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When Animal Housing and Strain Difference Matter: Cellular and Behavioral Studies in Mouse Olfaction

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WHEN ANIMAL HOUSING AND STRAIN DIFFERENCE MATTER: CELLULAR AND BEHAVIORAL STUDIES IN MOUSE OLFACTION

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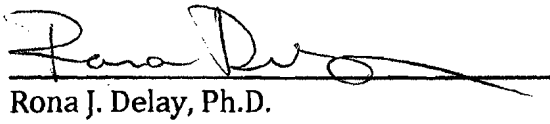

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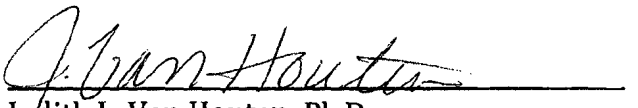

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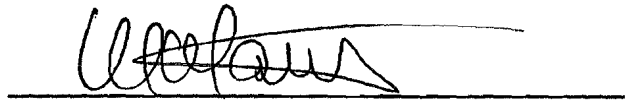

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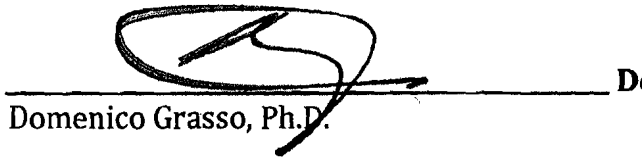

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ABSTRACT

In olfaction, the olfactory sensory neuron (OSN) is the point where chemical environment activates an electrochemical signaling that will result in odor perception. The OSNs in the main olfactory epithelium are part of a four part olfactory system that provides chemical sensory information, and can alter behavior through odor recognition, pheromone detection, and memory retrieval.

Here we studied the canonical odor transduction pathway, mediated by cAMP production and calcium influx, under conditions where mice were housed in either an odor-enriched, or standard animal housing environment. Very little is known about how odor exposure in the housing environment alters odor signaling in olfactory sensory neurons (OSNs). Using calcium imaging and perforated patch clamp recordings we examined the responses produced by activation of the odor-mediated cAMP signal transduction pathway. Our results showed that increased odor exposure corresponded to slower calcium recovery rates when the cAMP pathway was stimulated. In addition to C57BL/6 mice, we also tested mice without olfactory marker protein (OMP). OMP is a small cytosolic protein that is only expressed in mature OSNs. OMP has been shown to alter odor responses, however its role(s) in OSNs is still unknown. Here we report that both C57BL/6 and $OMP^{-/-}$ OSNs had slower calcium transient recovery rates in mice from odor enriched housing giving a longer-lasting response. A unique finding was that the $OMP^{-/-}$ OSNs from both odor-environments had shorter responses than their C57BL/6 odor matched counterpart. Using different stimuli to isolate the reasons for these differences, we found that odor-enrichment appeared to be altering events in the cilia, whereas $OMP^{-/-}$ OSNs had reduced responses to stimulation of adenylyl cyclase.

Higher order structures also play a role in olfactory detection and perception by fine-tuning the activity pattern in the olfactory bulb and directing attention to relevant odorants from descending cortical control. To study these aspects of olfactory function, behavioral methods are necessary to understand alteration in perception of odorants. We measured the detection threshold of C57BL/6 and CD1 mice to the odor geraniol and established that C57BL/6 and CD1 mice are quite similar in their detection abilities. To detect odor perceptual differences where only subtle differences may be we also used an odor-masking task that proved to be sensitive to strain differences between C57BL/6 and Balb/c mice. These studies help focus our testing procedures for olfactory impairment and function for future work.

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CHAPTER 1: INTRODUCTION

1. Olfactory system

Olfaction is an underappreciated sense in humans. Unlike hearing, sight and touch, an inability to smell (Anosmia) is not considered a "real" disability. However, olfactory sensation provides important information about the environment, and acts as a trigger for memory, especially emotional memory. The role of olfaction in human experience is easy to recognize from personal experience, and there are dangers associated with the inability to smell. Odors are often the first sign of danger and include things such as smoke, spoiled food, or the chemical smell of melting plastic. The role of odor as a trigger for memory is clear to anyone who has been brought back to childhood by the smell of a particular childhood food, of the smell of baby powder reminding which reminds you of your grandmother. Thus, odors not only enrich our gustatory experience or warn us of danger, but also gives depth to our experiences. Odors are comprised of complex mixtures of volatile and (particularly in pheromones) non-volatile molecules. When inhaled, these chemicals stimulate a set of cells expressing specific receptors for these molecules. The pattern of activation that occurs from the combined response of the thousands of cells produces a specific signal that says in the brain, "coffee" "ocean" "dirty socks". Perceptually different odors can activate overlapping populations of olfactory receptors. So how is it that animals detect and encode odor?

1.1 Olfactory stimuli

The olfactory sensory system is responsible for detecting and processing chemical sensory information from the environment. These include general odorants, pheromones, and in some species, carbon dioxide (review, Ma 2007). Specific cells within the olfactory system also appear to respond to changes in pressure and temperature (Grosmaitre et al., 2007; Mamasuew et al., 2008).

What humans generally think of as a single odor (such as chocolate) is usually a complex mixture of molecules. These mixtures can be broken down into their individual chemical components, each of which can have a specific odor such as amyl acetate smells like bananas. Pheromones are defined as chemical stimuli that are con specific, released and detected within a species and produces a stereotypic behavior. Pheromones may be a mixture of volatile and non volatile compounds including such things as small peptides released in urine (review, Johnston 2000; Cocke et al., 1993).

1.2 Olfactory sensory systems

In rodents, the neurons responsible for chemical detection in the olfactory system are divided into four components based on location and morphology. These are the Grueneberg ganglion (GG), septal organ (SO), vermeronasal organ (VNO), and main olfactory epithelium (MOE). All four areas are classified as part of the olfactory system based on their chemical receptor expression, axonal project to the olfactory bulb, and expression of olfactory marker protein, (review, Breer et al.,

2006). Each of the four parts of the olfactory sensory system appears to have different primary roles, although there is overlap between systems (review, Touhara and Vosshall, 2009).

Grueneberg ganglion and septal organ

The Grueneberg ganglion (GG) and septal organ (SO) are the least understood of the olfactory sensory systems. The GG is located at the very anterior tip of the nose in rodents (Figure 1). It appears to be involved in pheromone detection as it expresses a class of vomeronasal receptors (the V2r83) G-protein coupled receptors (GPCRs) (Fleischer, et al., 2006; review Ma, 2007). One role proposed for the Grueneberg ganglion cells is the detection of alarm pheromones, signaling the need for fight or flight (Brechbuhl et al., 2008). However it is unclear how these odors reach the GG at this point since anatomically they do not project to the nasal airspace.

The SO is located ventrally to the MOE and is surrounded by respiratory cells (Figure 1). The sensory neurons of the SO are bipolar, ciliated neurons (review, Ma, 2007). The septal sensory neurons are similar to the neurons of the MOE as they express some of the same olfactory receptors and signaling proteins. Moreover they are close in proximity to the MOE, although their axons project separately to the main olfactory bulb (Giannetti et al., 1992).

