufted cells (MT cells), project to primary olfactory cortical areas, such as the anterior olfactory nucleus, piriform cortex, olfactory tubercle and lateral entorhinal cortex, and the amygdala (Shipley et al., 2008). A schematic drawing of the organization of the early olfactory system is shown in **Figure 1**.

Sensory axon coalescence onto glomeruli has been found to rely on several mechanisms that contribute differently on a local and global scale, some of which likely related to activity. It is known that ORN type-convergence onto glomeruli is at least partly mediated by experience (Yu et al., 2004; Kerr and Belluscio, 2006; Imai and Sakano, 2007) and that activity plays a role in axon fate (Ming et al., 2002; Mombaerts, 2006; Sakano, 2010; Mori and Sakano, 2011). In axon growth, direction of the axon's growth cone is regulated by various chemical cues, diffusible chemoattractants, and repellants, in a series of discrete steps (Sanes and Jessell, 2000). Serizawa et al. (2006) found evidence in the mouse that correlation of neural activity mediated axonal attraction and repulsion by up- and down-regulation of a set of olfactory axon guidance cues, which suggested that axon sorting could be based on correlated neural activity.

It has been found at different levels of the brain, especially in the visual and auditory systems, that inputs are spatially embedded, so that the spatial structure of the nervous system reflects sensory stimuli within the environment, as well as the quality of the stimulus itself (cf. Udin and Fawcett, 1988; Singer, 1994; Malach et al., 2002). The spatial structure of representations in the brain has been observed in many parts of the brain to reflect locally subspaces of inputs that are behaviorally or perceptually relevant (e.g., Swindale, 2008; Humphries et al., 2010). In olfaction, discriminatory dimensions are still elusive (Sell, 2006; Haddad et al., 2008a), although several groups have found evidence for continuous spatial zones responsive for certain groups of odorants based on data accumulated using different techniques (e.g., Vassar et al., 1994; Meister and Bonhoeffer, 2001; Lodovichi et al., 2003; Mori et al., 2006, 2009; Johnson and Leon, 2007) and a systematic large-scale study of glomerular representations suggests that encoding is very local (Auffarth et al., 2011b). Evidence is now

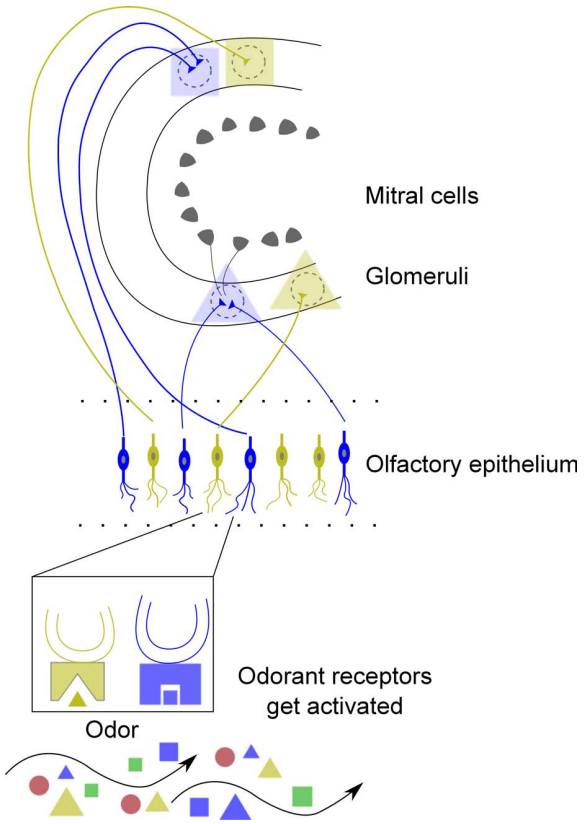

FIGURE 1 | Schematic of the early olfactory system including the olfactory epithelium and bulb. Each ORN expresses one OR which responds to different odorants (see **Figure 2**). In the first step of the sensory pathway, odorant molecules bind to ORs in the olfactory cilium which activate ORNs in the epithelium which in turn transduce the input signal into action potentials. ORNs expressing the same OR generally project to the same glomerulus (Bozza et al., 2002; Mombaerts, 2004) and connect to the principal neurons of the OB, the mitral, and tufted cells (MT cells). These principal neurons forward their output to higher-order brain regions. Bulbar interneurons, granule cells (not shown) receive lateral inhibition and feedback from piriform cortex (Haberly and Price, 1977). The colors and symbols indicate molecular features of odorants and a chemotopic convergence from ORN axons to glomeruli. According to odotope theory (Shepherd, 1987; Mori, 1995), individual MT cells transmit information about a range of odor molecules with related molecular structures (so called odotopes). Cleland et al. (2007) and Johnson and Leon (2000) argued that qualitative odor perception is determined by glomerular activity patterns. They described also that different glomerular activity patterns, elicited, e.g., by increased concentration can lead to qualitatively different odor percepts.

accumulating of behavioral relevance of molecular feature combinations and glomerular domains (e.g., Dielenberg and McGregor, 2001; Kobayakawa et al., 2007; Raman and Gutierrez-Osuna, 2009; Sakano, 2010). It has also been shown that perceptual differences can be predicted by glomerular spatial activity patterns (Uchida et al., 2000; Linster et al., 2001; Auffarth et al., 2011a).

We present a model of olfactory learning in humans in which competitive axonal wiring adaptations in the early olfactory system are a mechanism by which experiences are translated into memories expressed by structural changes of neurites and synapses. We set OR sensitivities as biologically relevant feature combinations in a set of real-world odorants. We cluster olfactory axons by an activity-dependent mechanism that results in a self-organization of glomeruli by the affinity of their corresponding ORs. After a description of the data used in our study and the model, we show results concerning the formation of glomeruli, the relationship between recruitment of glomeruli and concentration, and the match between glomerular responses and human perceptual ordering. For the last point, we compare codes of glomerular responses at population and spatial levels to human-rated perceptual similarities of odorant categories. The spatial code is a test of the hypothesis that distance between coding regions are relevant for behavior or perception. Finally, we discuss results in the context of olfactory information processing.

2. MATERIALS AND METHODS

As commented in the introduction, there is no simple scale or known dimensionality to olfactory perception. Therefore one solution to order odorants is to represent them by a large number of molecular descriptors. (Haddad et al., 2008a) presented a set of 32 physico-chemical descriptors, derived from an initial set of 1,664 descriptors, that were shown to reflect variability of the bulb and antennal lobe population responses. In simple terms, this means that odors that cause similar responses are proximal in this space and odors that elicit dissimilar responses are distant. They supplied a dataset of 447 odorants described by these 32 properties as supplementary material with their paper, which we use this study.

We extracted perceptual odorant descriptors from flavor net (Acree and Arn, 1998)[1], a public resource on volatile compounds that humans experience in their environment. Examples for these odor descriptors are sweet, camphoraceous, floral, or minty. We assigned these descriptors to categories defined by Zarzo (2008). These categories are florals, cleaner, foul, woody, medicinal, nutty/spicy, balsamic, fruity, alcohol, oily, herbacious, musk, vegetable, and green. From 238 compounds for which we had perceptual information, we could categorize 210 odorants into at least one of these 14 categories.

Our odorant receptors should be distributed to capture variance of the physical space and each be placed to recognize biologically relevant regions (compare Sánchez-Montañés and Pearce, 2002; Schmuker and Schneider, 2007 for similar concepts). We applied the fuzzy c-means algorithm (Bezdek, 1981) to draw cluster centers at locations in the 32-dimensional space. In this way, each OR responds to ligands that occupy a neighborhood in physical space as described by molecular descriptors. The closer the combination of molecular properties of an odorant to the center of the receptive field of the receptor, the higher the response. Each OR can be described by its center in the 32-dimensional space and its affinities to odorants based on the distance relation in the 32-dimensional space. OR–odorant affinity relationships are indicated by the circle radii in **Figure 2**.

We modeled ORN responses to ligands at a given concentration after Sandström et al. (2009a) as a sigmoidal function of the

[1]Available at http://www.flavornet.org/

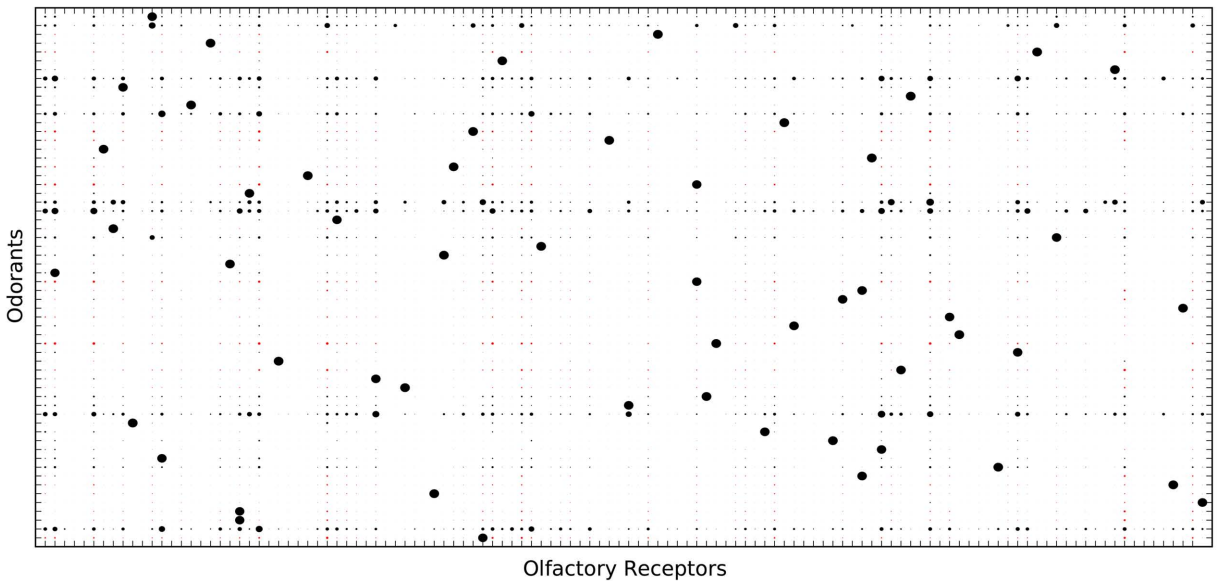

FIGURE 2 | Olfactory receptor-odorant affinity matrix. In total, 384 ORs were generated with graded affinity to each of the 447 odorants. Shown is a subset of 60 ORs and the 30 odorants to which these ORs have the highest affinity. Affinity is indicated by the radii of the circles. Black circles stand for excitatory OR response, red circles stand for inhibitory responses.

OR–ligand affinities. The response $R_i(C)$ of ORN i to a ligand at concentration C is expressed as the product of a term A_i that represents the amplitude and a term that includes the ligand responses of ORs. a_i is the affinity of receptor to the ligand and h is the gain (steepness) of the ORN response curve.

$$R_i(C) = A_i\left(1 - \frac{1}{e^{h_i a_i C}}\right) \tag{1}$$

The response of ORN population i is expressed as the product of a term μ_A and a term that includes the ligand responses of homogeneous ORs. The mean frequency of responses was taken to be as in this formula (adapted from Sandström et al., 2009a):

$$\mu_{R_i}(C) = \mu_A\left(1 - \frac{1}{e^{\mu_h a_i C}}\right) \tag{2}$$

We set the mean amplitude, μ_A, of ORNs to 1 and mean gain μ_h to 1.4. Please see **Figure 3** for an illustration of ORN dose-response curves.

We clustered ORN axon projections by a biomimetic method described in (Lansner et al., 2009). Using multi-dimensional scaling (MDS), axons can be put in a lower-dimensional space where their locations are defined by distance relations based on co-activation. In this way, the distances between glomeruli reflects regularities in the physical odor space. For distance relationships we calculated correlations between vectors of response activities of ORN populations to all odorants. We reduced the resulting matrix by MDS to three dimensions and obtained coordinate points corresponding to each olfactory axon bundle. MDS makes few assumptions about the structure of data and preserves the distance relationships among data samples. In total, this operation is similar in principle to the self-organizing map used as ORN convergence model used earlier (Gutierrez-Osuna, 2002; Schmuker and Schneider, 2007) and is consistent with the chemoaffinity hypothesis (Sperry, 1963).

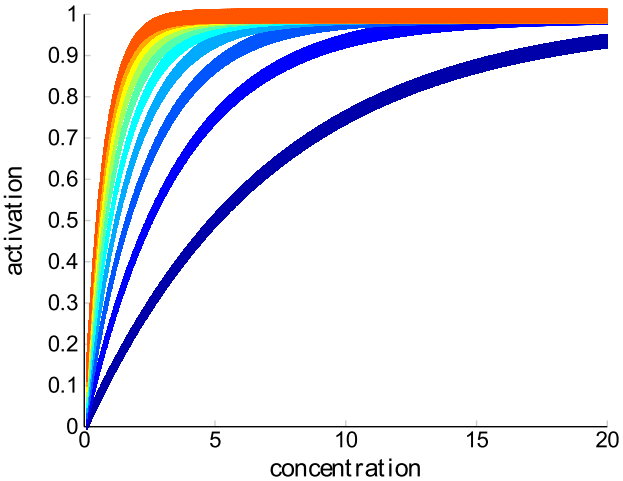

FIGURE 3 | Dose-responses of ORNs of different OR types (affinities) to a single ligand at linearly increasing concentrations. Compare to Eq. 2.

We first show how glomerular structures could arise from activity-dependent mechanisms. For this purpose, we explicitly modeled ORN populations using Eq. 1. In the following we used the population Eq. 2.

After having obtained the spatial distribution of glomerular responses, we determined the activation loci for odor categories by

the following procedure: We tested statistically for each glomerulus whether it showed significant differences with respect to the odor category by comparing responses to odors that belonged to an odor category with responses to odors that did not belong to the odor category. We did the comparison using the Wilcoxon ranked-sum test (also called Mann–Whitney U test). The test was applied within a bootstrap (Efron, 1982) resampling procedure in order to estimate distributions from small sample sizes and to account for unequal number of maps. We thresholded p-values at 5% significance. Thus, we found for each odor category, the glomeruli which are activated differentially. More details about this method are available in (Auffarth et al., 2011b).

We investigate how perceptual categories are represented in the olfactory bulb responses. Spatial coding refers to the situation, where specialized local encoders exist for certain information. This concept is opposed to population coding, where information is distributed in the responses of the population. We analyzed and compared glomerular responses to human perceptual categories.

Zarzo (Zarzo, 2008; Zarzo and Stanton, 2009) published analyses of two studies, perfumers' odor perception space (BH, Boelens and Haring, 1981) and cross-cultural odor similarity ratings (Chrea, 2004). In order to know how well the coding as identified by the coding centers reflected perceptual orderings as reported in the literature, we mined PCA plots in the two papers by Zarzo, which indicated perceptual distances in the first two principal components between odorant qualities. This provided us with a pair-wise distance matrix between two sets of perceptual odor categories.

We extracted two distance matrices, D_{Chrea} and D_{BHsmall}. *Chrea* corresponded to odor categories floral/cosmetic, cleaner, foul/musty, woody, medicinal, nutty/spicy, balsamic, and fruity. *BH small* corresponded to categories floral, woody, medicinal, balsamic, fruity. We compared matches to these perceptual spaces from both population activities of the entire glomerular layer and from spatial codes in order to see which reflected better these perceptual orderings.

In order to obtain population responses, we took the mean map over all activity maps corresponding to the same odor quality (cf. Rubin and Katz, 1999; Lin et al., 2006; Cleland et al., 2007). Thus, a population code for a given odor category A can be written as $\vec{v}_A = (\langle x_1 \rangle_A, \ldots, \langle x_{\text{nglom}} \rangle_A)$ where A is the set of odorants representing category A and $\langle x_i \rangle$ stands for the mean response of glomerulus i averaged over all odorants belonging to A. As an ordering between properties we calculated the Euclidean distances between these mean-maps, thus obtaining pair-wise distances between odor qualities based on population code, D_P.

As for the ordering between spatial zones, we applied the Hausdorff distance (cf. Alt et al., 2003), which calculates distances between two-dimensional shapes and therefore incorporates coding center distance (similar to Euclidean distances), but additionally information of shape, size, and orientation match. We applied the modified Hausdorff distance function (Dubuisson and Jain, 1994) between vertices of pairs of encoding zones. Vertices consisted of points that were found to be responsive to odor categories.

Informally, the Hausdorff distance is the farthest distance of closest points between two sets. Formally, given X and Y, two non-empty subsets of a metric space (M, d), their Hausdorff distance $d_H(X, Y)$ is defined as follows:

$$d_{\text{H}}(X, Y) = max \left\{ \sup_{x \in X} \inf_{y \in Y} d(x, y), \sup_{y \in Y} \inf_{x \in X} d(x, y) \right\}, \qquad (3)$$

with sup and inf representing the supremum and infimum, respectively.