Vermeronasal organ

The vermeronasal organ (VNO) is a bilateral tube like structure encapsulated by bone (Figure 1). The VNO is not directly in line with respiratory airflow and

requires chemicals to enter through a small duct connecting the VNO either to the nasal passages or oral cavity, depending on the species (see Farbman, 2000, and Johnson, 2000, for reviews). The VNO is present in most mammals including rodents, cats, dogs, horses and elephants but excluding humans (Johnson, 2000; Salazar et al., 1997; Garcia-Suarez et al., 1997; Johnson and Rasmuseen, 2002). The function of the VNO is primarily for pheromone detection, but it is also able to detect general odorants (Trinh and Storm, 2004; review, Keller et al., 2009). Like the septal neurons, the vermeronasal sensory neurons (VSNs) are bipolar neurons although their unmyelinated axons project to the accessory olfactory bulb (Figure 1)(review, Johnson, 2000). The VSNs express two classes of receptors on the microvilli, the V1Rs and V2Rs, which couple to the G-proteins (G_i and G_o respectively). Both of these receptor classes appear to stimulate phospho-lipase C (PLC) signaling cascades (Rünnenburger et al., 2002; Zhang, et al., 2010).

Main olfactory epithelium

The main olfactory epithelium (MOE) is the best characterized of the four olfactory sensory systems, and the largest olfactory sensory region in rodents. The olfactory sensory neurons (OSNs) are embedded in the epithelium covering the four turbinates (Figure 1) and the septum that separates the two nasal cavities. This is the primary odor-detection system. Odor molecules diffuse across the turbinates during respiration, or are actively brought into the nasal cavity by sniffing. In addition, odors coming from the oral cavity pass by the retronasal cavity (Herbert and Leininger, 1999).

The MOE consists of the olfactory sensory neurons (OSNs), supporting cells, and basal cells, which are stem cells that generate new olfactory sensory neurons and supporting cells (review, Farbman, 2000). In addition, the MOE contains Bowman's glands that are dispersed across the MOE and secrete mucus across the surface of the epithelium. This mucus suspends the cilia and provides the ionic gradient necessary for signal initiation (review, Ache and Restrepo, 2000). An OSN is a bipolar neuron that extends a single dendrite crowned by cilia into the mucus layer covering the epithelium, while its unmyelinated axon projects to the main olfactory bulb (Ache and Restrepo, 2000). Each OSN is thought to express only one of ~1000 separate olfactory receptors (OR) (Buck and Axel, 1991). OSNs expressing a given OR are scattered across the olfactory epithelium, and the axons of these neurons converge onto the same glomeruli (for each bulb) (review, Mombaerts, 1999). Although OSNs serve primarily to detect volatile odor compounds, there is broad evidence to support their ability to detect pheromones as well (Wang, et al., 2007; review, Keller et al., 2009). The OSNs are the focus of the work presented here.

1.3 Olfactory bulb

The olfactory bulbs are two bilateral globular structures that are directly adjacent the cribiform plate. The bulbs are covered with small glomeruli made up of the incoming axons from the MOE, which synapse onto the dendrites of Mitral and Tufted cells that are the main output neurons of the olfactory bulbs (review, Mori et

al., 1999; Matsutani and Yamamoto 2008). In rodents each glomerulus contains input from ~5,000-10,000 OSNs (review, Chen and Shepherd, 2005; Su et al., 2009). In mice OSNs expressing the same olfactory receptor converge onto only a few of the nearly 1800 glomeruli. The incoming signal from the OSNs is regulated through GABAergic or dopamanergic periglomerular cells that provide lateral inhibition through dendro-dendritic synapses across glomeruli (reviews, Lledo and Gheusi, 2003; Benignus and Prah, 1982). Deeper into the OB dendrites of mitral and tufted cells are laterally inhibited by the GABAergic granule cells. Further modulation of the olfactory bulb is provided from excitatory input descending from the piriform cortex onto the granule cells (Mombaerts et al., 2005; reviews, Strowbridge, 2009; Matsutani and Yamamoto, 2008).

1.4 Olfactory cortical projections

From the bulb, mitral and tufted cells project along the lateral olfactory tract branching off to target a range of cortical structures. Included in these cortical regions are the prefrontal cortex, perirhinal cortex, entorhinal cortex, hypothalamus and the amygdala (reviews Benignus and Prah, 1982; Haberly, 2001). Compared to other sensory system the olfactory system has the most direct access to the amygdala, a central part of the emotional processing of the brain (review McDonald, 1998). Higher cortical structures provide reciprocal feedback to the olfactory bulb, thereby modulating the incoming signals (reviews, Slotnick 2001; Benignus and Prah, 1982; Haberly, 2001; Matsutani and Yamamoto, 2008).

2. Signal transduction

2.1 cAMP signal transduction in olfactory sensory neurons

Odor detection begins in the cilia where odor molecules activate olfactory receptors (OR). These ORs are seven transmembrane receptors coupled to a unique olfactory guanosine triphosphate (GTP) binding protein, G_{olf} (Jones and Reed 1989; review, Schild and Restrepo 1998). G_{olf} shares 88% homology with $G_{s,}$ both of which stimulate adenylyl cyclase to increase cAMP production (Jones and Reed, 1998). The best characterized, and most abundant odor signal transduction pathway is a cAMP mediated pathway (Figure 3). Odor binding to the OR activates the attached G_{olf} that releases its alpha subunit (Buck and Axel, 1991). (review, Ebrahimi, and Chess, 1998; Spehr and Munger, 2009). The alpha subunit stimulates adenylyl cyclase III (ACIII) which increases production of cAMP resulting in the opening of the cyclic nucleotide gated (CNG) channel, a non-selective cation channel resulting in the entry of Na and Ca (Bakalyer and Reed, 1990; Belluscio et al., 1998; reviewed, Ache, and Restrepo, 2000). The influx of calcium through the CNG channel then activates a calcium dependent chloride channel. Depending on the intracellular (dendritic) chloride concentration, the chloride current either suppresses or amplifies the depolarization occurring from the cation influx (Reuter, et al., 1998; Dubin, and Dionne, 1994; Kleene, and Gesteland, 1991).

The cAMP mediated odor transduction pathway is the predominate signaling system in rodents. This has been supported by the lack of odor responses for both knockout mice for the CNGA2 subunit of the CNG channel, or ACIII (Lin, et al., 2004;

Wong et al., 2000). Most odors tested increase cAMP rather than IP3 following odor stimulation (Boekhoff and Breer, 1992). Further, calcium imaging studies have demonstrated that the phosphodiesterase inhibitor, IBMX, produced similar and overlapping activation with many odors (Leinders-Zufall, et al., 1998). OSNs contain other G-proteins (G_s, and G_o) and additional forms of AC (AC 2, 3 and 4 are all present in the cilia of OSNs). However, ACIII null mice are anosmic for a wide range of odors, failing to produce field potentials in response to odor stimulation, and show an inability to detect odors in several behavioral tasks (Wong et al., 2000).

The olfactory CNG channel is a tetramer comprised of three subunits (2) CNGA2, CNGA4 and CNGB1b (review Bradley et al., 2005). The CNGA2 subunits are necessary for channel activation, while the CNGA4 helps stabilize the open configuration, and CNGB1b is required for the desensitization to cAMP (Waldeck et al., 2009). A calmodulin (CaM) regulatory site is present on both the CNGB1b and A4 subunits (Bradley et al., 2001; review Truedeau and Zagotta, 2003) and the calcium free calmodulin, apocalmodulin, is constitutively bound to the channel (Bradley et al., 2004).

The calcium dependent chloride channel (Cl_{Ca}) is abundantly expressed in olfactory cilia, and acts as a powerful low-noise amplification of the CNG initiated depolarization (Reisert et al., 2003). An increase in ciliary intracellular calcium is sufficient to activate the calcium dependent chloride channels leading to a chloride current that makes up as much as 90% of the excitatory odor response in both field potential recordings and isolated OSNs (Frings et al., 2000, review; Nickell et al., 2006; Boccaccio and Menini 2007). Knowledge of the specific type of Cl_{Ca} channel located in

OSNs has not yet been established. Recently, two papers pertaining to this question came out, one excluding bestrophin-2 as the Cl channel involved in odor detection, and the other giving evidence for ANO2 as the likely candidate (Pifferi et al., 2009; Stephan et al., 2009). Pifferi et al., (2009) showed that bestrophin-2 was present in the olfactory cilia of mice but this loss did not alter field potential recordings or electrophysiology properties to odor stimulation or increased intracellular calcium. They propose that bestrophin-2 may act in response to osmotic change rather than odor signaling. In contrast, Stephan et al., (2009) demonstrated that Anoctamin 2 (ANO2) was abundant in OSN cilia membranes and when expressed in a cell culture system exhibited single channel conductance and current properties consistent with those reported for the channels in OSNs.

The odor stimulated signal in OSNs is amplified by the Cl_{Ca} channel due to a high intracellular chloride level within the cilia and dendritic knobs of OSNs. What maintains this high chloride level has been the subject of debate. It is well established that the Na, K, Cl co-transporter (NKCC1) is present and functional in OSNs (Keneko et al., 2004; Resiert et al., 2005). However NKCC1 is not solely responsible for maintaining a high intracellular Cl level since NKCC1 null mice still show little deficit or reduction in their chloride current. NKCC1-null mice are able to detect some odorants (Smith et al., 2008) and Nickell, et al., (2006) In NKCC1-null mice blocking chloride currents with the inhibitor DIDS indicated that odor-responses in field potential recordings still had a chloride component. Neither NKCC1 null, nor the $(Cl^-)/HCO_3^-$ exchanger 2 (AE2) null mice fully abolished the chloride response in field recordings, and RT-PCR showed that

the main olfactory epithelium has many forms of Cl transporters including members of the NKCC, KCC, and AE families (Nickell et al., 2007). It is therefore likely that several mechanisms are at work, and may be able to compensate for any single chloride transporter.

2.2 Non-cAMP mediated signaling in olfactory sensory neurons

As dominant as the cAMP pathway appears to be in the MOE of mammals, it is not the only signaling system. Both a cGMP and a PLC signaling system can modulate odor responses in mammalian OSNs. Within OSNs that use the cAMP mediated signal, a second cascade can be activated by soluble and membrane bound guanylyl cyclases (GC) located in the cilia, and regulated by calcium and PKA (Moon et al., 1998). In cell culture a secondary, long duration rise in cGMP occurs following odor stimulation (Moon et al., 2005). This may help regulate the olfactory signal as greater odor concentration increases the amount of cGMP produced (Moon et al., 2005). While a PLC/IP3 signaling cascade is established in invertebrates OSNs it is less understood in mammals (see reviews Ache and Restrepo, 2000; Ache and Zhainazarov, 1995). It appears more probable that in mammals the IP3 signaling cascade acts as a modulator rather than a primary odor transduction mechanism. In mammals the calcium response to odors appears to be attenuated by PLC and the PI3 kinase (PI3K) (Spehr et al., 2002). This is seemingly at odds with work done in frogs showing that PLC activity increased AC responses to odors and forskolin activation (Frings, 1993).

A subset of OSNs in the MOE also uses a guanylyl cyclase D (GC-D) signaling pathway for odor responses. Unlike ACIII + OSNs, the GC-D neurons express the cGMP signaling mirror of the cAMP pathway. The cGMP CNG subunit, CNGA3, is expressed rather than the cAMP CNGA2, and the cGMP stimulated phosphodiesterase 2 (PDE2) is present whereas its cAMP counterpart (PDE4A) is not (review Zufall and Munger, 2009). These neurons appear to project to the necklace glomeruli, a subset of glomeruli, and seem to be responsive to several peptides in mouse urine as well as CO_2 (Cockerham et al., 2009; reviews Breer et al., 2006; Ma, 2007). However, the full role of these neurons is still poorly understood.

2.3 Signal termination

Signal termination occurs with the removal of the odor molecule, potentially internalization of the receptor, degradation of cAMP, and removal of calcium. All of these must occur for the signal to be fully resolved.

The initial point of signal termination is the diffusion of odorants away from the receptors. With strong persistent odors, receptor internalization and recycling may occur, but is not well characterized in OSNs. Mashukova et al., (2006) demonstrated that β-arrestin2 binding and clathrin mediated endocytosis recycling of ORs occurs in cell cultures. Furthermore they showed that arrestin2 was present in OSNs, and inhibition of β-arrestin2 prolonged the response duration in an odor dependent manner.

The odor-mediated increase in $[cAMP]_i$ returns to baseline levels by hydrolysis of cAMP by phosphodiesterases (PDE). Two types of PDEs are known to be present in olfactory sensory neurons; a calcium/CaM stimulated PDE1C and the cAMP specific PDE4A (Yan et al., 1995; Cygnar and Zhao 2008; review, Goraya and Cooper, 2005). PDE1C is expressed in the cilia and PDE4A in the knobs and somas of OSNs. Mice that are knockouts for both of these PDEs show delayed onset and offset of field potential responses similar to those when occurring from application of a high concentration of the PDE inhibitor IBMX. This suggests that these are the primary PDEs in OSNs (Yan et al., 1995, Cygnar and Zhao 2008). Cygnar and Zhao (2008) nicely demonstrated the respective contributes of PDE1C and PDE4A through individual and then double knockouts of these PDEs. Their results demonstrated that when the PDE1C was not present, the duration of the response increased slightly, although the amplitude of the response was smaller.

Calcium plays many roles in OSNs, and it appears to be highly regulated. For cells to be able to respond to another stimuli, calcium levels must have sufficiently recovered for a large response to occur. In the cilia, calcium influx is terminated when the cAMP levels drop below the concentration required to activate the CNG channel (Song et al., 2008). Calcium clearance is also required to terminate the signal since calcium dependent chloride channels remain open as long as sufficient calcium is present (Kleen and Gesland 1991; Kleen 1993; Hallani et al.1998). Three primary mechanisms are used to control intracellular concentrations, the plasma

membrane ATPases (PMCA), sarco/endoplasmic reticulum Ca-ATPases (SERCA), and sodium/calcium exchangers (NCX).

In OSNs PMCAs and NCX are expressed throughout the cell. Weeraratne et al., (2006) showed that PMCA isoforms 1-4 are present, with PMCA1, 2 and 4 expressed in the cilia as well as the knob, soma and (except PMCA4) dendrite. Pyrski et al., (2007) using immuno staining and mRNA expression, found NCX1-3 with NCX1 showing the strongest label in OSNs. Additionally, they found mRNA for the potassium dependent sodium calcium potassium exchanger (NCKX). It may be conjectured that this almost overwhelming redundancy in calcium extrusion mechanisms could be necessary for greater signal mediated regulation of calcium handling.

Four genes make up the mammalian PMCA1-4 with two known RNA splice points (A and C). The result is that over 20 PMCA isoforms are known of, each of which has a different affinity for calcium, and slightly different forms of regulation (review, Strehler et al. 2007; Gromadzinska et al., 2001; Caride et al., 2007; review, Carafoli, 1994). PKA, PKC and CaM have all been shown to have stimulatory effects on PMCA activity, possibly through interacting means, where PKA and PKC phosphorylation of PMCA increases CaM binding, and therefore increases activity (Gromadzinska et al., 2001; Caride et al., 2007; review, Carafoli, 1994). CaM binding occurs in all four isogenes, however the effect and affinity for CaM depends on the splice variant (Caride et al., 2007). Thus PMCAs provide a system that may be finely tuned depending on its role in each area of the cell.