Thus, we obtained a matrix of pair-wise differences between properties based on coding maps, D_S.

We normalized each of the three matrices of pair-wise distances D_P, D_S to unit sum, and calculated the sum of the absolute error between both of them and D_{Chrea} and D_{BHsmall}.

We also added spatial and population information linearly with the same weight to see if combined they provided a better fit to the perceptual space. For the baseline, 100,000 sets of points were sampled from random uniform distributions. Then the distances from their pair-wise distances to perceptual space was calculated.

3. RESULTS

We generated populations of ORNs according to Eq. 1 to show how sensory axons can cluster together based on activity. This is demonstrated in **Figure 4**. **Figures 4A,B** illustrate how through the

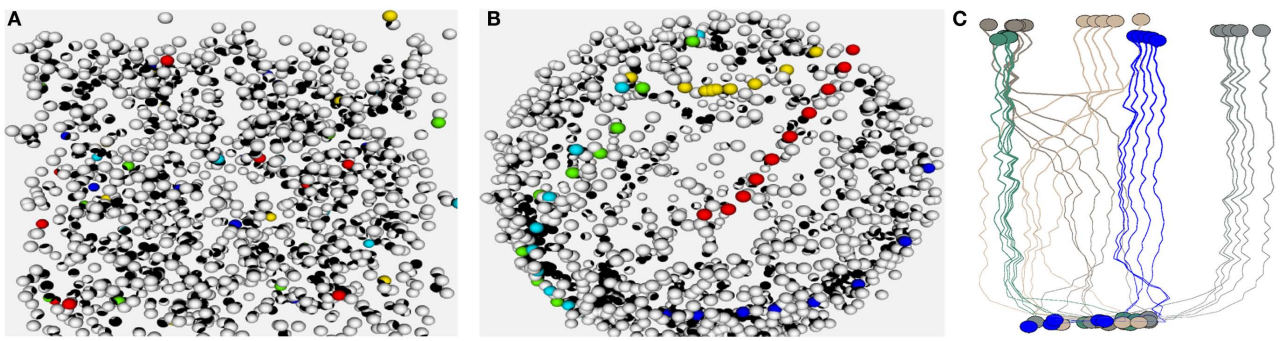

FIGURE 4 | (A,B) Show a 3D visualization of the ORN layer organization before and after calculating the projection. The visualization was done based on the Visualization Toolkit (VTK) C++ libraries (Schroeder et al., 1996). ORNs of five OR families are highlighted by color. In **(B)** you can see how ORNs of a certain type (indicated by color) cluster together. Subfigure **(C)** is an illustration of how olfactory axons project from epithelium (bottom) to glomeruli (top) in a small network consisting of five OR types and five ORNs per type. Colors indicate OR identity.

projection from epithelium to bulb, olfactory neurons can become ordered. **Figure 4C** shows on a toy example how ORNs of the same type converge onto the bulb. At each iteration odorants were presented to the network and the distance between olfactory axons was adjusted.

In the following, we applied Eq. 2 for concentration–responses of populations. In **Figure 5**, we show the effect of concentration on recruitment of glomeruli.

Table 1 gives an indication about how accurately the perceptual space is reflected in the glomerular responses. Distances, D_P were generated from the population code, based on the activity of all neurons. Distances between spatial zones, D_S, are the Hausdorff distances between coding regions. Both matrices were compared to perceptual orderings of odors. The numbers give the sum of the absolute error of fit. We added the normalized pair-wise distance matrices for population and spatial codes to combine them and also calculated the error of fit. This is annotated as *combined*. Error of fit from pair-wise distances between points drawn from a uniform random distribution is given as *baseline*. The numbers for baseline with variance are 0.65 ± 0.09 for Chrea and 0.65 ± 0.15 for BH.

Graphically you can compare in **Figure 6** the perceptual spaces and the two datasets to distances resulting from spatial and population code. The plots show the pair-wise distances dimensionality-reduced to two dimensions using the MDS algorithm. This means that distances between odor categories are maintained in the plots.

We found significant (Spearman rank) correlations (at the 5% significance threshold) between distances only for the BP data and the spatial code ($\rho = 0.6848$, $p = 0.04$), while other correlations were insignificant. Correlations between spatial and population codes were low and highly insignificant (Chrea: $\rho = 0.07, p = 0.71$; BP: $\rho = 0.04, p = 0.92$).

We looked at general patterns of distances between odor qualities and found that medicinal emerged as an odor quality that was especially well-situated in the codes over Chrea and BP, while fruity was ill-fitting.

4. DISCUSSION

Haddad et al. (2008a) presented a set of 32 physico-chemical descriptors that maximized correlations between variability of OB/antennal lobe activity responses and variability of the odorant descriptors. In our model, we placed OR receptive fields in the space captured by these molecular odorant descriptors and defined positions of ORN axon projections onto the bulb by dimensionality reduction of the OR–odorant affinity correlation matrix. Our model includes a population mean rate in response to a concentration.

We showed how activity-dependent mechanisms could serve to organize olfactory axons into glomerular structures (cf. **Figure 4**). Axons of receptor neurons of different types are intermixed as they grow toward the brain and they have to undergo a sorting process before arriving at their target glomeruli. In our model, olfactory axons cluster together over several iterations of the MDS algorithm.

We then showed how glomerular responses spatially broadened and saturated with increasing concentrations. It is well-established that increasing concentration leads to an increasing recruitment of glomeruli and thereby to a spatial broadening of local peaks (Johnson and Leon, 2000; Khan et al., 2010). **Figure 5** demonstrates how our model can account for this.

We then analyzed variability of glomerular responses over different odor categories. Data of odor categories were obtained by mapping odorant descriptors. We used a statistical method

Table 1 | Absolute error of fit between coding spaces (D_P, D_S) and perceptual spaces (D_{Chrea} and D_{BHsmall}).

	D_{Chrea}	D_{BHsmall}
D_S	0.51	0.24
D_P	0.60	0.55
Combined	0.43	0.34
Baseline	0.65	0.65

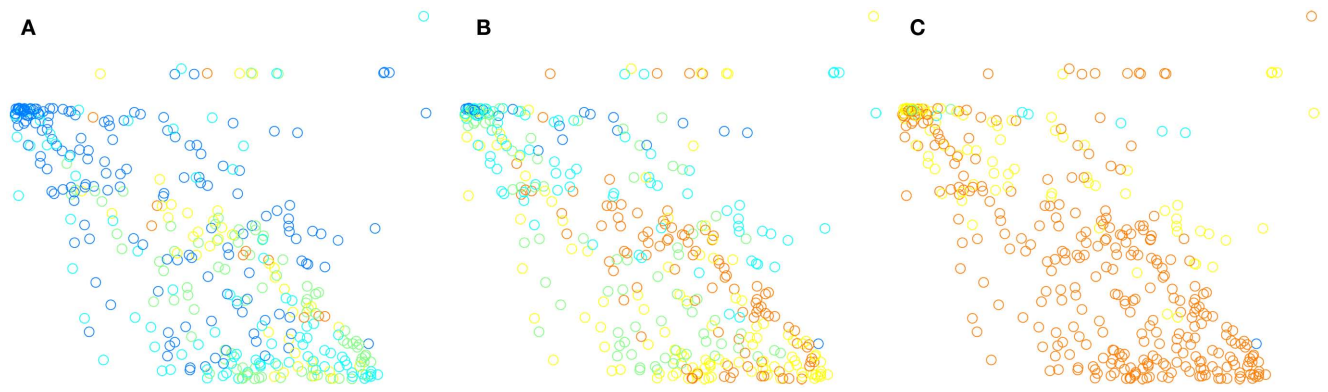

FIGURE 5 | Glomerular activations at three different concentrations. **(A)** shows glomerular responses at a low concentration, **(B)** at a medium concentration, and **(C)** at a high concentration. Plots are shown in two dimensions for clearer illustration (the actual space is in three dimensions). The color map starts from dark blue for small activations and includes shades of blue, cyan, green, yellow, and red, and ends with dark red for high values. At low concentration few glomeruli are activated (indicated by red). As concentration increases more glomeruli are activated, until the glomerular map becomes very unspecific and saturated.

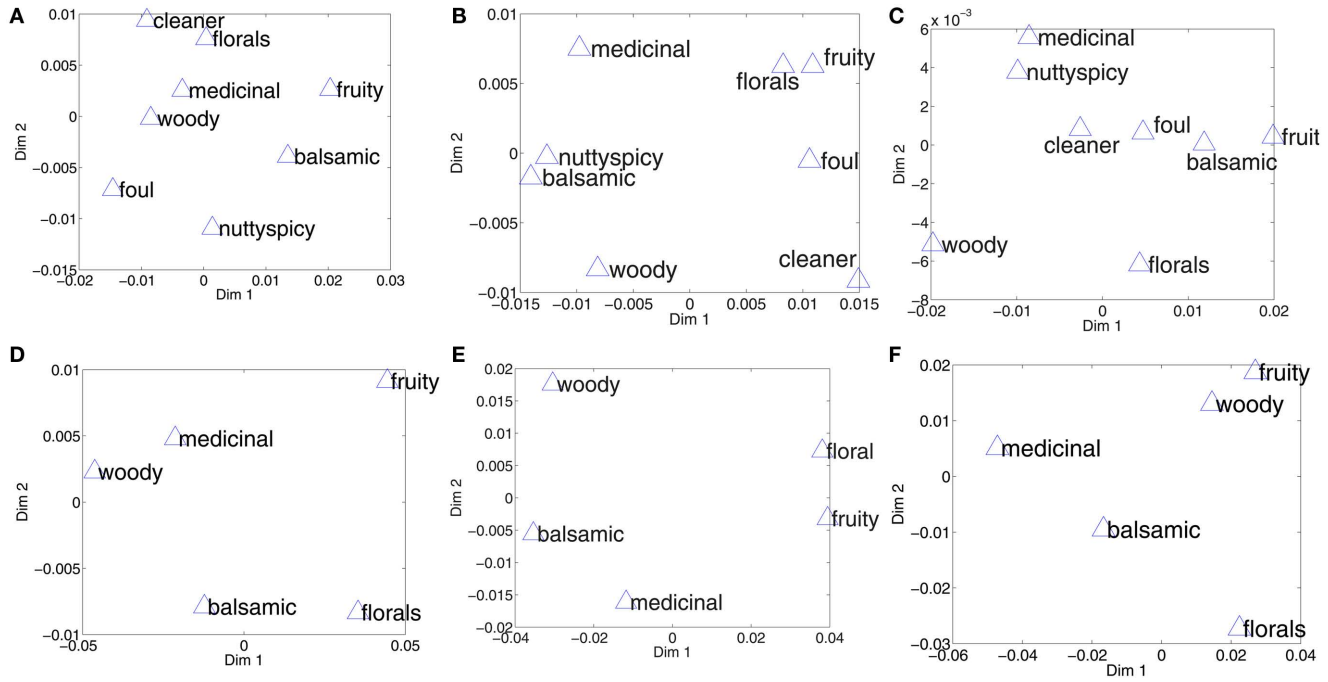

FIGURE 6 | Perceptual spaces constructed from pair-wise distances reduced to two dimensions by multi-dimensional scaling. (A) Shows the perceptual space in Chrea (2004), as per Zarzo (2008). **(B)** Shows the space as given by the distances from spatial encodings. **(C)** Shows the space as given by distances between activations of the entire glomerular layer, independent of spatial arrangement of glomeruli. The lower panel compares the perceptual space, **(D)** for the *BH small* data set, and the distances between the categories in the spatial encoding, **(E)**, and the population encoding, **(F)**.

to calculate receptive fields corresponding to odor categories for each glomerulus. Then, we compared how well a spatial code and population code matched human perceptual experiences.

Table 1 gives an indication about how accurately the perceptual space is reflected by the two different coding methods. Distances D_P are the distances between population codes, and D_S are distances between zones that showed differential activation in response to odor categories. Both were compared to perceptual orderings of odors. The errors between the perceptual space and the spatial encoding space is smaller compared to the population coding space for both data sets. Both population and spatial coding spaces performed better than a baseline generated from random points. For the *Chrea* data set, some categories have close distances from each in both the perceptual space and the spatial encoding space, such as for example medicinal–floral, balsamic–woody, and nutty/spicy–balsamic, which do not appear in the population coding space. Still, the match between the perceptual space and the spatial encoding space is not exact, as, e.g., the category cleaner show, which is misplaced in both encoding spaces.

For the smaller data set *BH small*, the absolute error was significantly smaller for the spatial encoding, but still quite high for the population code. This is reflected, e.g., by the concave pentagon structure in the population coding space (see **Figure 6F**) in comparison to the convex pentagon structure in the perceptual and spatial encoding space (compare **Figures 6D,E**).

Our results therefore suggest that spatial coding has a stronger relation to perception, but we also find a match better than baseline for population coding of perceptual categories. The combined codes integrating population and spatial codes, matched better in the case of the smaller set of odor descriptors and worse for Chrea. Therefore no clear conclusion can be drawn with regard to whether population and spatial coding complement each other.

4.1. TRANSLATING PHYSICAL REGULARITIES INTO SPATIAL MAPS

It is not clear yet how the early olfactory system translates information about molecules into a space relevant for perception and action. As discussed in the next paragraphs, some research groups found regularities in the physical odor space relative to perceptual, especially hedonic values, others found representations in the olfactory bulb (and insect antennal lobe) for representations of perceptual categories.

Khan, Haddad, Sobel, and colleagues suggested (Khan et al., 2007; Haddad et al., 2008b) that olfactory pleasantness corresponds to a natural axis of maximal discriminability among biologically relevant molecules and that the olfactory system has evolved to exploit regularities in the odor space. Where exactly physico-chemical properties are mapped to perceptual qualities is unclear, however a perceptual ordering of representations has been found previously in the piriform cortex (Howard et al., 2009). We found for our model of the OB that main orientations of perceptual and encoding spaces matched, however internal distances over these two spaces were different, which suggests that this mapping could occur earlier.

It is known that some odors are associated with specific molecular properties, e.g., putrid to amines and Doleman (1998) suggested that increased sensitivities to amines could constitute

an evolutionary adaptation for detecting decaying food and toxic gases.

We show in this paper that a model of cortical projections (Lansner et al., 2009) can be extended to explain the emergence of topography from statistics of naturally occurring odors. The application of this principle based on coalescence by co-activation gives rise to a topographic map where the distance of the components in the topographic representation is a function of the dependencies of the components.

We hope that our model can provide insights into the formation of the olfactory bulb map and internalizations of environmental regularities, even though the match between glomerular activity and human perceptual space could be improved by tuning parameters of the receptor affinity distribution or by including top-down projections. It could be that odorant receptors and projections to the OB are optimized to make environmental regularities more prominent. For example Geisler and Diehl (2002) proposed that the design of perceptual systems is optimized according to statistics of natural stimuli and evolutionary fitness. More particular for olfaction, Nei et al. (2008) discussed that part of molecular changes in chemoreceptor repertoires constitutes an adaptation of organisms to different environments. However, it was suggested (Abbott and Luo, 2007) that olfactory receptors are not optimal either and Sánchez-Montañés and Pearce (2002) demonstrated that optimal stimulus estimation arises from a local randomized mechanism for receptor specificity generation.

4.2. SPATIAL CODING IN THE OLFACTORY BULB

There are arguments for spatial, temporal, and spatio-temporal coding in the OB (cf. Leon and Johnson, 2009). For example, Wilson and Stevenson (2006) argued for the plausibility of population coding in the olfactory bulb. Rubin and Katz (1999) showed that maps of similar molecules were more correlated than maps of different molecules. (Haddad et al., 2010) analyzed population activity of glomeruli and MT cells from different studies and found that the first principal component was correlated to approach or withdrawal in animals and to odorant pleasantness in humans. They also argued that for reasons of robustness, speed, and in the light of experimental evidence, it is plausible that global and local coding schemes could work together.

A relationship between olfactory bulb activations and perceptual representations was also found on a spatial level. In fact, many studies suggest a spatial encoding (e.g., Vassar et al., 1994; Meister and Bonhoeffer, 2001; Mori et al., 2006; Johnson and Leon, 2007). There is evidence that distance between spatial zones could have a relationship to behavior. Laska and Teubner (1999) found in a forced-choice test that discrimination ability of subjects between homologous odors was correlated to differences in carbon chain length and Auffarth et al. (2011b) confirmed that different glomerular areas in the olfactory bulb are activated depending on carbon chain length and found contiguous olfactory bulb coding sites for several properties. In the same study, it was found that classification of molecular properties using a support-vector machine on activation data, for most compared properties, the spatial zones for coding were small and compact.