NCX is part of the solute carrier (SLC) gene family (SLC8), with 3 genes present in mammals (NCX1-3) (review Blaustein and Lederer, 1999). NCX exchanges sodium for calcium, and can run in both directions depending on the intracellular and extracellular concentrations of these ions (review Blaustein and Lederer, 1999). NCX activity is electrogenic with a stoichiometry of 1 calcium ion for 3 sodium ions. It appears to use the same binding pocket for both sodium and calcium leading to confusion over the exact mechanism that allows its bidirectional activity (reviews, Balaustein and Lederer, 1999; Lytton 2007). NCX extrusion mechanisms are dependent on the driving force of sodium. Also, when calcium increases inside the cell after depolarization, the rate of extrusion also increases. NCX may also run in reverse if extracellular sodium is sufficiently reduced (reviewed Balaustein and Lederer, 1999).

Although not involved in calcium removal from the cilia, SERCA also removes calcium from the cytoplasm. SERCA sequesters calcium into the SR/ER. SERCA expression in OSNs has not been directly studied in OSNs, but candidates include the ubiquitous SERCA2 and possibly SERCA3 both of which have been shown in the brain and epithelial tissue (review, Baba-Aissa et al., 1998). SERCA mediated calcium removal in OSNs has been demonstrated by Saidu et al., (2009) who showed that PMCA, NCX, and SERCA all contributed in equal measures to cAMP increased calcium levels in mouse OSN dendritic knobs.

It is not yet known what roles each of these calcium extruders plays within OSNs. While NCX, PMCAs, and SERCA have been shown to contribute to calcium

recovery in the knob of mouse OSNs (Saidu et al., 2009) the contribution to cilia calcium removal is still unclear. Castillo et al., (2007) using calcium transport assays and electrophysiology recordings from toad and rat cilia found that PMCA 1 and 4 appeared to be the isoforms responsible for calcium recovery in the cilia. In salamanders, PMCAs also appeared to be the major calcium extrusion mechanism in the cilia (Antolin et al., 2010). In the frog however, Resiert and Matthews (1998) found that NCX appeared to be primarily responsible for ciliary calcium removal. So far it is unclear if the dominant calcium extrusion mechanisms are species specific as well as location specific in OSNs.

In other cell types PMCAs and NCX play different roles in the cell. In neurons, PMCA and NCX appear to localize to different areas. NCX is primarily expressed in areas where the ER is close to the plasma membrane, whereas PMCAs are more evenly distributed across the cell membrane (Blaustein et al., 2002). At the synapse, both NCX and PMCA are highly expressed, however the PMCAs are most abundant in the active zones of the pre-synaptic terminal, whereas NCX expression is higher in regions adjacent to the active zone (Blaustein et al., 2002). In addition to neurons, regional distributions differ for PMCAs and NCX in mammalian sperm, which share some striking similarities to OSNs including expression of a subset of ORs that also activate ACIII (Spehr et al., 2006). In sperm, PMCAs are expressed in the flagella while NCX is expressed in the head of the sperm (Wennemuth et al., 2003). Collectively these reports suggest that NCX and PMCAs serve different functions within the cell, presumably due to their respective calcium removal properties.

These may help determine what the role of PMCAs and NCX have in OSNs, and help identify how signal termination is regulated through calcium removal.

2.4 Adaptation

Adaptation decreases the sensitivity (responsiveness) to a stimulus when the stimulus is either present for a long time, or with repetitive stimulation. In olfaction, adaptation is extremely important to odor detection since many odors remain present long after they are detected. Research on adaptation within OSNs takes one of two forms: short-term adaptation where an odorant is presented for a brief period (~100 ms) and then re-stimulated (resulting in a smaller response), or longer adaptation where an odor is presented for >several seconds and then repeated. Long duration adaptation is also produced when the odorant is kept constant and an additional response is only elicited when a higher concentration of the same odor is presented.

In general, short-term adaptation has been found to be due to regulation of the CNG channels through the calcium/CaM feedback that desensitizes them to cAMP. Kurahashi and Menini (1997) first demonstrated the importance of the CNG channel in short-term adaptation, and this was further clarified by Boccaccio et al., (2006) who used forms of caged cAMP analogs, and caged calcium to demonstrate that neither the PDEs nor the calcium dependent chloride channels were responsible for adaptation. Since apocalmodulin is constitutively present on CNG

channel subunits CNGA4 and CNGB1b fast calcium mediated feedback can occur (Bradley et al., 2004). It is now fairly clear that CNGB1b appears to be involved in closing the channel (Song et al., 2008; Waldeck et al., 2009), while CNGA4 is responsible for regulating the sensitivity to cAMP via calcium/CaM regulation, and therefore adaptation (Munger et al., 2001; Waldeck et al., 2009).

Long-term adaptation can be seen with sustained odor exposure. Reisert and Matthews (2001) simultaneously recorded calcium transients in the cilia and current responses in the soma of salamanders. With sustained (30 seconds) of odor, the calcium and current responses showed an oscillating response that decreased over time. This form of adaptation, like short-term adaptation, still relies on the CNG channel sensitivity, however it is also now well established that calcium/CaM stimulatory effects on the calcium calmodulin dependent kinase II (CaMKII) phosphorylates ACIII thereby reducing cAMP production (Wei et al., 1998; Leinders-Zufall et al., 1999). The gradual decrease in the amplitude of responses seen in sustained odor stimulation occurs as ACIII activity is progressively reduced.

The calcium clearance mechanisms mentioned previously are also likely to play a role in the rate and duration of adaptation since calcium levels dictate the feedback on the CNG channels, and the stimulatory effect on CaMKII. Thus, ciliary calcium control is essential for proper signaling, and adaptation.

3. Olfactory marker protein (OMP)

Olfactory marker protein (OMP) was identified as a marker for all of the mature sensory neurons of the olfactory system over thirty years ago. Immunostaining showed that this protein was highly enriched in all parts of the OSNs from the cilia, all the way to the axon terminals in the olfactory bulb (Monti-Graziadei et al., 1977). Since that time, OMP has been a useful indicator for differentiating between adult and immature OSNs, but identification of the function of OMP in sensory neurons has remained open for debate. The work of Frank Margolis' lab in collaboration with others has been the primary driving force for understanding OMP. While much has been learned, the actual function of OMP remains elusive.

3.1 OMP in development and neurogenesis

Early work demonstrated that OMP showed a developmental link, since its expression and transport was greatest in younger mice or in olfactory epithelial that were regenerating after injury (Kream and Margolis, 1984). Carr et al., (1997) demonstrated that OMP acts in a mitogenic capacity, increasing the proliferation of supporting cells and neuronal precursors. Furthermore, severing the connections to the olfactory bulb resulted in massive OSN death, however small populations of OMP+ cells remained. Of those remaining cells Carr et al. (1997) found that OMP expression was higher than in non-bulbectomized OSNs. What was unique about those OMP+ cells that survived is not known. Since the OE was analyzed at time

points from 3 days to 6 months after bulbectomy, the OSNs present with high OMP expression would have been born both before and after loss of the target. More recently, OMP has been suggested to play a more compensatory role in OSNs, which was supported by David Coppola's lab using naris occlusion (unilateral blocking of the nasal cavities). OMP expression was greater in the occluded (odor-deprived) side of the olfactory epithelium (Waguespack et al., 2005). Collectively these data show that OMP increases in response to either target depletion (bulbectomy) or reduced exposure to odor stimuli; the signals that regulate this apparent compensatory function are not known.