Leon and Johnson (2009) examining arguments for temporal and spatial coding, concluded that much of the available data is actually inconsistent with hypotheses related to temporal coding and rather support a spatial coding scheme. They argued that in rodents spatial patterns of glomerular activities and perceptual similarities are related. They suggested that perceptually driven behavior could serve as a starting point to evaluate the two coding schemes. We think that our study is a first step in that direction.

Studies in rats and mice have shown that different types of behavior, e.g., defensive behavior toward predators, aversion, or attraction toward food, can be related to the chemical categories of odorants emitted by the odor source (Dielenberg and McGregor, 2001) and that glomeruli coding for these categories are organized in domains or clusters in the OB. A study by Kobayakawa et al. (2007) suggested that the OB of mice consists of at least two different functional modules, one for innate odor responses and one for (associatively) learned odor responses. Similar spatial behavioral organization is also known to occur in insects (e.g., Semmelhack and Wang, 2009).

FUNCTION OF TOPOGRAPHY

Studies of odorant coding in the OB show that odor codes are represented on the levels of glomeruli and M/T cells by spatio-temporal codes (cf. Laurent, 1997; Leon and Johnson, 2009). Our model of activity-dependent self-organization of the glomerular layer suggests that there could be information about perceptual categories on spatial and population levels. It is not clear if topography on the olfactory bulb is key to a function (Zou et al., 2009). However, it was known that changing locations of glomeruli can result in behavioral impairments in mice, in spite of persistent physiological activations (Adam and Mizrahi, 2010). This could imply the existence of readout mechanisms that rely on spatial codes.

It could be speculated that local patterns of odor categories constitute an instance of the minimization of wiring length in cortical networks (cf. Chen et al., 2006) with functional implications (cf. Thivierge and Marcus, 2007). Practically topography could constitute an anatomical basis to sharpen MT responses over periglomerular pathways. Yaksi and Wilson (2010) provide evidence that local circuity between glomeruli in the antennal lobe, the insect analog of the OB, could serve for gain control by both contrast enhancement and increase of sensitivity. In favor of this functionality speaks also the length of periglomerular axons, which reach only a few glomeruli far (Shepherd et al., 2004).

One hypothesis for the generation of receptive fields on the MT layer is that competitive inhibitory mechanisms between MT cells could facilitate a mechanism for odorant concentration (see Sandström et al., 2009b) based on the highly variable response properties of ORNs expressing the same OR (Grosmaitre et al., 2006). In fact, a viral tracer study (Willhite et al., 2006) suggests a columnar organization by receptor type reaching from glomerular to deep granular layers (Willhite et al., 2006). This could indicate a local proximity which would be expected to underly circuits optimized for wiring length.

Evidence is accumulating that ORs and glomeruli are internalizations of environmental regularities (e.g., Dielenberg and McGregor, 2001; Hommel et al., 2002; Khan et al., 2007; Kobayakawa et al., 2007; Semmelhack and Wang, 2009; Sakano, 2010). In this model of axonal convergence we could account for

at least part of the organization of representations. Together with earlier evidence for spatial continuous maps for perceptual categories (Auffarth et al., 2011a), results are consistent with other studies to show a functional compartmentalization and a spatial organization in the OB. This could indicate organizing principles that could serve to efficiently convey behaviorally relevant information to higher stages, e.g., the amygdala or piriform cortex. Thus, higher brain regions seem to sample from glomeruli in spatial domains of the OB to receive behaviorally relevant information which triggers innate behavior, e.g., aversion due to fox urine or aggression due to male mouse odors.

The establishment of a functional architecture relies on process outgrowth and synapse formation. Connectivity is defined by molecular cues and neural activity (Katz and Crowley, 2002). While neural activity could be generated spontaneously in early phases of development, in later phases it is then crucially dependent on sensory experience, so that connectivity is defined by different forms of input, such as intrinsic, sensory, and other, such as coming from cognitive or motor areas. It is known that top-down projections from higher stages influence the dynamics in the OB (Fuentes et al., 2008) and therefore they could shape the glomerular map in a way to simplify the readout of behaviorally relevant information. The formation of topographic organization in our model relies exclusively on the input side, however other factors could be integrated as adaptations to the distance matrix, which is fed into the MDS algorithm.

CONCLUSION

We presented a model of olfactory perceptual coding at the glomerular level. Although the machinery responsible for axon guidance is much more complex than that presented here, we hope that progress of functional understanding may be facilitated by keeping our model as simple as reasonably possible. We used realistic, ecologically relevant odorant data and showed how from simple principles glomeruli form and spatial maps emerge with receptive fields specialized on a combination of physico-chemical features and odor categories. We showed in our model how OR affinities govern the formation of a topographic map in the OB and how the emergent coding domains for receptive molecular ranges reflect a perceptually relevant categorization.

We found that glomerular regions responsive to odor categories have relative spatial distributions over the bulb that represent a qualitatively good match between the odor ratings by humans. Therefore, our findings confirm previous studies which suggest a spatial coding at the olfactory bulb. This could suggest that the OB encodes perceptual categories.

ACKNOWLEDGMENTS

The authors thank Simon Benjaminsson for implementing a C++ library for simulation of neural networks. This work was supported by the EU Seventh Framework Programme (FP7/2007-2013), project NEUROCHEM under grant agreement no. 216916. We want to thank both reviewers for their insightful comments.

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

Received: 22 August 2011; accepted: 22 September 2011; published online: 11 October 2011.

Citation: Auffarth B, Kaplan B and Lansner A (2011) Map formation in the olfactory bulb by axon guidance of olfactory neurons. Front. Syst. Neurosci. ***5****:84. doi: 10.3389/fnsys.2011.00084*

frontiers in
SYSTEMS NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 09 May 2012
doi: 10.3389/fnsys.2012.00030

Psychophysical properties of odor processing can be quantitatively described by relative action potential latency patterns in mitral and tufted cells

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Electrophysiological and population imaging data in rodents show that olfactory bulb (OB) activity is profoundly modulated by the odor sampling process while behavioral experiments indicate that odor discrimination can occur within a single sniff. This paper addresses the question of whether action potential (AP) latencies occurring across the mitral and tufted cell (M/TC) population within an individual sampling cycle could account for the psychophysical properties of odor processing. To determine this we created an OB model (50,000 M/TCs) exhibiting hallmarks of published *in vivo* properties and used a template-matching algorithm to assess stimulus separation. Such an AP latency-based scheme showed high reproducibility and sensitivity such that odor stimuli could be reliably separated independent of concentration. As in behavioral experiments we found that very dissimilar odors ("A vs. B") were accurately and rapidly discerned while very similar odors (binary mixtures, 0.4A/0.6B vs. 0.6A/0.4B) required up to 90 ms longer. As in lesion studies we find that AP latency-based representation is rather insensitive to disruption of large regions of the OB. The AP latency-based scheme described here, therefore, captures both temporal and psychophysical properties of olfactory processing and suggests that the onset patterns of M/TC activity in the OB represent stimulus specific features of olfactory stimuli.

Keywords: model, latency code, olfaction, discrimination times, *in vivo*, behavior

INTRODUCTION

In rodents the sense of smell is of critical importance. This combined with its somewhat simple functional architecture has made the olfactory system an ideal model system to examine the neural basis of sensory processing in mammals. Nevertheless, the detailed mechanisms of olfactory processing and the neural processes underlying it remain largely unknown. Behavioral approaches offer an excellent means of constraining models of olfactory processing (Linster and Cleland, 2004; Cleland and Linster, 2005). Studies in rodents using go/no go odor detection and discrimination tasks for example indicate that lesions that encompass large parts of the olfactory bulb (OB) do not produce a dramatic phenotype at least for simple tasks (Lu and Slotnick, 1998). Furthermore, generally in rodents odor discrimination is very rapid, occurring in less than 200–250 ms within a single sniffing bout (Uchida and Mainen, 2003; Abraham et al., 2004; Rinberg et al., 2006). There also exists a speed-accuracy trade-off such that discrimination between highly similar odorants requires additional time, in the range of 70–100 ms (Abraham et al., 2004; Rinberg et al., 2006). These overall discrimination times include both the sensory transduction, which might require several tens or even hundred milliseconds (Duchamp-Viret et al., 1999; Carey et al., 2009), and the motor and cognitive components of the discrimination task. It is thus likely that the processing time in the OB is actually substantially less than the time window defined by the behavioral discrimination task.

Voltage-sensitive dye and calcium imaging experiments indicate that odors activate not only a specific spatial pattern of glomerulus activity but that activation is strongly modulated by the sniff-cycle. Inputs to the OB are strongly shaped by the respiration cycle in anesthetized as well as awake animals (Spors et al., 2006; Verhagen et al., 2007; Wesson et al., 2008; Carey et al., 2009). Moreover, the sequence of glomerular activation is also odor specific and virtually concentration invariant (Spors and Grinvald, 2002; Spors et al., 2006). Many studies using extracellular recordings in anesthetized and awake animals found a strong coupling of bulb activity to the respiration cycle even as frequencies as high as 10 Hz (Adrian, 1950; Macrides and Chorover, 1972; Buonviso, 2006; Cury and Uchida, 2010; Carey and Wachowiak, 2011). Intracellular *in vivo* recordings have shown that individual mitral/tufted cells (M/TCs) display a prominent sub-threshold membrane potential oscillation synchronous with the sniff cycle

Abbreviations: AP, action potential; EPSP, excitatory postsynaptic potential; InF, integrate-and-fire; ISI, inter-spike interval; MC, mitral cell; M/TC, mitral/tufted cell collectively referring to projection neurons; OB, olfactory bulb; TC, tufted cell.

(Charpak et al., 2001; Cang and Isaacson, 2003; Margrie and Schaefer, 2003). M/TC suprathreshold activity is thus structured across a single sampling cycle such that those cells firing more action potentials (APs) begin to fire consistently earlier than those discharging fewer APs (Margrie and Schaefer, 2003).

Many features of the psychophysics of odor discrimination and detection are exquisitely captured in simple models relying on a static pattern of all-or-none glomeruli (Koulakov et al., 2007). Since rhythmic odor sampling is phylogenetically highly conserved and defines activity early in the olfactory pathway it seems desirable that working models of olfactory processing should incorporate this active and dynamic process (Künsting and Spors, 2009). To examine whether the temporal structure of OB activity across a sampling cycle might contain information that could account for the known psychophysical properties of olfactory processing we built a large-scale model of the OB. We have constrained the discharge patterns of M/TCs, based on *in vivo* measurements of AP latencies, inter-spike intervals (ISIs) and AP distributions within a sniff cycle. Furthermore, we ensure that odor concentration dependence and the distribution of activity follow that measured for individual neurons and the OB network. As this scheme quantitatively reproduced both similarity-dependent discrimination times and robustness against lesioning, we suggest that in the OB, odor processing relies on the *patterns of AP onset* across the network of M/TCs.

MATERIALS AND METHODS

Our explicit model for the OB was built with a focus on accurate reproduction of *in vivo* single M/TC discharge patterns with particular attention to the onset of AP activity. Since these data reflect contributions of both sensory and local OB activity we did not include any additional explicit sources of inhibition (see also Discussion). To facilitate quantitative comparison with behavioral data, we built an OB network of realistic size with 2400 glomeruli with 25 M/TCs each. Varying the number of M/TCs per glomerulus did not significantly alter the results (data not shown). To maintain computational feasibility it was thus necessary to keep the single-cell model simple and efficient. We proceeded in three steps: Firstly, based on whole-cell recordings *in vivo* we adjusted parameters of a leaky integrate-and-fire (InF) neuron to match the measured onset latencies, ISIs and other single-cell parameters. Secondly, using the measured distribution of AP firing for odor-evoked activity we determined the distribution of input currents corresponding to an odor stimulus. These currents were then related to binding affinities using simple sigmoid relations. Thirdly, we tested our model by comparing its concentration dependence with published electrophysiological and imaging measurements. All simulations were performed using Matlab 6.5 (The MathWorks, Natick, MA, USA) with InF neuron models in the csim_lifnet simulation environment (T. Natschläger, available at http://www.igi.tugraz.at/tnatschl/csim_lifnet/).

CONSTRAINING SINGLE-CELL PARAMETERS

M/TCs were modeled as Inf neurons to allow for networks of realistic size of spiking neurons. Gaussian noise was added resulting in a membrane potential variance of 0.20 ± 0.04 mV (mean $\pm$ SD, $n = 10$, Schaefer et al., 2006). Background synaptic input consisted of 100 Hz Poisson inputs with excitatory postsynaptic potential (EPSP) amplitude of 0.58 mV and a decay time constant of 10 ms. A 4 Hz oscillation with a peak-to-peak amplitude of 10 mV was mimicked by sinusoidal current injection (Schaefer et al., 2006). Varying oscillation frequency between 2 and 10 Hz did not significantly alter the findings (data not shown). AP discharge in M/TCs was measured for constant current injection between 0 and 0.18 nA resulting in 1.6 ± 3.3 APs (range 0–15) per cycle [second cycle, see (Lengyel and Erdi, 2004); $n = 10{,}000$] and the four parameters (membrane time constant, AP threshold, AP reset voltage, and refractory period) adjusted to fit ISI (**Figures 1A,B**) latency (**Figures 1A,C**) and the distributions of APs within an oscillation cycle (**Figure 1A**) as observed *in vivo* (Margrie and Schaefer, 2003). This resulted in an AP threshold of 15 mV, a refractory time constant of 4 ms, a membrane time constant of 30 ms, and a reset voltage of 10 mV.

CONSTRAINING STIMULUS PARAMETERS

In order to constrain odor stimulus parameters, the AP distribution in response to odors was analyzed. A distribution of input currents was determined that reproduced the measured cumulative AP discharge probability observed *in vivo*

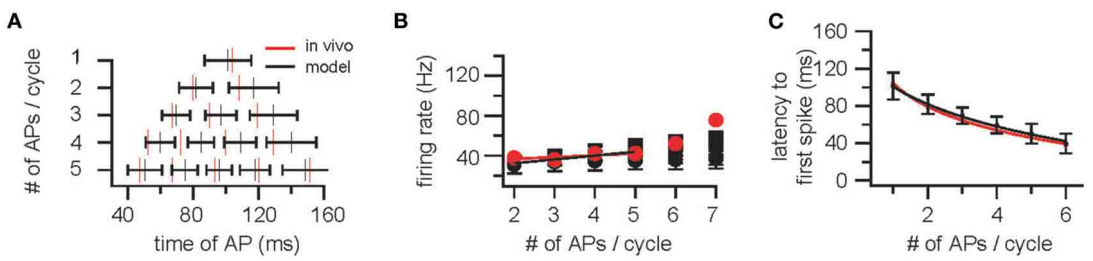

FIGURE 1 | Construction and validation of an OB model based on parameters determined *in vivo* – cellular constraints. (A) Top: Example trace of the membrane potential of a mitral/tufted cell (M/TC) showing respiration synchronized sub-threshold oscillations in an awake mouse. Scale bar is 200 ms and 20 mV. The beginning of each respiration cycle is indicated by *open circles*. Below: A plot of action potential distribution in M/TCs for sniff cycles that evoke between 1 and 5 APs. **(B)** Instantaneous firing rate [inverse of the inter-spike interval (ISI)] and the latency to action potential (AP) onset **(C)** plotted against the number of APs evoked per sniff cycle. *Red markers and lines* indicate data obtained from M/TC whole-cell recordings *in vivo* [Margrie and Schaefer (2003)]. *Black* indicates the cellular responses for the integrate-and-fire (InF) neurons used.

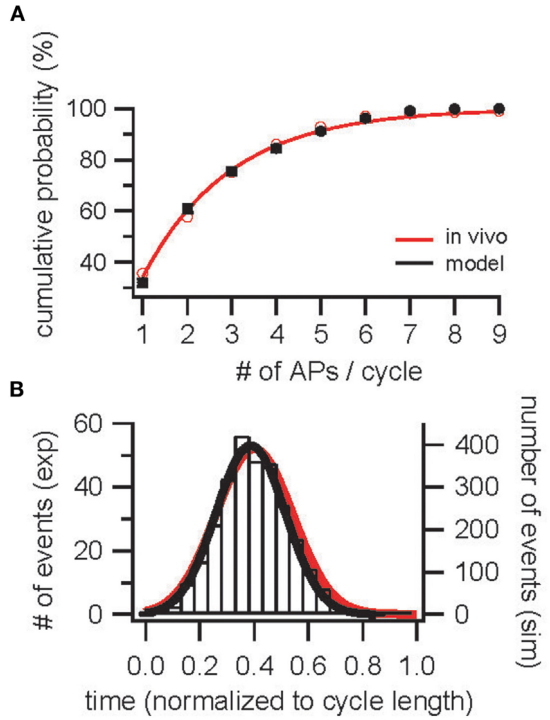

FIGURE 2 | Construction and validation of an OB model based on parameters determined *in vivo*—population constraints. (A) Cumulative probability for the occurrence of 1–9 APs per sniff cycle during odor-evoked AP discharge in InF neurons (*black*) and *in vivo* [red; Margrie and Schaefer (2003)]. **(B)** Distribution of APs across a sniff cycle in simulation (*black*) and *in vivo* [*red*, Margrie and Schaefer (2003)].