3.2 OMP structure and interactions

Olfactory marker protein (OMP) is a highly conserved ~16.5-19 kD acidic cytoplasmic protein and is present in a range of animals including mice, rats, fish, and humans (Keller and Margolis 1975,1976; Buiakova et al., 1994; Baldisseri et al., 2002; Celik et al. 2002). OMP resembles a β-Clam fold, suggestive of a protein binding structure (Baldisseri et al., 2002). Indeed, OMP is known to dimerize with itself and can bind with Bex1 and 2 (Buiakova et al., 1994; Behrens et al., 2002). The OMP homo-dimer has a stronger affinity for Bex1/2 than the monomer, however the duration of this binding is brief. Interestingly, OMP with Bex appears to cross into or out of the nucleus, a novel finding opening up additional possible roles for OMP in cellular regulation (Behrens et al., 2002). Unfortunately, the ability of OMP to interact with Bex has not clarified the OMP function since the function of Bex is not

yet fully understood. The X-chromosome linked genes, Bex 1-3 (Brain Expressed X-linked) were first identified in 1999 and their high expression in several brain regions, and the olfactory system including the olfactory epithelium and vomeronasal organ have brought them into interest for olfactory function (Brown and Kay, 1999; St. John and Key 2005). The OMP/Bex interaction may be key in determining a means for OMP to interact with signaling or other cellular functions. Indeed, the Margolis lab has suggested that OMP/Bex interaction may be important for calcium signaling following their paper demonstrating that Bex1 has a CaM binding site and binds CaM when Ca is present (Koo et al., 2007; Kwon et al., 2009).

3.3 Odor mediated activity, detection, and perception in OMP-null mice

What part does OMP have in odor detection? Two widely sited studies by Youngentob and Margolis using the odorant propanol showed that OMP was necessary for odor detection. OMP-null mice tested for odor detection sensitivity with the odor propanol showed a significantly higher threshold for the odor (Youngentob and Margolis, 1999). This was followed up by the same authors with a demonstration that partial rescue with an OMP recombinant adenoviral vector to express OMP in the OMP-null mice decreased the threshold to propanol; thereby showing a partial behavioral rescue was possible by reintroducing OMP to the OE (Youngentob et al, 2004). However, these mice are far from anosmic, and the differences in their olfactory sensation of most odors may be slight perceptual differences rather than an inability to make the detection. Youngentob et al. (2001)

demonstrated this high capacity for odor discrimination and detection using five different odors. They found that both OMP-null and wild type mice responded correctly above 85% of the time with similar accurate detection between both groups. However, the *type* of errors that were made were different between OMP-null and wild type mice so that a wild type mouse may be more likely to miss odor A if odor C was presented first, OMP-null mice would be more likely to miss odor A if B was presented first. This suggests that the perceptual qualities (or intensities) of the odors are altered in OMP-null mice. Support of this idea was further demonstrated by the activity pattern that intact olfactory epithelia showed in response to several different odors. The intensity and localization of areas responding to each odor was more distributed across the OE in the OMP-null mice than those of control mice (Youngentob et al., 2003).

3.4 OMP in odor signaling

The search for odor perceptual/detection differences in OMP-null mice was motivated by work showing that the OE responded to odors in an altered way when OMP was missing. These OMP-null mice were first reported to have physiological differences by Buiakova et al., (1996) who found a longer recovery response in electroolfactogram (EOG) recordings from the OMP-null mice, and substantially smaller second odor responses. This result was both confirmed and further strengthened by work showing that this characteristic EOG response could be mostly rescued by adenoviral vector reintroduction of OMP+ cells into the OE (Ivic

et al., 2000). Interestingly, Ivic et al., (2000) also showed that heterozygous $OMP^{+/-}$ mice were not different from wild type mice suggesting that reduced OMP expression was sufficient for normal function (at least with these methods).

The role of OMP in odor detection suggests that encoding of that information should also be altered. Indeed, since OMP appears to be synthesized in the soma and then transported to all areas of the neuron in a "slow transport" fashion (Kream and Margolis 1984) it is reasonable to wonder what role it has in action potential and olfactory bulb signals. Oddly, OMP-null mice have larger diameter axons however the compound action potential conduction velocity is nearly identical between wild type and OMP-null mice (Griff et al., 2000). As for the olfactory bulb, they are ~15% less dense (weight for size) than the wild type mice, and the axons from OMP-null mice fail to properly innervate their glomeruli and show "overshooting" of their target synapses (Griff et al., 2000; St John and Key, 2005).

Thus far the physiology described here is from populations of cells, not individual neurons. To understand the role OMP has in signal transduction, Reisert et al., (2007) characterized the properties of isolated OSNs to odors and to the phosphodiesterase (PDE) inhibitor IBMX. IBMX increases cAMP and thereby mimics an odor stimulus in OSNs. Using large suction electrode recordings where only the cilia and knob are exposed to the bath, Reisert et al., found that brief odor stimulation matched the previously reported kinetics of the field potentials from EOG recordings. Like the EOGs, the brief odor exposure showed slower recovery (time from peak to return to baseline) period for OMP-null OSNs, and a sustained

odor (60sec) produced repetitive slow wave responses, unlike the multiple transient currents in wild type OSNs. Surprisingly, 50ms IBMX stimulation failed to illicit a slow response in OMP-null OSNs. Indeed, these responses were identical to their wild type controls in recovery, however their amplitude was significantly higher. What causes a slow recovery period in these OMP-null OSNs? Kwon et al., (2009) have proposed that NCX mediated calcium extrusion is reduced in OMP-null OSNs. This is based on their calcium imaging recordings from dendritic knobs of epithelial sheets where they found that calcium recovery was slower after IBMX stimulation (increasing cAMP), high external K Ringers to depolarize the cells, or caffeine to release calcium from intracellular calcium stores. All three conditions showed slower calcium recovery in the OMP-null responses suggesting that calcium extrusion was the regulatory point. NCX was proposed as the prime target due to slower onset and termination of a calcium rise in the knobs with low-external Na solution (which reverses the exchanger causing it to increase intracellular calcium). These results are, however, at odds with those data reported by Resiert et al., (2007) who did not see slower recovery when the stimulus was IBMX rather than an odor. To understand these contradictory results, further studies on the calcium handling and odor responses in $OMP^{-/-}$ are needed. While Reisert et al., (2007) proposed the OMP must be acting upstream of the CNG channel, presumably at the level of ACIII, Kwon et al., (2009) proposed that NCX (downstream of the CNG channel) is the target. The proposal by Kwon et al., (2009) that there is protein-protein interaction between OMP, Bex, and CaM (and possibly others) may help

explain these studies. The differences between the results of these two studies suggest that calcium regulation occurs at multiple points. Thus, depending on the methodology used, the interaction occurring between OMP, Bex and CaM may differ given that their interaction could regulate many steps in OSN function.

One of the questions that may become most relevant is whether the effects seen in OMP-null mice are due to the lack of OMP during development, or from odor detection/signaling differences, since OMP appears to act as both a mitogen and a modulator of odor-transduction (Kream and Margolis, 1984; Carr et al., 1997; Buiakova et al., 1996; Reisert et al., 2007; Kwon et al., 2009).