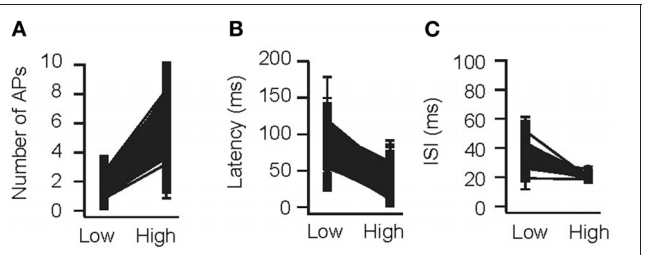

FIGURE 3 | Concentration dependence in the model. Validation of the OB model for variable odor concentrations. An odor was presented to the OB model at concentrations of 0.3 ("low") and 3 ("high") times relative to the concentration used in **B** (see methods). The number of APs per cycle **(A)**, latency to the first AP **(B),** and average ISI **(C)** were determined.

in cells that responded to an odor (**Figure 2A**, Margrie and Schaefer, 2003) resulting in a distribution of input currents of $[(i \times 0.53 + 0.3)^{4.9} + 0.3] \times 0.18$ nA with i distributed equally between 0 and 1. In **Figure 2A**, mean and SDs are plotted for 10 repetitions with different random seeds and 1000 cells each. As a first control, the resulting AP distribution was compared to the measured AP distribution (**Figure 2B**).

To assess the correspondence of odor affinities and concentrations to the distribution of activity in ORNs, a simple sigmoid relation with glomerulus-independent parameters was assumed (Meister and Bonhoeffer, 2001): $R = R_{max}\ [C/(C + k_i)] + b$. "$b$" is the response threshold (current needed for 0.2 APs; $b = 0.035$ nA); $R_{max} + b$ equals the maximal current [current required to evoke 12 APs (Margrie and Schaefer, 2003); here 0.169 nA]. From this, we determined the binding coefficients, k_i, resulting in the AP distribution of **Figure 2**.

CONCENTRATION-DEPENDENCE OF RESPONSES

As a next control, odors were presented at two concentrations (0.3 and 3 relative to **Figure 2**) and number of APs, ISI, and latency to the first spike were measured (**Figure 3**). Cang and Isaacson (2003) reported a two-fold increase in the number of APs per cycle (from 2.1 to 4), a 60 ms decrease in mean onset latency and a non-significant decrease in ISI. In the model, the number of APs increased from 1.51 ± 0.80 to 6.16 ± 2.21 APs; the latency to onset decreased from 95.4 ± 20.1 to 41.5 ± 19.9 ms ($313/500$ cells spiked at both concentrations), whereas the ISI did not change substantially (34.8 ± 11.0 vs. 20.2 ± 2.2 ms, $111/500$ cells spiked with more than 1 AP at both concentrations; simulations were repeated 10 times with different random seeds). This is in excellent agreement with whole-cell recordings *in vivo* (Cang and Isaacson, 2003).

To obtain qualitative insight into population activity in the model, we created images of activity, corresponding to 200 glomeruli (the number of glomeruli that could be visualized using intrinsic imaging of the dorsal surface; Meister and Bonhoeffer, 2001). The largest number of glomeruli activated by an individual odor as measured by Ca^{2+} imaging of presynaptic activity was 60 out of the approximately 150 glomeruli visible in this study (Wachowiak and Cohen, 2001). Thus, an odor consisted of an activity pattern mapped onto 40% of the glomeruli, with the above described distribution of input currents. To qualitatively compare the model to imaging results at different concentrations, we varied concentrations over four orders of magnitude and converted the resulting M/TC activity (number of APs/cycle) in gray scale levels and mapped them on a scheme of an OB (**Figure 4**). Two hundred glomeruli were randomly distributed as dots in an ellipse with 50 and 30 pixels radius and Gaussian noise ($\sigma = 0.1$) was added to every data point. The resulting image was filtered by a 7×7 Gaussian filter with two passes, clipped to the ellipse and smoothened with a 3×3 Gaussian filter. In agreement with intrinsic imaging studies (Rubin and Katz, 1999) at low concentrations only few distinct glomeruli are activated; at higher concentrations widespread activity occurs.

RESULTS

Sub-threshold oscillations in M/TCs are synchronized to sniffing and are a hallmark of the early olfactory system (Schaefer and Margrie, 2007; Wachowiak, 2011). Across an individual sampling or oscillation cycle instantaneous firing rate of M/TCs is barely affected by changes in input strength (Cang and Isaacson, 2003; Margrie and Schaefer, 2003). Overall activity of a given M/TC is, however, accurately reflected by its onset latency (Margrie and Schaefer, 2003; Kepecs et al., 2006) that are highly odor-specific and reproducible (Junek et al., 2010). Could these latencies account for the rapid but stimulus dependent discrimination times that are difficult to reconcile with a code relying on

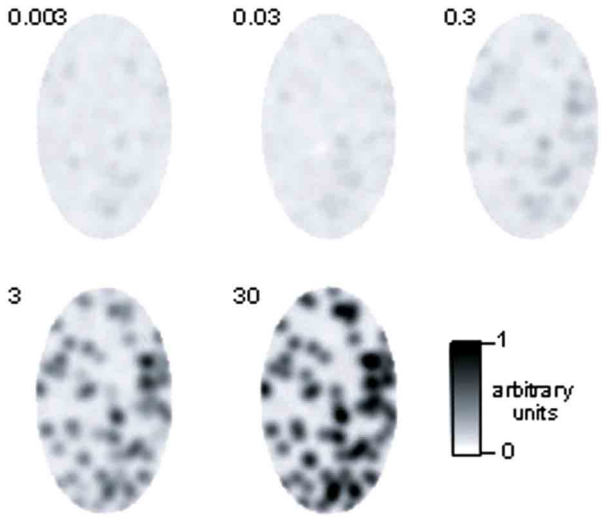

FIGURE 4 | Population response to an odor at different concentrations in the model. The depicted region corresponds to the dorsal part of the OB that has been visualized using intrinsic imaging methods [Rubin and Katz (1999); Uchida et al. (2000); Meister and Bonhoeffer (2001)]. M/TC activity was converted to gray scale values as described in the methods.

"counting" the number of APs discharged during a cycle (Margrie and Schaefer, 2003)? From a simple sketch of AP latencies across the M/TC population (**Figure 5**) it is apparent that—in the case where very dissimilar odorants evoke spatially non-overlapping activation patterns—odor discrimination based on the onset of activity could be very rapid (**Figure 5**, left column): For example, where odor A evokes AP discharge early in M/TC #1 and 4 (open arrowhead), odor B evokes APs only late or not at all. Conversely, odor B results in very short onset latencies in M/TC #3 (filled arrowhead) that is activated weakly and late in a cycle by odor A. Thus, for these very different stimuli only a brief period (gray bar and dotted line) would be needed to discriminate odor A from B based on the activity onset pattern.

If, however, the response to two similar odors (that evoke highly overlapping patterns; such as binary mixtures, 0.6A/0.4B [Mix 1] and 0.4A/0.6B [Mix 2]) is compared, M/TC #1 may discharge early for both odors (**Figure 5** right column, open vs. filled arrowheads). Averaging over a large number of repetitions or alternatively a large number of cells, a very small difference in the initial onset latencies might become apparent. Thus, for "one-sniff" odor discrimination, many cells or glomeruli (including those with delayed onsets, e.g., #5, #6) would be needed to reliably separate very similar stimuli. Hence, while simple discriminations could be performed quickly, difficult separations would require the activity of many late firing M/TCs (Schaefer and Margrie, 2007).

To obtain quantitative evidence for this hypothesis, we built an OB model with 2400 glomeruli containing 25 M/TC each (described in detail in the methods). An activity onset vector for the M/TC network was generated for each odor (**Figure 6A**). Reproducibility and similarity was determined by correlating these onset vectors (**Figures 6B**,**C**). The analysis was then

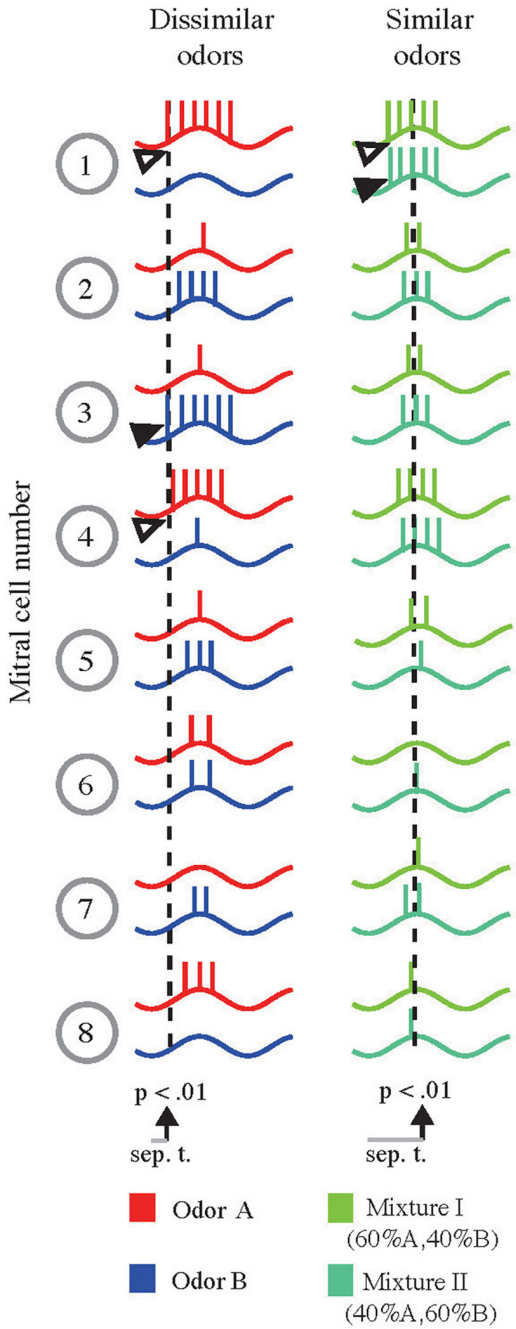

FIGURE 5 | Schematic representation of the proposed AP latency-based scheme and emerging similarity-dependent separation times. Left column: Sketch of the membrane potential of eight M/TCs in response to two different odors (*red and blue*) with little spatial overlap. For dissimilar odors arrows indicate M/TCs that respond either weakly for odor A and strongly for odor B (*solid arrow*, M/TC #3) or vice versa (*open arrows*, M/TC #1 and #4). Based on AP latencies in the strongly activated M/TCs reliable stimulus separation may be achieved early during the evoked response (indicated by the *dotted line*). **Right column**: Same M/TCs in response to two similar odors (binary mixtures of A and B, Mixture I [60%A, 40%B], light green; Mixture II [40%A, 60%B], dark green). Due to the overall similarity of M/TC responses, information from many more cells (including those with delayed onsets e.g., M/TC #5 and #6) must be obtained to allow for stimulus separation (*dotted lines*).

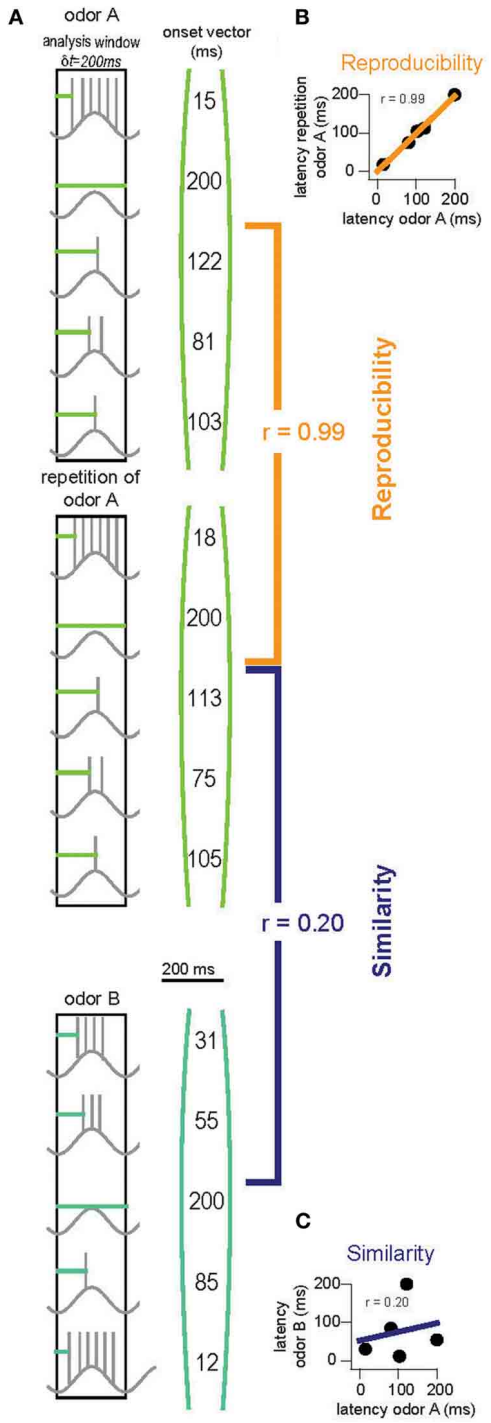

FIGURE 6 | Quantifying stimulus separation in the OB. (A) Onsets of activity (*horizontal light and dark green lines*) of all M/TCs are collected into "onset vectors" (*right*). Each odor is presented twice with different random seeds for noise generation. "Reproducibility" is calculated by correlating onset vectors with a repetition of the same odor [orange, **(B)**]. "Similarity" is determined by correlating onset vectors for responses to two different odors [purple, **(C)**]. This is repeated eight times with different random seeds for odor and noise generation. Separation of two odors is ensured when the resultant reproducibility values are significantly higher than the similarity values (unpaired *t*-test).

restricted to, for example, the first 200 ms of activity. That is only onset values for those M/TCs discharging within 200 ms were taken into account. For all other M/TCs, ceiling values (200 ms) were employed for the correlation. This time window was then varied and correlations of onset vectors re-calculated until the entire sampling period was encompassed. Using a template-matching scheme we find that within the first 30 ms of activity the latency patterns for repetitions of the same odor are already highly reproducible and that their correlation improves with time (**Figure 7A**; 1 way ANOVA $F_{(8,\ 63)} = 534$, $p < 10^{-5}$). In contrast, the overall M/TC onset pattern evoked by dissimilar odors revealed no significant correlation over the entire sampling period (**Figure 7A**; $r = -0.008 \pm 0.013$).

To determine the time-course of AP latency-based stimulus separation we first presented dissimilar odor pairs (e.g., A vs. B). In this case we find that the reproducibility of the M/TC responses is sufficiently large to reliably separate stimuli very early in the evoked response (**Figure 7A**; $p < 10^{-4}$ at 30 ms). In contrast, the time required to reliably differentiate the responses to very similar odors (binary mixtures; 0.4A/0.6B vs. 0.6A/0.4B) was substantially longer (**Figure 7B**; $p > 0.1$ for $\delta t < 90$ ms). Quantifying stimulus separation times for pairs ($n = 10$) of both very dissimilar and similar odors shows that while less than 30 ms of OB activity can be enough for easy separation tasks (30 ± 1 ms; range 25–33 ms) up to 90 ms longer is needed to perform the more difficult separation of similar odors (**Figure 8**, 89 ± 2 ms; 82–118 ms). Both similarity dependence and absolute separation times (**Figure 8B**) are consistent with the behaviorally observed stimulus-dependent discrimination times in rodents. The stimulus-dependence of separation times was also largely independent of the absolute odor concentration ($R^2 = 0.12$, $p > 0.05$). This is again consistent with concentration-independent discrimination times observed experimentally (Uchida and Mainen, 2003; Abraham et al., 2004).

A second observation for the olfactory system is that basic odor discrimination appears rather insensitive to partial deletions of the OB. Such studies indicate that disruption of large regions of the OB fail to produce a dramatic behavioral phenotype (Lu and Slotnick, 1998). In some animals, discrimination of very different

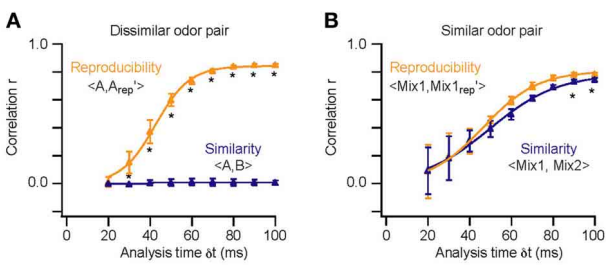

FIGURE 7 | Stimulus reproducibility and similarity in the model. Similarity and reproducibility for repetitions of a dissimilar [**A** vs. **B**; **(A)**] and similar odor pair [Mix1:60A/40B vs. Mix2:40A/60B; **(B)**] as a function of the analysis time window (δt). *Asterisks* (*) highlight times at which "reproducibility" was significantly higher than "similarity" ($p < 10^{-4}$). *Error bars* indicate SD for the eight repetitions of each comparison. Thick lines are sigmoidal fits of the correlation scores.

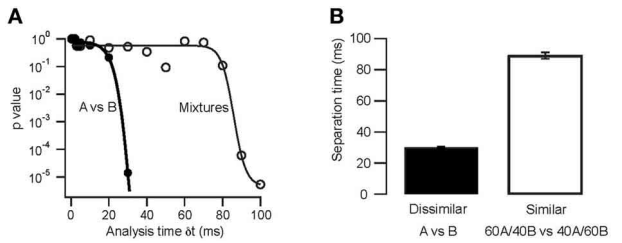

FIGURE 8 | Separation times for dissimilar and similar odor pairs in the model. (A) Stimulus separation (*p* value of the comparison of reproducibility and similarity) as a function of analysis window time δ*t*. Thick lines are sigmoidal fits. **(B)** Separation times (time at which reproducibility was significantly higher than similarity) for dissimilar and similar odor pairs. *Error bars* indicate s.e.m. from 10 odor pairs (eight repetitions each as in **A**).

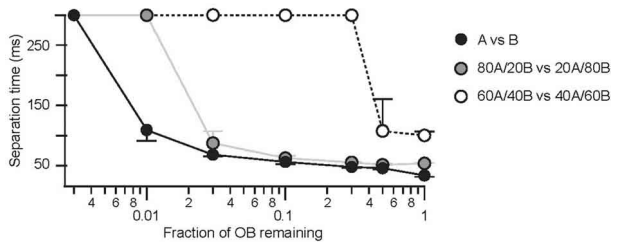

FIGURE 9 | The AP-latency scheme predicts robustness against large OB lesions. Separation times as a function of the size of a "virtual OB lesion" for dissimilar (*black*), intermediate (*gray*), and similar (*open*) odor pairs. Medians and their respective quartiles are plotted.

odors was partially impaired only if lesions impacted on more than 80% of the OB. However, when more difficult discriminations were considered lesioning 50–90% of the OB was at least partially effective (Lu and Slotnick, 1998). To determine whether the AP latency-based coding scheme might also account for these observations we introduced virtual lesions that randomly removed between 50 and 99.7% of the OB. Firstly we find that AP latency-based discrimination is very robust against lesions of the OB. For very dissimilar odorants discrimination could still occur with less than 5% of the intact OB network. For more similar odors 50% of the OB was sufficient to achieve successful odor discrimination, in agreement with previous behavioral observations (Lu and Slotnick, 1998). In addition, we can make the prediction that separation times for discrimination tasks of both easy and intermediate difficulty depended critically on the size of the OB network (**Figure 9**).

DISCUSSION

Here we propose a simple scheme of odor representation in the OB that relies on the onset latencies of APs in M/TCs across an individual sampling cycle. At the single-cell level, the model used to test this is based on whole-cell recordings from M/TCs *in vivo*. The patterns of activity in M/TCs in response to odor stimuli indicate that the overall level of excitation in an individual M/TC is accurately predicted by the onset of APs across each respiration cycle (Margrie and Schaefer, 2003). Our simulations here show that such an AP latency-based scheme can account for a number of temporal and psychophysical features of odor processing. Firstly, it is concentration invariant and mirrors the similarity-dependent discrimination times observed in rodents (Uchida and Mainen, 2003; Abraham et al., 2004; Rinberg et al., 2006). The latency-based scheme also reproduces the behaviorally observed robustness against extensive OB lesions (Lu and Slotnick, 1998) and predicts that the time required to discriminate odors will gradually increase with lesion size. Thus, our data not only account for existing behavioral observations but also predict that the overall odor discrimination time is sensitive to the size of the OB network, a prediction that could be tested in automatized behavioral assays (Schaefer and Claridge-Chang, 2011) where animals with differing lesion size would be systematically tested on a battery of odors with varying similarity.

We speculate that the relative onset latency of a glomerulus or M/TCs contained therein reflects the relative activation intensity of a given ORN channel or functional module (Cang and Isaacson, 2003; Margrie and Schaefer, 2003; Bhandawat et al., 2005; Spors et al., 2006). It has been suggested that lateral inhibition between such functional modules may provide a mechanism of enhancing subtle differences between the activity patterns evoked by different odor stimuli (Yokoi et al., 1995; Urban, 2002; Leon and Johnson, 2003; Cleland and Linster, 2005). Such inhibition either through direct GABAb modulation of the ORNs by juxtaglomerular cells (Aroniadou-Anderjaska et al., 2000) or via the granule cell mediated pathway (Urban, 2002) could be used to enhance differences between the onset latencies of glomerular units (Margrie and Schaefer, 2003). Indeed, alterations to the OB network through Cre-mediated excision of Glutamate receptors resulted in altered olfactory learning and discrimination (Shimshek et al., 2005). More targeted modifications, however, through virus-mediated, ablation of the AMPA receptor subunit GluA2 specifically in the granule cell layer resulted in increased Ca influx in granule cells, and increased inhibition. Odor discrimination learning, however, was left un-altered as were odor discrimination times for simple odor discrimination tasks. Ablating the NMDA receptor subunit NR1 and thus decreasing Ca influx and decreasing inhibition similarly left performance on simple tasks unaltered (Abraham et al., 2010). This indicates that indeed for simple odor discriminations, inhibition in the OB is not needed. For highly similar odor pairs, that require overall longer for accurate discrimination (Abraham et al., 2004), increasing and decreasing inhibition did indeed decrease and increase odor discrimination times (Abraham et al., 2010) indicating a role of inhibition in shaping late activity and thus potentially contributing to the exact latencies of M/TCs active later in a respiration cycle. Irrespective of the exact source of AP latency differences (Cang and Isaacson, 2003; Margrie and Schaefer, 2003; Bhandawat et al., 2005; Buonviso, 2006; Spors et al., 2006; Cury and Uchida, 2010; Carey and Wachowiak, 2011; Shusterman et al., 2011) we suggest that such activity in M/TCs is sufficient to explain several psychophysical properties of the mammalian olfactory system. The role of inhibition might become more prominent in situations where expectation as mediated by cortical inputs onto GCs begins to modulate odor representation (Koulakov and Rinberg, 2011).

A key feature structuring AP latencies and latency difference is the rhythmic sniff-coupled drive that results in sniff-coupled sub-threshold oscillations in M/TCs and consequently in sniff-coupled rhythmic AP discharge. While most electrophysiological recordings so far have been made in anesthetized rodents (e.g., Adrian, 1950; Macrides and Chorover, 1972; reviewed in Buonviso, 2006), recently data has also been acquired in awake animals. While initially it was suggested that the increased sniff frequency in the awake preparation might result in reduced sniff coupling, recent experimental data with careful alignment of unit recording data to sniff measurement again indicates that M/TCs can indeed tightly couple to the underlying sniff rhythm (Cury and Uchida, 2010; Carey and Wachowiak, 2011; Shusterman et al., 2011) consistent with the fact that mice can indeed be behaviorally trained to distinguish inputs at different phases of the sniff cycle (Smear et al., 2011). While these data indicate that AP discharge is indeed tightly coupled to sniffing in the awake animal as well, it remains to be seen whether mechanistically mitral and tufted cells (M/TCs) display similar sniff coupled sub-threshold oscillations in the awake animal that would further strengthen a robust latency encoding of input strength (Hopfield, 1995; Margrie and Schaefer, 2003; Schaefer and Margrie, 2007).

How might M/TC onset latencies be read out? A detailed understanding of the connectivity between OB and piriform cortex on a single-cell level is at present lacking (Miyamichi et al., 2010; Ghosh et al., 2011; Sosulski et al., 2011). Generally, tufted cells project to more anterior parts of olfactory cortex including anterior olfactory nucleus and olfactory tubercle, whereas mitral cells additionally innervate the entire piriform cortex including posterior parts as well as olfactory amygdala (Haberly and Price, 1977; Orona et al., 1984; Nagayama et al., 2010). While most studies indiscriminately investigate M/TC coding properties, these heterogeneous projection patterns might find functional correlates in odor encoding as well (Nagayama et al., 2004). Notably, evidence is mounting for strong direct excitatory drive onto tufted cells, whereas mitral cells seem to be activated either indirectly or with increasing threshold (Gire and Schoppa, 2009; Najac et al., 2011). This might suggest that tufted cells are particularly suited to relay a rapid snapshot of the olfactory environment.

However, implementing any realistic readout mechanism based on known anatomical and physiological properties is, at present, difficult. Recent work indicates that projections from the OB to PCx as well as the odor-evoked patterns in the PCX do not show any specific topography (Stettler and Axel, 2009; Choi et al., 2011; Sosulski et al., 2011). Minimal stimulation studies (Franks and Isaacson, 2006) and *in vivo* recordings (Wilson, 1998; Poo and Isaacson, 2009) show that many M/TCs as well as recruitment of recurrent excitation in PCx may be necessary to produce the observsed compound EPSPs observed in piriform cortex (Franks et al., 2011). This, together with electroencephalogram recordings, that suggest a substantial temporal heterogeneity of mono- and di-synaptic delays (Ketchum and Haberly, 1993), offers a potential substrate for coincidence-based readout (Hopfield, 1995; White et al., 1998; Margrie and Schaefer, 2003). Due to feed-forward inhibitory circuits, such detection mechanisms might be further sharpened (Perez-Orive et al., 2002; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2010) thereby increasing the sparseness of M/TC readout. Thus, although an AP latency based code in M/TCs is sufficient to explain stimulus-dependent discrimination times, concentration invariance, and the olfactory systems robustness against lesioning, the mechanism underlying downstream readout of such activity is yet to be determined.

ACKNOWLEDGMENTS

We thank Alexander Arenz, Diogo Pimentel, and Moritz Helmstaedter for discussion. This work was supported by the Max-Planck-Society, the BMBF, DFG SPP1392, the Gottschalk-Stiftung (Andreas T. Schaefer), the Wellcome Trust and the MRC (MC_U1175975156; Troy W. Margrie). Andreas T. Schaefer is a member of the ExcellenzCluster CellNetworks.

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

Received: 03 January 2012; accepted: 09 April 2012; published online: 09 May 2012.

Citation: Schaefer AT and Margrie TW (2012) Psychophysical properties of odor processing can be quantitatively described by relative action potential latency patterns in mitral and tufted cells. Front. Syst. Neurosci. ***6****:30. doi: 10.3389/fnsys.2012.00030*

frontiers in
SYSTEMS NEUROSCIENCE

REVIEW ARTICLE
published: 16 September 2011
doi: 10.3389/fnsys.2011.00078

Chemosensory learning in the cortex

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Taste is a primary reinforcer. Olfactory–taste and visual–taste association learning takes place in the primate including human orbitofrontal cortex to build representations of flavor. Rapid reversal of this learning can occur using a rule-based learning system that can be reset when an expected taste or flavor reward is not obtained, that is by negative reward prediction error, to which a population of neurons in the orbitofrontal cortex responds. The representation in the orbitofrontal cortex but not the primary taste or olfactory cortex is of the reward value of the visual/olfactory/taste input as shown by devaluation experiments in which food is fed to satiety, and by correlations of the activations with subjective pleasantness ratings in humans. Sensory-specific satiety for taste, olfactory, visual, and oral somatosensory inputs produced by feeding a particular food to satiety is implemented it is proposed by medium-term synaptic adaptation in the orbitofrontal cortex. Cognitive factors, including word-level descriptions, modulate the representation of the reward value of food in the orbitofrontal cortex, and this effect is learned it is proposed by associative modification of top-down synapses onto neurons activated by bottom-up taste and olfactory inputs when both are active in the orbitofrontal cortex. A similar associative synaptic learning process is proposed to be part of the mechanism for the top-down attentional control to the reward value vs. the sensory properties such as intensity of taste and olfactory inputs in the orbitofrontal cortex, as part of a biased activation theory of selective attention.

Keywords: sensory-specific satiety, taste, olfaction, selective attention, biased activation, orbitofrontal cortex, insular taste cortex, cognitive modulation

INTRODUCTION

The aim of this paper is to describe some of the principles of chemosensory learning in the cerebral cortex. The focus is on the mechanisms that are present in primates including humans. One of the reasons for this focus is that the taste and related pathways in non-human primates are similar to those in humans (Norgren, 1984; Rolls and Scott, 2003; Rolls, 2005; Rolls and Grabenhorst, 2008; Small and Scott, 2009), and thus evidence from these sources is particularly relevant to understanding taste and olfactory processing in humans. For example, in primates the taste pathways project from the nucleus of the solitary tract directly to the taste thalamus (Beckstead et al., 1980) and thus to the primary taste cortex in the anterior insula (Pritchard et al., 1986). There is no known pontine taste area in primates (Norgren, 1984; Rolls and Scott, 2003; Rolls, 2005; Small and Scott, 2009), whereas in rodents there is a pontine taste area that then sends onward connections to a number of subcortical areas including the hypothalamus and amygdala (Rolls and Scott, 2003). In contrast, in primates the taste processing is directed straight to the primary taste cortex (from the nucleus of the solitary tract via the thalamus), which then has onward connections to the cortical taste hierarchy of the orbitofrontal cortex, which contains the secondary taste cortex (defined by its direct anatomical projections from the primary taste cortex; Baylis et al., 1995), which in turn projects to the anterior cingulate cortex which is thus a tertiary taste cortical area (Rolls, 2008a) (**Figure 1**). The primary taste cortex in primates is the source of connections to subcortical structures such as the amygdala. It has been suggested that this cortically dominated taste connectivity in primates including humans is related to the great development of cortical processing in primates including humans, so that the unifying design is to bring all sensory modalities to the cortex for processing, and then after one or several mainly unimodal cortical areas for computations, to then bring the different sensory pathways together, with one key convergence area being the orbitofrontal cortex, as shown in **Figure 1** (Rolls, 2005, 2008b; Rolls and Grabenhorst, 2008).

Another key reason for focusing on taste and related processing in primates including humans is that the principles of operation with respect to taste reward, olfactory reward, and the control of appetite, appear to be rather different from those in rodents. For example, in macaques there is no reduction of the neuronal responses to taste stimuli in the primary taste cortex in the anterior insula (Yaxley et al., 1988) and adjoining frontal opercular cortex (Scott et al., 1985) as hunger is reduced to zero by feeding to normal, physiological, self-determined, satiety. (The same holds for the nucleus of the solitary tract; Yaxley et al., 1985.) Thus taste reward (whether one works to obtain a taste, i.e., has an appetite for a taste) is not represented in the primary taste cortex, or at any earlier stage of taste processing, including the taste receptors. Instead, neuronal activity in the macaque primary taste cortex reflects the concentration of a tastant, and what the taste is (sweet, salt, bitter, sour, umami) as shown by information theoretic and related analyses of the neuronal activity (Baylis and Rolls, 1991; Rolls et al., 1996a, 2010a; Kadohisa et al., 2005; Rolls and Treves, 2011). The same is the case in humans, in that functional magnetic resonance neuroimaging (fMRI) investigations show that