4. Odor exposure in the olfactory system

4.1 Odor exposure and environment on human olfaction

Humans are not generally dependent on their olfactory system in the way they are on sight, touch or hearing. Anosmia is not considered a disadvantage in the way that blindness or deafness would be. Nevertheless, the importance of olfactory function in humans can be found from several examples. Those lacking the ability to smell are most at a disadvantage for flavor where much of "taste" is in fact olfactory cues, thus leading to possible poor nutrition as well as a general decrease in the richness of food consumption. More serious perhaps are the dangers presented by the inability to detect warning odors, such as smoke, spoiled food, a gas leak, and so on. Moreover, understanding human olfaction can help in the diagnoses of diseases that show olfactory impairment. Alzheimer's disease, Parkinson's disease, multiple

sclerosis, and schizophrenia have all been linked to olfactory aberration (review, Strous and Shoenfeld, 2006). This raises the question of how do these diseases differ in their olfactory abnormalities, and can they be used as a diagnostic tool if the reason for olfactory impairment is clarified?

Like all animals, humans are exposed daily to a multitude of odors, but if odor exposure alters odor perception and discrimination is not yet well understood. Gabba (2006) in a review of the literature on workers exposed to industrial chemicals such as chromium, acrylates, and metals, found that increased thresholds or minor to severe olfactory impairment was common. However, the lack of a standardized olfactory test used to assess olfactory function makes it impossible to infer the degree and type of dysfunction between chemical exposures. Interestingly, working in a strong odor environment does not appear to impair olfactory acuity. Hummel, et al., (2004) found that people (mostly women) working at perfume counters had no difference in odor threshold or recognition before or after work compared with matched controls. Further the perfume group was significantly better at odor discrimination (although if it was due to experience detecting differences or a change in odor perception was not clear).

4.2 Odor enrichment and deprivation in rodents

Sensory experience or deprivation has long been known to modify sensory neurons (Leventhal and Hirsch 1983; review, Tian 2004) as well as cortical systems (reviewed, Grubb and Thompson, 2004; Fox and Wong, 2005). Olfaction provides a

particularly interesting system in which to study sensory dependence since neurogenesis continues throughout adult life in the olfactory neurons of the epithelium and in the bulb (review, Farbman, 1994). In the bulb, "odor-enrichment" has been shown to improve memory for odorants, increased progenitor cells in the main olfactory bulb (Rochefort, et al., 2002) and lead to increased interneuron survival and apparent increased memory for odors previously presented to mice (Rochefort and Lledo, 2005). Odor-enrichment also increases cell survival in the olfactory bulb and the piriform cortex (Woo et al., 2006; Shapiro et al., 2007). Alternatively, naris occlusion – used as a model of odor-deprivation – causes decreased granule cell neurogenisis, survival and arborization in the bulb (Corotto et al., 1994; Saghatelyan et al., 2005). Whether the changes seen in the olfactory bulb to odor exposure is due to a net change in afferent signal from the olfactory epithelium, or if it is due to a change in the signaling within individual sensory neurons in the epithelium is unclear. Odor enrichment or odor-limited experience in the olfactory epithelium (OE) has been less extensively studied; however, field potential recordings in the naris occluded side of the OE show increased amplitude, and time to recover, than non-occluded OE responses (Waggener and Coppola, 2007). Moreover, a role for odor in regulating gene transcription in olfactory sensory neurons (OSNs) has been suggested by Moon et al. (1999) who showed that odors induce phosphorylation of the transcription factor cAMP-responsive binding protein (CREB).

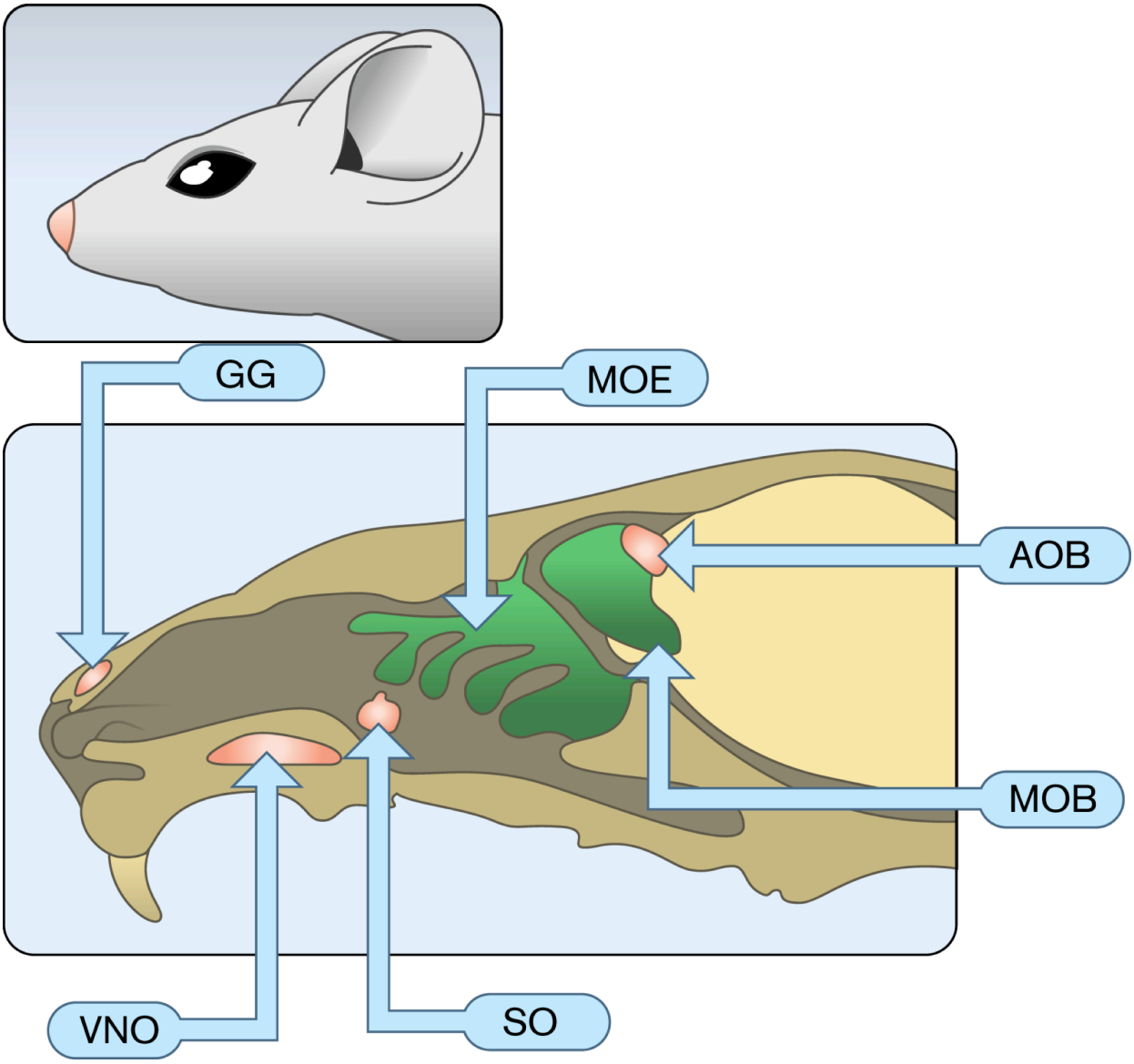

Figure 1. Top: The animal model (mouse), used for all experiments facing the same direction as the bottom illustration of the olfactory sensory system. Bottom: illustration of the olfactory system in rodents. In pink the Gruenberg ganglia (GG) veromonasal organ (VNO), septal organ (SO), and accessory olfactory bulb (AOB). In green, the main olfactory epithelium (MOE), and main olfactory bulb (MOB).