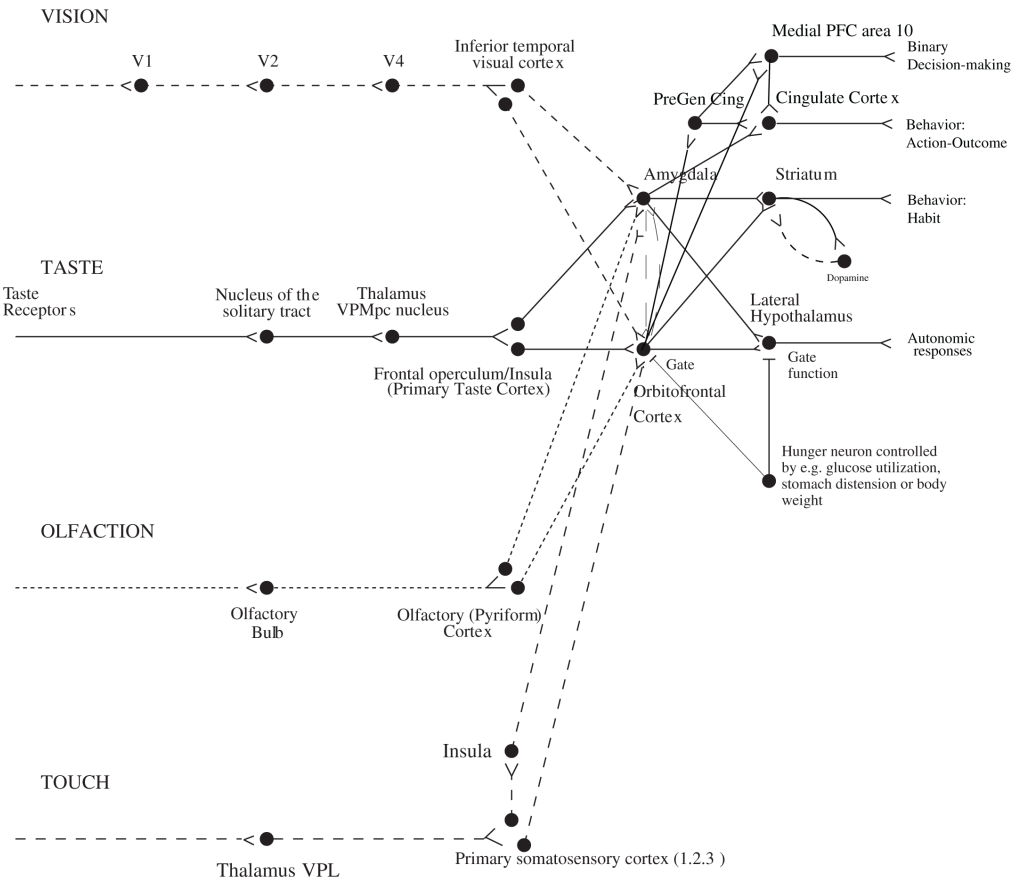

FIGURE 1 | Schematic diagram showing some of the gustatory, olfactory, visual, and somatosensory pathways to the orbitofrontal cortex, and some of the outputs of the orbitofrontal cortex, in primates. The secondary taste cortex, and the secondary olfactory cortex, are within the orbitofrontal cortex. V1, primary visual cortex; V4, visual cortical area V4; PreGen Cing, pregenual cingulate cortex. "Gate" refers to the finding that inputs such as the taste, smell, and sight of food in some brain regions only produce effects when hunger is present (Rolls, 2005). The column of brain regions including and below the inferior temporal visual cortex represents brain regions in which what stimulus is present is made explicit in the neuronal representation, but not its reward or affective value which are represented in the next tier of brain regions, the orbitofrontal cortex, and amygdala, and in areas beyond these. Medial PFC area 10, medial prefrontal cortex area 10; VPMpc, ventral posteromedial thalamic nucleus.

the subjective correlate of activations in the primary taste cortex is the intensity of the taste, not its pleasantness (Grabenhorst and Rolls, 2008; Grabenhorst et al., 2008a) [which is the subjective correlate of reward value (Rolls, 2005; Rolls and Grabenhorst, 2008; Grabenhorst and Rolls, 2011)]. In contrast, in rodents there is evidence that satiety stimuli such as food in the gut can decrease neuronal responses to taste stimuli even in the nucleus of the solitary tract (Rolls and Scott, 2003). [It is worth noting that these studies in rodents often do not use self-determined, that is physiological levels of, satiety, but instead use set quantities of satiety stimuli (and the studies may also be performed under anesthesia). In those cases effects may be being investigated that are outside the physiological range. In addition, it is found that the pleasantness of food reliably goes to zero when humans eat to self-determined satiety, Rolls et al., 1981, and, correspondingly, in macaques, neurons that respond to food reward simply stop responding to the food when self-determined satiety is reached; Burton et al., 1976; Rolls et al., 1986, 1989; Critchley and Rolls, 1996a.]

For these reasons, investigations of the neurophysiology of chemosensory processing in macaques may be particularly relevant to studying the fundamental principles of the neural processing including learning in the chemosensory system that occur in humans. These studies are complemented in the following by fMRI studies in humans, which however cannot reveal the details of the neural mechanisms, which can only be understood at the neuronal level (Rolls, 2008b; Rolls and Treves, 2011). I highlight key points about this chemosensory processing and learning in each of the following sections.

TASTE IS A PRIMARY REINFORCER, AND MOST OLFACTORY STIMULI ARE NOT

A primary reinforcing stimulus is a stimulus that is rewarding or punishing without learning. Taste is a primary reinforcer, in that for example the first time that a sweet taste is encountered it will be accepted, and the first time that a bitter taste is encountered it will be rejected (Rolls, 2005). The mechanism is that genes specify taste receptors, and these must be connected by labeled lines

to parts of the brain where they are then represented in terms of their reward value which reflects the gene-specified taste receptors from which they receive inputs (Rolls, 2005). The first stage in the primate taste system at which this occurs is in the secondary taste cortex in the orbitofrontal cortex (see above and Rolls, 2005; Rolls and Grabenhorst, 2008). This probably applies to all five tastes, sweet, salt, bitter, sour, and umami.

Most olfactory stimuli are not primary reinforcers. Their reward or punishment value is learned by association with a primary reinforcer such as a taste by mechanisms that will be described below. Exceptions to the general principle are for example pheromones that may attract other individuals (including the odors involved in major histocompatibility gene effects), probably some odors that promote disgust produced for example by rotting food, possibly some odors associated with food such as maltol, and some odors that may signal danger such as burning-related odors, though here the effects may be at least in part trigeminal (unpleasant somatosensory sensation) or learned by association with trigeminal stimuli (Rolls, 2005).

This summary (with evidence provided in the literature, e.g., Rolls, 2005, 2012) provides a background for some of the principles described in the next few sections.

TASTE VALUE CAN BE ALTERED BY ASSOCIATIVE LEARNING

Although taste is a primary, gene-specified, reinforcer, its value can be relearned by association with a strong primary reinforcer, such as energy intake in the processes known as conditioned appetite and conditioned satiety (Booth, 1985), and such as sickness (nausea). The classic example is taste aversion learning, in which for example a salty taste of lithium chloride is avoided after it has been ingested and sickness has followed. Most of this research, described elsewhere (Scott, 2011), has been performed in rodents, and appears to involve changes to neural encoding as early as the nucleus of the solitary tract which however depends on mechanisms in the gustatory cortex for the learning. This is an interesting and unusual example of associative learning in that there can be a long delay of up to several hours between the taste (the conditioned stimulus) and the sickness (the unconditioned stimulus). This is possible in the taste system, where foods are eaten at periods often separated by long intervals, so that there is no confusion about which taste it was that caused the sickness. This is not possible with for example visual-to-sickness learning, for there is usually a continuing succession of visual stimuli before the sickness occurs, and there is no easy way to relate the particular visual stimulus that caused the sickness with the sickness. Indeed, rodents show neophobia (fear of new foods), and implement a strategy of selecting one of a set of new foods to eat, so that sickness, if it follows, can be associated with that particular food. If all the new foods were eaten early on, there would be no way to determine which one caused the sickness. This learning mechanism depends on the amygdala in rats (Rolls and Rolls, 1973).

OLFACTORY-TO-TASTE ASSOCIATION LEARNING

This is an example of stimulus–reinforcer association learning. In macaques, neurons in the primary taste cortex in the anterior insula are not activated by olfactory stimuli (Verhagen et al., 2004). The primary taste cortex is not therefore the site of olfactory-to-taste association learning. (We do not typically find activations in the human primary taste cortex in the anterior, taste, insula by odors. However, if some activations are reported in some studies, they may reflect the effects of cortico-cortical back projections from multimodal areas such as the orbitofrontal cortex that are being used for memory recall, Rolls, 2008b, for example of a taste associated with an odor. Such memory recall and related top-down attentional effects must be relatively weak so as not to dominate bottom-up sensory processing, as analyzed quantitatively elsewhere; Renart et al., 1999b; Deco and Rolls, 2005a,b; Rolls, 2008b.)

Taste and olfactory pathways first come together anatomically in the primate brain in the orbitofrontal cortex (see **Figure 1**; Carmichael et al., 1994; Price, 2006) where bimodal neurons are found that respond to both odor and taste stimuli (Rolls and Baylis, 1994; Critchley and Rolls, 1996b). These bimodal neurons reflect olfactory-to-taste association learning (olfactory discrimination learning) in which one odor is paired with one taste (e.g., glucose), and a second odor with a different taste (e.g., salt, which is mildly aversive). This is shown to be a learned effect by the fact that when the olfactory-to-taste pairing is reversed, these neurons reverse the olfactory stimuli to which they respond (see **Figure 2**; Rolls et al., 1996b). This type of associative learning is how flavors are formed, where flavors are defined by olfactory–taste combinations.

In the case of umami, such olfactory-to-taste association learning appears to be key to the pleasantness of umami (Rolls, 2009). Monosodium glutamate as a taste is not very pleasant, but when combined with a savory pleasant odor (such as vegetable), can become very pleasant (McCabe and Rolls, 2007). (The odor must be consonant: in these experiments the effect of combining rum odor with monosodium glutamate was to produce a flavor that was quite unpleasant.) The combination of monosodium glutamate and vegetable odor produced supralinear activations (greater than the sum of those produced by the taste and odor separately) in the part of the brain that represents the pleasantness of odors and taste, the orbitofrontal cortex (McCabe and Rolls, 2007). That is the explanation of how umami can make a food pleasant: by a combination of monosodium glutamate and a consonant odor. That will have been learned in a lifetime of experience of eating foods rich in glutamate and/or inosine monophosphate such as tomatoes, mushrooms, meat, and human mother's milk (Rolls, 2009).

In humans, olfactory–taste convergence occurs in the orbitofrontal cortex and in the region that is intermediate between it and the primary taste and olfactory cortices, the agranular insula, at the far anterior end of the insula in what is topologically related to the orbitofrontal cortex (De Araujo et al., 2003).

The reversal of olfactory-to-taste association learning is a relatively slow process which takes often 40–60 trials for the reversal to occur (Rolls et al., 1996b). This is consistent with the utility of maintaining neurons that represent particular flavors because of previously learned combinations of odorants and tastants.

VISUAL-TO-TASTE ASSOCIATION LEARNING IN THE ORBITOFRONTAL CORTEX

Neurons with visual responses to the sight of food are found in the lateral hypothalamus (Rolls et al., 1976). These neurons probably receive their inputs from neurons in the orbitofrontal cortex,

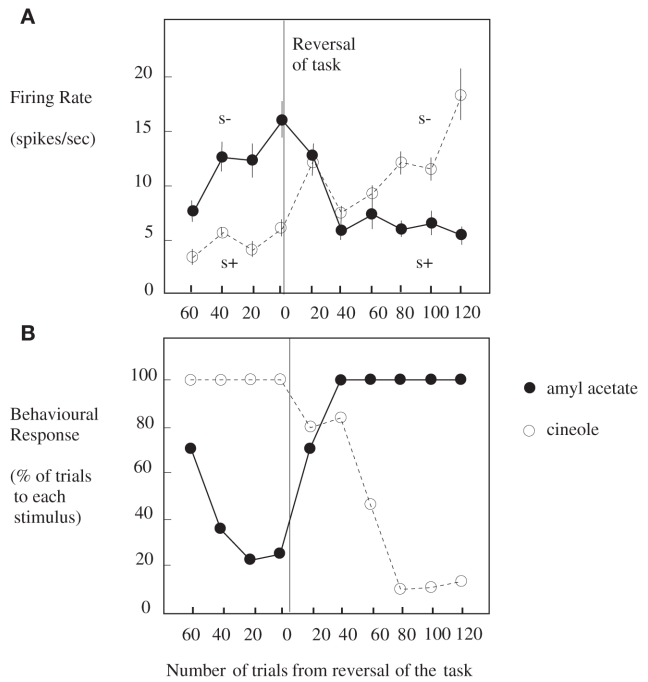

FIGURE 2 | Orbitofrontal cortex: olfactory-to-taste association reversal. **(A)** The activity of a single orbitofrontal cortex olfactory neuron during the performance of a two-odor olfactory discrimination task and its reversal is shown. Each point represents the mean poststimulus activity of the neuron in a 500-ms period on approximately 10 trials of the different odorants. The SE of these responses are shown. The odorants were amyl acetate (closed circle; initially S−, punished with a taste of salt) and cineole (o) (initially S+, rewarded with fruit juice flavor). After 80 trials of the task the reward associations of the stimuli were reversed. This neuron reversed its responses to the odorants following the task reversal. **(B)** The behavioral responses of the monkey during the performance of the olfactory discrimination task. The number of lick responses to each odorant is plotted as a percentage of the number of trials to that odorant in a block of 20 trials of the task. (After Rolls et al., 1996b).

where we also discovered neurons that respond to the sight of food (Thorpe et al., 1983), and to taste (Thorpe et al., 1983; Rolls et al., 1989, 1990; Critchley and Rolls, 1996b). The orbitofrontal cortex neurons that respond to the sight of food do so by visual-to-taste association learning, as shown by the fact that they reverse their responses when the visual-to-taste contingency is reversed in a visual discrimination task (Thorpe et al., 1983; Rolls et al., 1996b). The mechanism probably involves in part pattern association learning, and its decrement by synaptic long-term depression when the contingency is reversed (Rolls, 2005, 2008b).

But association learning is not all that there is to the learning, for the reversal can take place in one-trial (see **Figure 3**). In particular, in the Go–NoGo visual discrimination task on a trial on which the reward contingencies are reversed, the following occurs. When one stimulus is shown which indicates that taste reward (glucose or fruit juice) should be obtained but instead saline is delivered, the monkey licks to the other stimulus which has been recently associated with saline, and obtains reward (Thorpe et al., 1983; see **Figure 3**). This is termed serial reversal learning set, and can occur after repeated experience with reversal has been obtained. The effect cannot therefore involve visual–taste association learning, but in this case involves the switch of a rule (about which of the two visual stimuli is currently associated with reward).

This type of reversal trial produces remarkable activity in a population of orbitofrontal cortex neurons that respond when the expected reward is not obtained (Thorpe et al., 1983; **Figure 3**). They thus respond to an expectation–outcome mismatch that is negative. We thus term them error neurons (Thorpe et al., 1983), or negative reward prediction error neurons (Rolls, 2008b, 2011b; Rolls and Grabenhorst, 2008; Grabenhorst and Rolls, 2011). Consistent effects are found in humans (Kringelbach and Rolls, 2003).

The rapid reversal requires a rule which indicates which of the visual stimuli is currently associated with reward. We hypothesize that the negative reward prediction error neurons, which maintain their firing for 8–10 s after the non-reward event (Thorpe et al., 1983; see **Figure 3**) in what is likely to be an attractor state (Rolls, 2008b), are important in the reversal. We believe that they reset, by inhibition through inhibitory interneurons, short-term memory rule-encoding attractor networks in the same brain region. After the inhibition, the attractor that emerges from the noisy (Poisson) firing of the neurons is the attractor for the opposite rule, because it is showing less synaptic or neuronal adaptation than the neurons in the network that represent the recently active rule (Deco and Rolls, 2005c).

An integrate-and-fire computational model which illustrates how the rapid reversal learning could be implemented is shown in **Figure 4** (Deco and Rolls, 2005c). In the lower module, stimuli are mapped from sensory neurons (level 1, at the bottom), through an intermediate layer of conditional object–reward combination neurons with rule-dependent activity, to layer 3 which contains reward/punishment neurons. The mapping through the intermediate layer can be biased by the rule module inputs to perform a direct or reversed mapping. The activity in the rule module can be reversed by the error signal which occurs when an expected reward is not obtained. The reversal occurs because the attractor state in the rule module is shut down by inhibition arising from the effects of the error signal, and restarts in the opposite attractor state because of partial synaptic or neuronal adaptation of the previously active rule neurons.

The operation of this system is facilitated by the conditional reward neurons, which respond to a reward stimulus only when one rule applies. These neurons for example respond to a green stimulus when it is associated with taste reward, but not to a blue stimulus when it is associated with taste reward (Thorpe et al., 1983; Rolls, 2008b; **Figure 5**). The importance of these conditional reward neurons is that they can be biased on (or off) by the rule neurons. For example, if a green stimulus is seen, and the "green is reward" rule attractor is firing and biasing the "conditional green is reward" neurons, then the "conditional green is reward" neurons will win the competition and be activated, and in turn activate the "go" or "reward" neurons at the output stage (**Figure 4**). A fuller description is provided elsewhere (Deco and Rolls, 2005c; Rolls, 2008b).

It is significant in terms of brain design that in the orbitofrontal cortex where these multimodal olfactory-to-taste

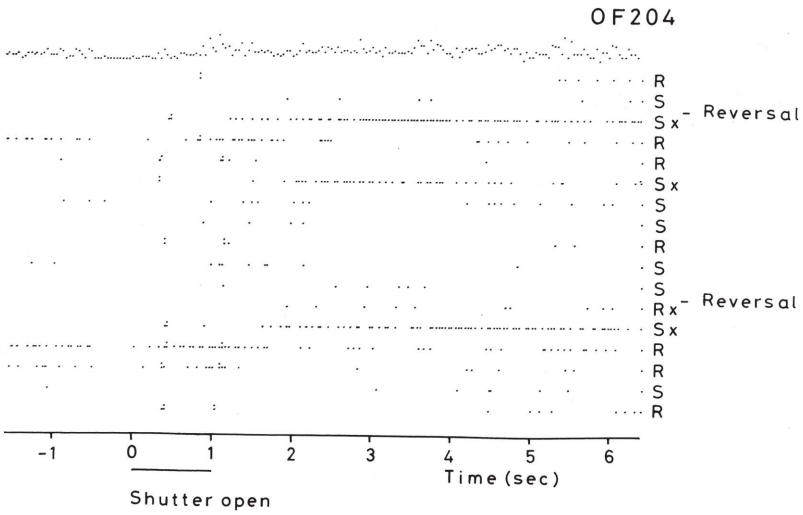

FIGURE 3 | Visual discrimination reversal for sweet taste reward vs. the aversive taste of salt (NaCl). Negative reward prediction error neuron: responses of an orbitofrontal cortex neuron that responded only when the monkey licked to a visual stimulus during reversal, expecting to obtain fruit juice reward, but actually obtained the taste of aversive saline because it was the first trial of reversal. Each single dot represents an action potential; each vertically arranged double dot represents a lick response. The visual stimulus was shown at time 0 for 1 s (labeled "shutter open"). The neuron did not respond on most reward (R) or saline (S) trials, but did respond on the trials marked x, which were the first trials after a reversal of the visual discrimination on which the monkey licked to obtain reward, but actually obtained saline because the task had been reversed. It is notable that after an expected reward was not obtained due to a reversal contingency being applied, on the very next trial the macaque selected the previously non-rewarded stimulus. This shows that rapid reversal can be performed by a non-associative process, and must be rule-based. (After Thorpe et al., 1983).

and visual-to-taste convergences and learning occur, it is the reward value of the olfactory/visual/taste combination that is represented, as shown by experiments in which the neuronal response to the particular food eaten decreases to zero during feeding to satiety (Rolls et al., 1989; Critchley and Rolls, 1996a; Kringelbach et al., 2003).

This orbitofrontal cortex association learning system is very important in behavior, for damage to it in macaques (Butter, 1969; Iversen and Mishkin, 1970) and humans (Rolls et al., 1994; Hornak et al., 2004) impairs reversal learning and may be very important in the behavioral changes that follow damage to the human orbitofrontal cortex (Rolls, 2005, 2008b).

The responses of amygdala neurons are much less specifically tuned to respond to the sight of particular foods, and reversal of the responses of amygdala neurons is much more difficult to obtain, and is much slower than the one-trial reversal found in the orbitofrontal cortex (Sanghera et al., 1979; Rolls, 2005; Wilson and Rolls, 2005). The fact that if primate amygdala neurons reverse they do so slowly was confirmed in a trace conditioning procedure [in which there is a delay between the end of the conditioned stimulus (a visual image) and the unconditioned stimulus (an air-puff to the eye, or a liquid)] in which if neurons reversed it took 30–60 trials (Paton et al., 2006). The evidence thus indicates that primate amygdala neurons do not alter their activity as flexibly and rapidly in visual–reinforcer reversal learning as do orbitofrontal cortex neurons (Rolls, 2008b). The rodent amygdala is involved in the neophobia to new foods, which gradually becomes replaced by investigation and acceptance over time (Rolls and Rolls, 1973).

LEARNING OF NEW OLFACTORY–TASTE AND ORAL TEXTURE–TASTE REPRESENTATIONS BY COMPETITIVE LEARNING IN THE ORBITOFRONTAL CORTEX

Each orbitofrontal cortex neuron responds to a different combination of taste and oral texture stimuli. The taste stimuli that may be combined in this way include sweet, salt, bitter, sour, and umami; and the oral somatosensory stimuli include viscosity, fat texture, gritty texture, capsaicin, fatty acids such as linoleic and lauric acid, and oral temperature (Rolls et al., 2003, 2010a; Verhagen et al., 2003; Kadohisa et al., 2004, 2005). This encoding of information by different neurons is to some extent independent, which enables the total information to increase approximately linearly with the number of neurons involved in the population, a very powerful neural code (Rolls, 2008b; Rolls et al., 2010a; Rolls and Treves, 2011). Part of the basis for this representation may be the random sampling by each neuron of the different inputs being received in a cortical area (Rolls, 2008b). That process is likely to be facilitated by competitive learning, which, because of the inhibition implemented by the cortical inhibitory interneurons, helps the neurons to learn to respond to different combinations of their inputs (Rolls et al., 2006; Rolls, 2008b).

The same two processes may contribute to the non-linear separation of the olfactory and taste inputs to neurons in the orbitofrontal cortex. Evidence for such non-linear processing is that after feeding to satiety with fruit juice, a neuron may no longer respond to fruit juice, but does still respond to one of the components, sweet taste (Rolls et al., 1989; see **Figure 6**, which illustrates that the responses can become sometimes a little larger to other

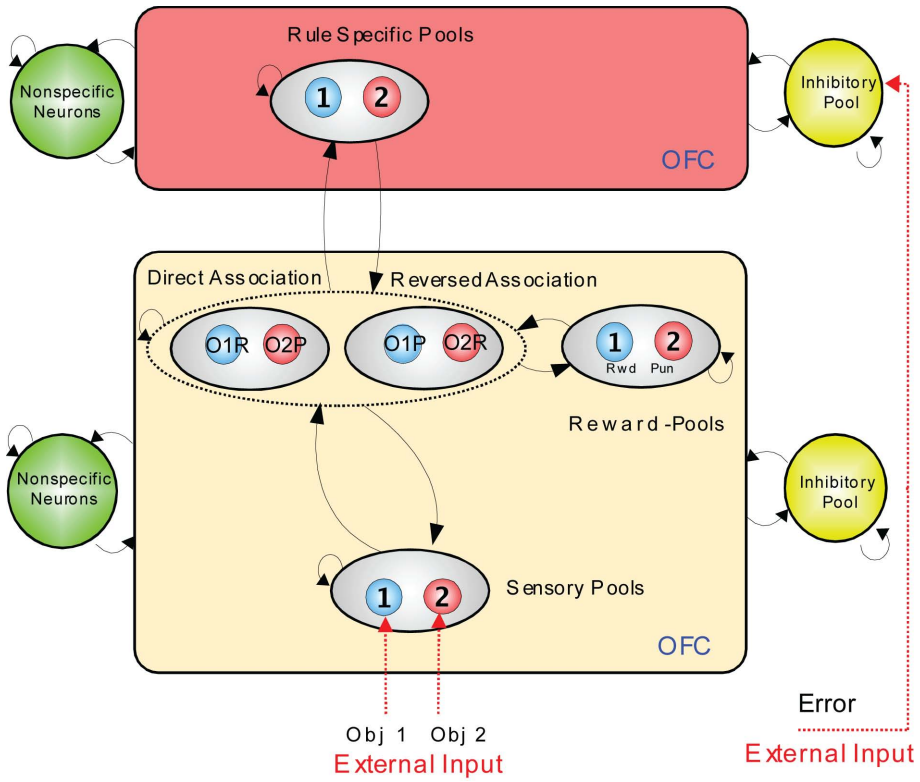

FIGURE 4 | Visual–taste discrimination reversal: a model. There is a rule module (top) and a sensory – intermediate neuron – reward module (below). Neurons within each module are fully connected, and form attractor states. The sensory – intermediate neuron – reward module consists of three hierarchically organized levels of attractor network, with stronger synaptic connections in the forward than the backprojection direction. The intermediate level of the sensory – intermediate neuron – reward module contains conditional reward neurons that respond to combinations of an object and its association with (e.g., taste) reward or punishment, e.g., object1–reward (O1R, in the direct association set of pools), and object1–punishment (O1P in the reversed association set of pools). The rule module acts as a biasing input to bias the competition between the object–reward combination neurons at the intermediate level of the sensory – intermediate neuron – reward module. The model was implemented with integrate-and-fire neurons. OFC, orbitofrontal cortex. (After Deco and Rolls, 2005c).

stimuli after one food has been fed to satiety). Indeed, the fact that neurons can respond in this specific way to combinations of their inputs, so that a neuron may respond optimally to a particular flavor, is an important part of the mechanism of sensory-specific satiety (Rolls et al., 1989; Rolls, 2005, 2008b).

LEARNING AS A MECHANISM FOR SENSORY-SPECIFIC SATIETY

Sensory-specific satiety, discovered during lateral hypothalamic neuronal recordings (Rolls, 1981; Rolls et al., 1986), is the process by which the reward value, and its correspondent, human subjective pleasantness, of the flavor of a particular food decreases to zero after the food has been eaten to satiety, but remains relatively high for other foods not eaten in the meal (Rolls et al., 1982, 1983a,b, 1984; Rolls and Rolls, 1997; Rolls, 2005). Sensory-specific satiety is reflected in the responses of orbitofrontal cortex neurons that respond to the taste, odor, sight, and/or oral texture of foods (Rolls et al., 1989; Critchley and Rolls, 1996a; see example in **Figure 6**), and is also reflected in activations in the human orbitofrontal cortex with fMRI neuroimaging (Kringelbach et al., 2003). The taste neurons in this population are found throughout a wide medial as well as lateral extent of the orbitofrontal cortex (Rolls et al., 1989, 1990, 1996a, 2003; Rolls and Baylis, 1994; Critchley and Rolls, 1996c; Verhagen et al., 2003; Kadohisa et al.,

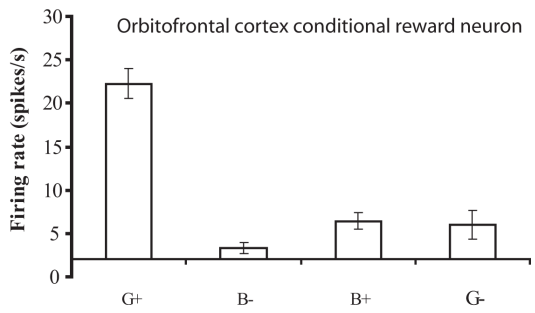

FIGURE 5 | A conditional reward neuron recorded in the orbitofrontal cortex which responded only to the Green stimulus when it was associated with reward (G+), and not to the Blue stimulus when it was associated with reward (B+), or to either stimuli when they were associated with a punisher, the taste of salt (G− and B−). The mean firing rate ± SEM is shown. (After Thorpe et al., 1983).

FIGURE 6 | Sensory-specific satiety effects in an orbitofrontal cortex neuron with visual, olfactory, and taste responses, showing the visual, flavor, and olfactory responses measured separately before and after feeding to satiety with blackcurrant juice. The solid circles show the responses to blackcurrant juice. The olfactory stimuli included apple (ap), banana (ba), citral (ct), phenylethanol (pe), and caprylic acid (cp). The spontaneous firing rate of the neuron is shown (sp). (After Critchley and Rolls, 1996a).

2004, 2005; Rolls, 2008a), as has been confirmed (Pritchard et al., 2007, 2008; Rolls, 2008a). The orbitofrontal cortex projects to the lateral hypothalamus, and provides a route for hypothalamic neurons to also show sensory-specific satiety effects (Rolls, 1981; Rolls et al., 1986).

Sensory-specific satiety effects are not found in the macaque primary taste cortex (Rolls et al., 1988; Yaxley et al., 1988) or inferior temporal visual cortex (Rolls et al., 1977), and the mechanism for sensory-specific satiety is thus implemented in the orbitofrontal cortex, which receives direct inputs from both these structures (Rolls, 2005, 2008b).

The mechanism of sensory-specific satiety that is proposed is a simple type of learning, in which the neurons in the orbitofrontal cortex that respond to relatively specific foods gradually show habituation of their responses over a time period of approximately 10–15 min of stimulation by the food in the mouth, while it is being eaten. The mechanism may involve synaptic adaptation of the afferent inputs to the neuron that are activated by a particular food, for the neuron can still respond after satiety to other foods that have not been eaten in a meal (see example in **Figure 6**). Sensory-specific satiety generalizes a little to similar foods, but not to dissimilar foods, reflecting the somewhat distributed encoding used by the neurons, which allows the similarity of stimuli to be reflected in neuronal responses that utilize dot-product decoding (Rolls, 2008b; Rolls and Treves, 2011). In the case of sensory-specific satiety, the generalization to other foods thus reflects the similarity (dot-product or correlation) between the firing rate vectors that activate the synaptic weight vector on a neuron (Rolls, 2008b).

Sensory-specific satiety can occur in part if the food is not swallowed, but only chewed or even only smelled for 10–15 min (Rolls and Rolls, 1997). The mechanism thus does not rely on food entering the stomach or intestines, though full satiety only occurs if that is the case, showing that gastro-intestinal feedback is necessary for full satiety (Rolls, 2005).

Although the proposed mechanism thus involves synaptic adaptation, the process is not at all the same as sensory adaptation, in that there is no effect of satiety on neuronal responses at stages before the orbitofrontal cortex (Rolls et al., 1988; Yaxley et al., 1988), and in that subjective ratings of the intensity of food hardly change after feeding to satiety, whereas the subjective pleasantness decreases to zero (Rolls et al., 1983b; Rolls and Rolls, 1997).

FLAVOR–PLACE LEARNING IN THE HIPPOCAMPUS

The primate anterior hippocampus (which corresponds to the rodent ventral hippocampus) receives inputs from brain regions involved in flavor reward processing such as the amygdala and orbitofrontal cortex (Suzuki and Amaral, 1994; Carmichael and Price, 1995a,b; Stefanacci et al., 1996; Pitkanen et al., 2002; Price, 2006). The primate hippocampus contains spatial view neurons, which respond to spatial locations "out there" being viewed (Rolls et al., 1997, 2005; Robertson et al., 1998; Georges-François et al., 1999; Rolls, 1999; Rolls and Xiang, 2006). To investigate how this affective input may be incorporated into primate hippocampal function, we (Rolls and Xiang, 2005) recorded neuronal activity while macaques performed a flavor reward-to-place association task in which each spatial scene shown on a video monitor had one

location which if touched yielded a preferred fruit juice reward, and a second location which yielded a less preferred juice reward. Each scene had different locations for the different rewards. Of 312 hippocampal neurons analyzed, 18% responded more to the location of the preferred reward in different scenes, and 5% to the location of the less preferred reward. When the locations of the preferred rewards in the scenes were reversed, 60% of 44 neurons tested reversed the location to which they responded, showing that the reward–place associations could be altered by new learning in a few trials. The majority (82%) of these 44 hippocampal reward–place neurons tested did not respond to object–reward associations in a visual discrimination object–reward association task, showing that the hippocampal representation is specialized for flavor–place rather than object–flavor representations.

Thus the primate hippocampus contains a representation of the reward associations of places "out there" being viewed, and this is a way in which reward information can be stored as part of an episodic memory (Rolls and Xiang, 2005; Rolls, 2008b, 2010b). There is consistent evidence that rewards available in a spatial environment can influence the responsiveness of rodent place neurons (Hölscher et al., 2003; Tabuchi et al., 2003).

TOP-DOWN COGNITIVE MODULATION OF TASTE, OLFACTORY, AND FLAVOR REPRESENTATIONS INVOLVES LEARNING

If a cognitive, high level, indeed verbal, label is used to describe an odor, the odor can be rated as more subjectively pleasant than when the label indicates that it is unpleasant (De Araujo et al., 2005). In a study of the underlying neural mechanisms with fMRI, we showed that when an olfactory stimulus, isovaleric acid (with a smell somewhat like brie) was delivered with a visual word label indicating that it was cheese, the activations in the orbitofrontal cortex were greater to the odor than when the label was body odor (De Araujo et al., 2005). We showed that this was an interaction between the top-down cognitive label and the bottom-up olfactory input, for the difference of the activations was much greater with the label and the odor present than with the labels alone (De Araujo et al., 2005). We have shown similar cognitive modulation of the pleasantness of taste (umami, monosodium glutamate) and flavor (umami, monosodium glutamate plus vegetable odor) in the orbitofrontal cortex (Grabenhorst et al., 2008a; **Figure 7**).

These findings are of great interest, for they show that high level cognitive influences descend down into the first part of the human taste, olfactory, and flavor brain systems in which the reward value is made explicit in the representation. The cognition appears to actually modulate the neural representation that is related to subjective pleasantness.

The question arises about how the top-down (cognitive) signal connects to the correct neurons in the orbitofrontal cortex so that when the verbal indication is of good value, then the reward representation is enhanced, and when the verbal indication is of poor value, the reward effects produced by the bottom-up input are not enhanced. This requires a matching between the top-down and the bottom-up signals. How could this be achieved?