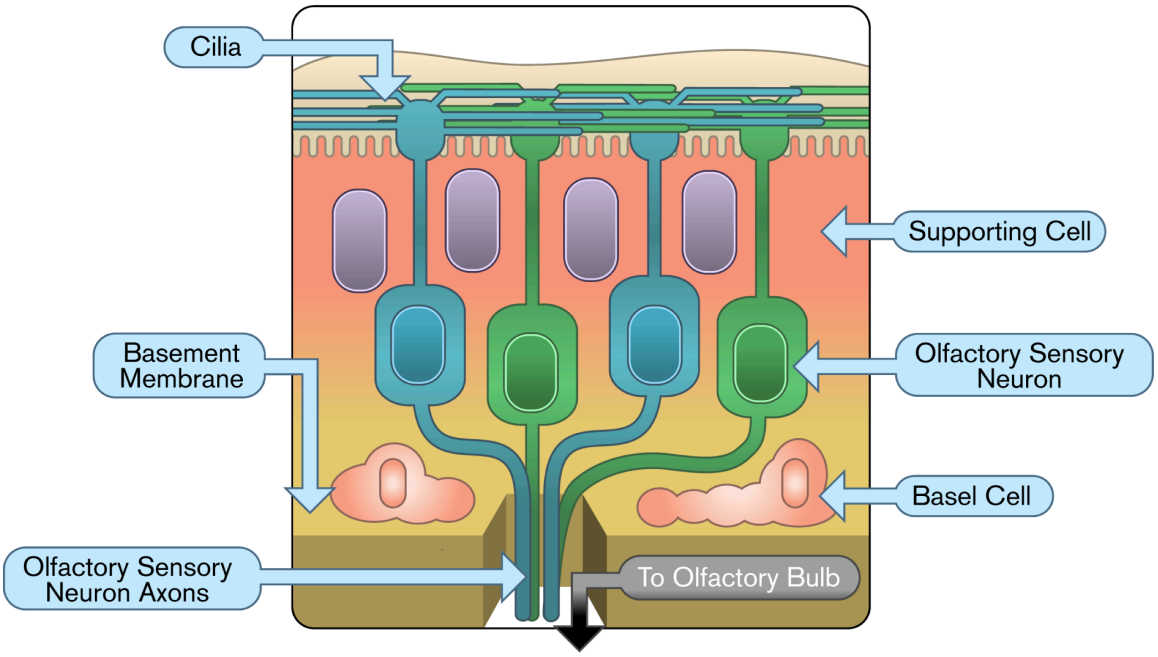

Figure 2. An illustration of a cross section of the olfactory epithelium with the cilia at the top in the mucus membrane, supporting cell layer, and olfactory sensory neurons somas, and the basement membrane with basal cells. Olfactory sensory neurons expressing the same olfactory receptor (presented here in green and blue) have unmyelinated axons that converge on their way to the olfactory bulb.

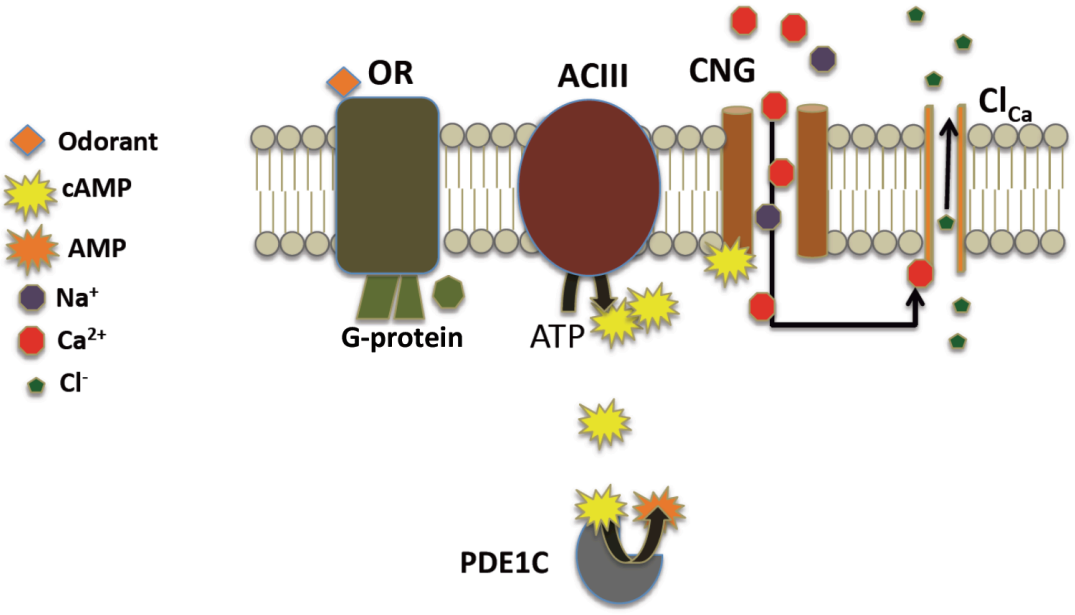

Figure 3. The cAMP mediated signal transduction pathway of olfactory sensory neurons. From left to right: an odorant bind the olfactory receptor (OR) activating the a G-protein, the alpha subunit stimulates adenylyl cyclase III (ACIII) thereby increasing cAMP production. The cAMP binds the nonselective cyclic nucleotide gated (CNG) channel allowing influx of Na^+ and Ca^{2+}. The Ca^{2+} then opens a calcium dependent chloride channel (Ca_{Ca}) where Cl^- can amplify the signal provided the chloride concentration is sufficiently high internally.

CHAPTER 2

An odor-enriched environment alters the response of isolated olfactory sensory neurons in two strains of mice, C57B/6 and OMP$^{-/-}$

Abstract

Odors activate olfactory sensory neurons (OSNs) leading to an increase in cAMP that open cyclic nucleotide gated (CNG), non-selective cation channels. The subsequent calcium increase opens calcium dependent chloride channels which can further depolarize the cell depending on the $[Cl-]_i$ level. In order to study the effect odor environment has on the primary signaling pathway of OSNs, we used C57BL/6 mice and olfactory marker protein (OMP) null mice. OMP is a cytoplasmic protein with an undetermined function in OSNs. Using two odor-environments classified as "standard " or "odor-enriched" we found that OSNs from the standard environment, when stimulated with IBMX/FSK to activate the cAMP mediated odor transduction pathway, produced faster rates of calcium recovery than from OSNs in the odor-enriched environment. This fast recovery was even more apparent in OSNs from $OMP^{-/-}$ mice. Depolarization with a high K solution to increase $[Ca]_i$ without the cilia failed to mirror the calcium recovery seen with IBMX/FSK stimulation. This suggests the effect seen was due to differences occurring in the signal transduction pathway rather than overall cellular calcium extrusion. Our results suggested that standard $OMP^{-/-}$ OSNs were limited in calcium influx. Using perforated patch clamp recordings less than 10% of the $OMP^{-/-}$ OSNs from the standard housing condition showed measurable responses, whereas all OSNs from the other 3 conditions responded ~50% of the time. Stimulation with IBMX to inhibit PDE or FSK to stimulate AC were used to increase $[cAMP]_i$. The amplitudes of the responses and the calcium recovery times when these two stimuli were used separately suggests that the OSNs lacking OMP are much less responsive to AC stimulation. Our work supports previous findings that OMP is involved in signal transduction. In addition we also show that odor-environment can alter the odor response.