I propose that the mechanism is analogous to that which we have described in relation to the recall of memories from the hippocampus to the neocortex in our theory of hippocampal function (Rolls, 1989, 2008b, 2010b), and for which we have a quantitative analysis (Treves and Rolls, 1994; Rolls, 1995). The hypothesis is as follows, and is described with the help of **Figure 8**, which describes a related mechanism, that for the top-down biasing of activity in affective vs. sensory systems in the brain for taste, flavor, olfactory, etc., representations. When there is a rewarding taste present as a bottom-up input that is causing orbitofrontal cortex neurons to fire, and simultaneously there is a cognitive top-down set of afferents (originating in language or related cortical areas) to the orbitofrontal cortex some of which are active reflecting cognitive processing that a good taste is present, then the active synaptic afferents labeled s1 in **Figure 8** show synaptic modification by associative, Hebb-like, long-term potentiation onto the active neurons reflecting the good bottom-up input. This associative synaptic modification is what sets up the correct relation between the cognitive top-down input and the bottom-up input. Other neurons, which might be activated by bottom-up bad tastes, odors, or flavors, would similarly become associated by synaptic modification of other synapses (for example s2, s3, or s4 in **Figure 8**) with the corresponding top-down cognitive input to the orbitofrontal cortex representing the unpleasant or aversive nature of the bottom-up taste, etc., stimulus. Then later, after the learning, the top-down cognitive inputs that enhance reward value would enhance the activity of just those neurons that represented a good taste, etc. If the top-down reward value input was not present, there would be less activation produced by the bottom-up input, in the same way that we have analyzed for attention (Deco and Rolls, 2005b).

This mechanism is analogous to the memory recall mechanism, in that the top-down signal (in that case from the hippocampus) activates the correct neurons back in the neocortex, because of prior associative synaptic modification when both the bottom-up and top-down inputs were present (Rolls, 1989, 1995, 2008b, 2010b; Treves and Rolls, 1994).

Studies of the neuronal mechanisms of attention show that the top-down input cannot be very strong, or else it dominates the bottom-up perception, which must not be disconnected from the world (Renart et al., 1999a,b; Deco and Rolls, 2005b). Given that fact, the modulatory effects of these top-down signals are most evident when the bottom-up input is weak or ambiguous (as in the case of the isovaleric acid "brie-like" odor; De Araujo et al., 2005), for otherwise the bottom-up input then dominates the system and there is little or no attentional or cognitive modulation that can be observed (Deco and Rolls, 2005b).

TOP-DOWN ATTENTIONAL MODULATION OF TASTE, OLFACTORY, AND FLAVOR REPRESENTATIONS INVOLVES LEARNING

If humans are asked to pay attention to pleasantness so that they can later rate the pleasantness of an odor, then activations related to pleasantness are enhanced in the orbitofrontal (secondary olfactory) cortex (Rolls et al., 2008). Selective attention to intensity enhances representations in other cortical areas (Rolls et al., 2008).

If humans are asked to pay attention to pleasantness so that they can later rate the pleasantness of a taste (umami), then activations related to pleasantness are enhanced in the orbitofrontal (secondary taste) cortex (Grabenhorst and Rolls, 2008; **Figure 9**).

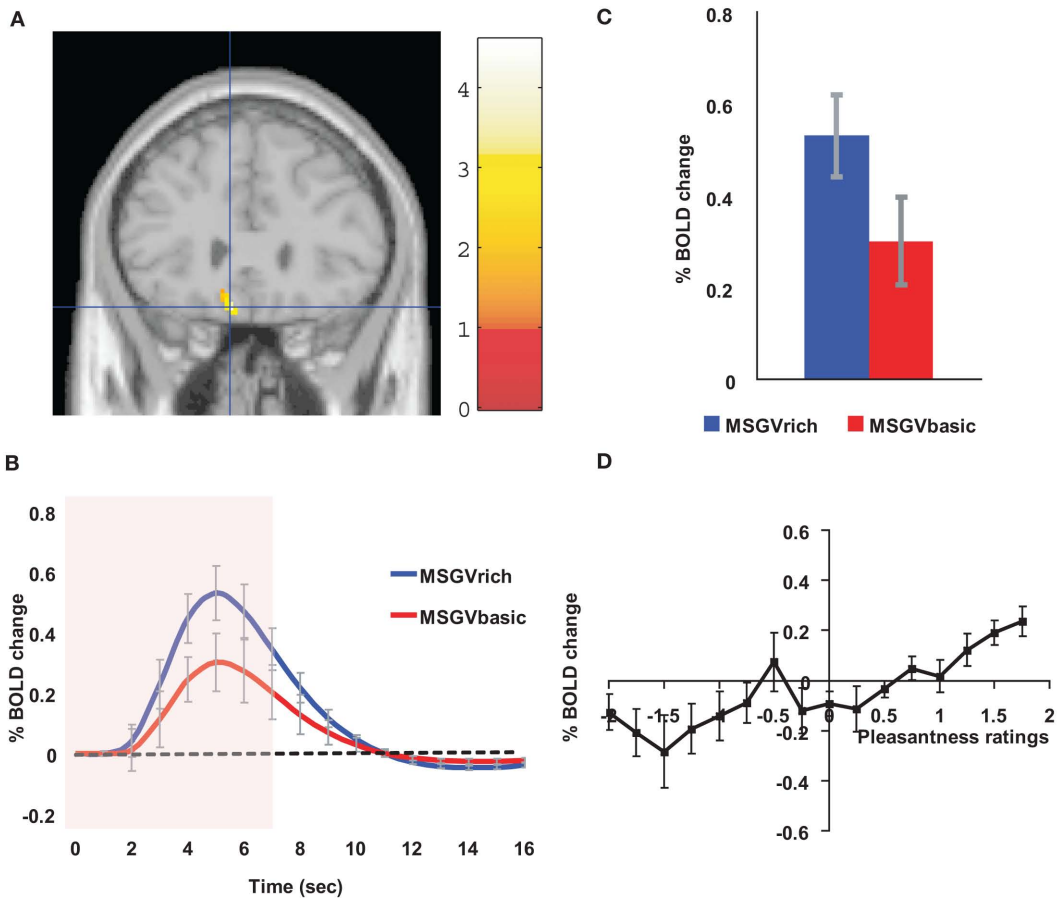

FIGURE 7 | Cognitive modulation of flavor reward processing in the brain. (A) The medial orbitofrontal cortex was more strongly activated when a flavor stimulus was labeled "rich and delicious flavor" (MSGVrich) than when it was labeled "boiled vegetable water" (MSGVbasic) ([−8 28 −20]). (The flavor stimulus, MSGV, was the taste 0.1 M MSG + 0.005 M inosine 5′-monophosphate combined with a consonant 0.4% vegetable odor.) **(B)** The timecourse of the BOLD signals for the two conditions. **(C)** The peak values of the BOLD signal (mean across subjects ± SEM) were significantly different ($t = 3.06$, df = 11, $p = 0.01$). **(D)** The BOLD signal in the medial orbitofrontal cortex was correlated with the subjective pleasantness ratings of taste and flavor, as shown by the SPM analysis, and as illustrated (mean across subjects ± SEM, $r = 0.86$, $p < 0.001$). (After Grabenhorst et al., 2008a).

Selective attention to intensity enhances representations in the primary taste cortex in the anterior insula (Grabenhorst and Rolls, 2008).

There is the same problem as for cognitive modulation of affective representations. How is a top-down signal originating from the level of language made to correspond with the correct bottom-up signals? The mechanism that I propose for attention is analogous to that which I proposed for cognitive modulation, that the top-down signal that is appropriate becomes associated by associative synaptic modification with the bottom-up signals when both are present. The circuitry for this is schematized in **Figure 8**, which shows the model we have proposed to accommodate these findings, the top-down biased activation model of selective attention (Grabenhorst and Rolls, 2010). The crucial synaptic modification for the correct correspondence to be set up is that between the top-down connections, and the neurons that receive the bottom-up input, labeled s in **Figure 8**.

BEYOND REWARD VALUE TO DECISION-MAKING

Representations of the reward value of food, and their subjective correlate the pleasantness of food, are influenced by associative learning, and by top-down cognitive and attentional control, as described above. But after the reward evaluation, a decision has to be made about whether to seek for and consume the taste, olfactory, flavor, oral texture, or other type of reward. We are now starting to understand how the brain takes decisions as described in *The Noisy Brain* (Rolls and Deco, 2010), and this has implications for whether a reward of a particular value will be selected (Rolls, 2008b, 2011a; Rolls and Grabenhorst, 2008; Rolls and Deco, 2010; Grabenhorst and Rolls, 2011). A tier of processing beyond the orbitofrontal cortex, in the medial prefrontal cortex area 10, becomes engaged when choices are made between odor stimuli based on their pleasantness (see **Figure 1**; Grabenhorst et al., 2008b; Rolls et al., 2010b,c,d). The choices are made by a local attractor network in which the winning attractor

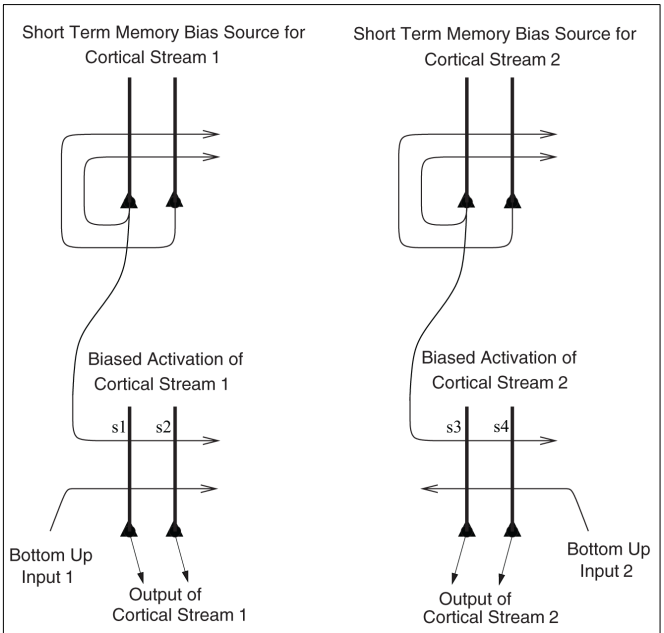

FIGURE 8 | Biased activation theory of top-down selective attention. The short-term memory systems that provide the source of the top-down activations may be separate (as shown), or could be a single network with different attractor states for the different selective attention conditions. The top-down short-term memory systems hold what is being paid attention to active by continuing firing in an attractor state, and bias separately either cortical processing system 1, or cortical processing system 2 via synapses labeled *s*. This weak top-down bias interacts with the bottom-up input to the cortical stream and produces an increase of activity that can be supralinear (Deco and Rolls, 2005b). Thus the selective activation of separate cortical processing streams can occur. In the example, stream 1 might process the affective value of a stimulus, and stream 2 might process the intensity and physical properties of the stimulus. The outputs of these separate processing streams then must enter a competition system, which could be for example a cortical attractor decision-making network that makes choices between the two streams, with the choice biased by the activations in the separate streams. (After Grabenhorst and Rolls, 2010).

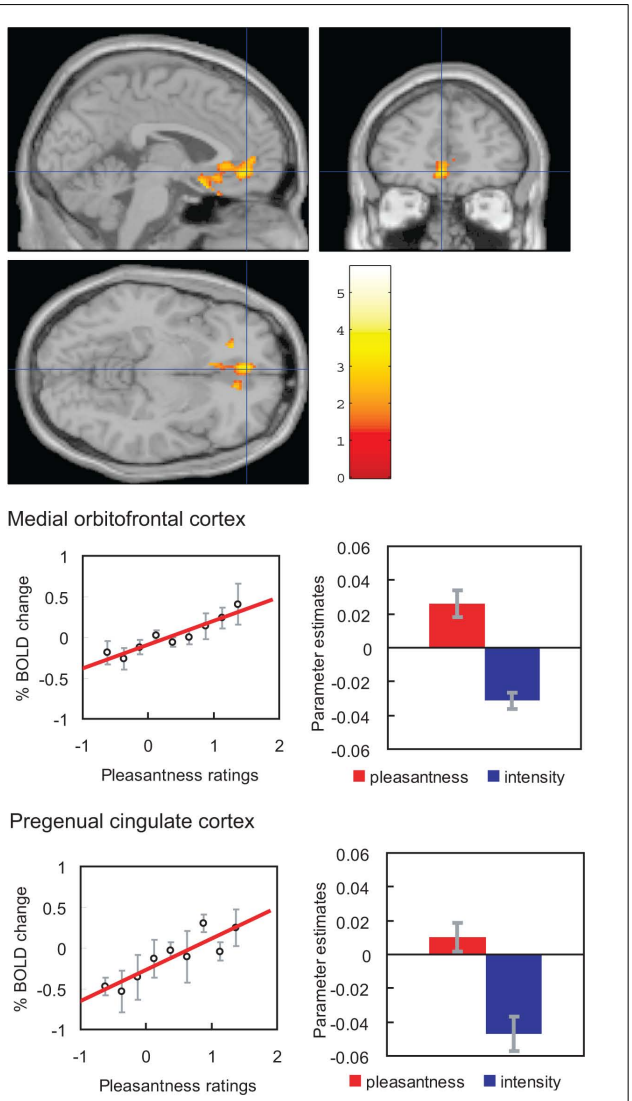

FIGURE 9 | Effect of paying attention to the pleasantness vs. the intensity of a taste stimulus. Top: a significant difference related to the taste period was found in the medial orbitofrontal cortex at [−6 14 −20] $z = 3.81$ $p < 0.003$ (toward the back of the area of activation shown) and in the pregenual cingulate cortex at [−4 46 −8] $z = 2.90$ $p < 0.04$ (at the cursor). Middle: medial orbitofrontal cortex. Right: the parameter estimates (mean ± SEM across subjects) for the activation at the specified coordinate for the conditions of paying attention to pleasantness or to intensity. The parameter estimates were significantly different for the orbitofrontal cortex $t = 7.27$, df = 11, $p < 10^{-4}$. Left: the correlation between the pleasantness ratings and the activation (% BOLD change) at the specified coordinate ($r = 0.94$, df = 8, $p \ll 0.001$). Bottom: pregenual cingulate cortex. Conventions as above. Right: the parameter estimates were significantly different for the pregenual cingulate cortex $t = 8.70$, df = 11, $p < 10^{-5}$. Left: the correlation between the pleasantness ratings and the activation (% BOLD change) at the specified coordinate ($r = 0.89$, df = 8, $p = 0.001$). The taste stimulus, 0.1 M monosodium glutamate, was identical on all trials. (After Grabenhorst and Rolls, 2008).

state represents the decision, with each possible attractor state representing a different choice, and the neurons in each of the possible attractors receiving inputs that reflect the evidence for that choice. (The attractor network is formed in a part of the cerebral cortex by strengthening of the recurrent collateral excitatory synapses between nearby pyramidal cells using associative synaptic modification. One group of neurons with strengthened synapses between its members can form a stable attractor with high firing rates, which competes through inhibitory interneurons with other possible attractor states formed by other groups of excitatory neurons; Rolls, 2008b, 2010a. The word attractor refers to the fact that inexact including incomplete inputs are attracted to one of the states of high firing that are specified by the synaptic connections between the different groups of neurons. The result in this non-linear system is that one attractor wins, and this implements a mechanism for decision-making with one winner; Wang, 2002, 2008; Rolls, 2008b; Rolls and Deco, 2010). The decisions are probabilistic as they reflect the noise in the competitive non-linear decision-making process that is introduced by the random spiking times of neurons for a given mean rate that reflect a Poisson process (Rolls and Deco, 2010; Rolls et al., 2010c).

The costs of each reward need to be subtracted from the value of each reward to produce a net reward value for each available reward before the decision is taken (Rolls, 2008b; Rolls and Grabenhorst, 2008; Grabenhorst and Rolls, 2011). The reasoning or rational system with its long-term goals (introducing evidence such as "scientific studies have shown that fish oils rich in omega 3 may reduce the probability of Alzheimer's disease") then competes with the rewards such as the pleasant flavor of food (which are gene-specified, Rolls, 2005, though subject to conditioned effects, Booth, 1985; Rolls, 2005) in a further decision process which may itself be subject to noise (Rolls, 2005, 2008b; Rolls and Deco, 2010). This can be described as a choice between the selfish individual or "phene" (standing for phenotype) and the selfish gene (Rolls, 2011a, 2012).

In this context, the findings described in this paper about chemosensory learning and top-down cognitive and attentional effects on the taste, olfactory, and more generally reward systems in the brain are important advances in our understanding of how reward value is represented in the brain and is influenced by learning, and how decisions between those reward values are reached in attractor networks that themselves involve associative learning to set up the correct attractor states.

ACKNOWLEDGMENTS

This research was supported by the Medical Research Council. The participation of many colleagues in the studies cited is sincerely acknowledged.

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

Received: 18 August 2011; accepted: 27 August 2011; published online: 16 September 2011.

Citation: Rolls ET (2011) Chemosensory learning in the cortex. Front. Syst. Neurosci. ***5****:78. doi: 10.3389/fnsys.2011.00078*