Introduction

Olfactory marker protein (OMP) is the hallmark for all mature olfactory sensory neurons. OMP is a highly conserved ~19 kD cytoplasmic protein expressed throughout the enter cell (Keller and Margolis 1975, 1976; Buiakova et al., 1994; Baldisseri et al., 2002; Celik et al. 2002). Although the function of OMP is unknown, its structure suggests it is a binding protein (Baldisseri et al., 2002). Electroolfactograms (EOGs), and single cell recordings of $OMP^{-/-}$ olfactory sensory neurons (OSNs) show slower responses to odorants (Buiakova et al. 1996; Reisert et al., 2007). Kwon et al., (2009) demonstrated that OMP appears to effect the activity of the sodium calcium exchanger (NCX). However behaviorally $OMP^{-/-}$ mice are not anosmic (Youngentob et al. 2001; personal observation) although Youngentob and Margolis (1999) showed that they have a higher threshold for the odorant propanol compared to wild type mice. Additionally the activity pattern of the olfactory epithelium in response to odorants is broader in $OMP^{-/-}$ OSNs than in wild type mice (Youngentob et al., 2003). Thus, the absence of OMP does not eliminate the ability to detect odors, although it appears to modulate odor responses.

In addition to the studies showing a role in altering the response to odors, OMP also has been shown to have a developmental and regenerative role. Expression of OMP is higher in the olfactory epithelium (OE) of young mice, as well as in the OSNs that are regenerating after damage (Kream and Margolis, 1984). Additionally OMP expression in unilateral naris occluded mice, increases in the side of the OE that is occluded (Waguespack et al. 2005). OMP added to olfactory

neurons in culture increases progenitor cell proliferation (Carr et al., 1997), and $OMP^{-/-}$ OSN axon terminals have higher incidence of overshooting the glomeruli targets in the olfactory bulb (St John and Key, 2005). Given the broad range of effects seen in $OMP^{-/-}$ mice it is probable that OMP has multiple roles in OSNs. In order to examine the role of OMP in the odor-signaling pathway we used OMP-GFP ($OMP^{-/-}$) mice to study the cAMP odor-mediated signal transduction pathway in two different odor-environments.

The dominant odor-transduction pathway in mice is the cAMP mediated pathway (reviews, Ebrahimi, and Chess, 1998; Ache, and Restrepo, 2000; Lin, et al., 2004). Odor signaling starts with an odorant binding to specific olfactory receptors (ORs) expressed in the cilia of OSNs (Buck and Axel, 1991; Zhang and Firestein, 2002). Odor binding to the receptor activates the G-protein, G_{olf} (review, Ebrahimi, and Chess, 1998). That, in turn stimulates adenylyl cyclase III (ACIII) thereby increasing cAMP production (Wong et al. 2000). Binding of cAMP opens the cyclic nucleotide gated (CNG) channel, a non-selective cation channel allowing influx of Na and Ca (Zufall, et al., 1994; review, Ache, and Restrepo, 2000; McClintock, 2000). Influx of calcium through the CNG channel then activates a calcium dependent chloride channel which, depending on the intracellular chloride concentration, can amplify the depolarization occurring from the cation influx (Reuter, et al. 1998; Dubin, and Dionne, 1994; Kleene, and Gesteland, 1991).

It is not known if the odor environment alters the odor-transduction pathway in the cilia of OSNs. However, sensory experience or deprivation has long been known to modify sensory neurons (Leventhal and Hirsch 1983; review, Tian 2004) as well as cortical systems (review, Grubb and Thompson, 2004; Fox and Wong, 2005). Olfaction provides a particularly interesting system to study sensory influence since neurogenesis continues throughout adult life in the olfactory neurons of the epithelium and in the bulb (review, Farbman, 1994). In the bulb, "odor-enrichment" has been shown to improve memory for odorants, increase progenitor cells in the main olfactory bulb (Rochefort, et al., 2002), increase interneuron survival, and an increased memory for odors previously presented to mice (Rochefort and Lledo, 2005). Odor-enrichment also increases cell survival in the olfactory bulb and the piriform cortex (Woo et al., 2006; Shapiro et al., 2007).

Alternatively, naris occlusion – used as a model of odor-deprivation – causes decreased granule cell neurogenisis, survival and arborization in the bulb (Corotto et al., 1994; Saghatelyan et al., 2005). Odor enrichment or odor-limited experience in the olfactory epithelium (OE) has been less extensively studied. EOG recordings from the naris occluded side compared to the non-occluded side of the OE show increased amplitude, and increased time to recover when stimulated with odors (Waggener and Coppola, 2007). Moreover, a role for odor in regulating gene transcription in OSNs has been suggested by Moon et al. (1999) who showed that odors induce phosphorylation of the transcription factor cAMP-responsive binding protein (CREB). CREB activation is not surprising given that the majority of odors

stimulate a cAMP mediated signaling pathway in OSNs (review Schild and Restrepo 1998; Lin, et al., 2004).

Understanding how sensory experience effects sensory signaling and the role of OMP in odor-signaling led us to ask three questions: 1) does odor environment alter the cAMP mediated signaling transduction pathway? 2) Is OMP acting on the signal transduction pathway or the termination of the signal? 3) Are $OMP^{-/-}$ OSNs more sensitive to odor environment? Using calcium imaging and perforated patch clamp recordings with OSNs from C57BL/6 and $OMP^{-/-}$ mice, we found that odor-environment altered the calcium recovery when stimulating the cAMP mediated pathway. OSNs from an odor-enriched environment showed a slower calcium recovery. Further, $OMP^{-/-}$ mice showed little AC activation. Although the calcium transients were slower both for OSNs from the odor-enriched environments and for wild type OSNs, we found no evidence that these effects were synergistic, rather they appear to be due to independent effects.

Material and Methods

Mouse Housing Conditions All mice were housed in accordance with IACUC guidelines. Food and water access was *ad libitum*, and mice were on a 12hr light/dark cycle. Mice were housed in either 1) standard animal care conditions with HEPA filter lids and high throughput filtered air ("standard"), or 2) odor-enriched (open wire top lids with food grade odors added daily). The odor-enriched mice were exposed (using a drop of the odor placed in a tea-ball on the cage each day) to one of 20 commercially available odors (vanilla, sarsaparilla, lemon, coffee, chocolate, almond, holy basil, ginkgo biloba, coconut, maple, orange, anise, banana, wild cherry, peppermint, strawberry, rum, spearmint, raspberry, and Echinacea extracts). In addition, odor-enriched mice received carrot pieces or sunflower seeds once a week.

Mouse Strains Mice were either C57Bl6 or OMP-GFP ($OMP^{-/-}$), a kind gift from Peter Mombaerts. OMP-GFP mice are a knockout of OMP by replacing it with GFP under the OMP promoter on a C57Bl6 background (Potter, et al., 2001). GFP fluorescence was not detected in calcium imaging experiments (pilot data not shown) and GFP has been used previously with Fura-2 imaging with no apparent calcium buffering effect (Billing-Marczak et al., 1999).

Euthanasia Mice were sacrificed and the experiments carried out in accordance with University of Vermont animal care guidelines (IACUC). The olfactory epithelial tissue was removed, cut into small pieces and placed in a divalent cation-free

Ringers solution containing ~0.5mg/mL papain (Calbiochem, La Jolla, CA) for 15 minutes. Cells were kept in high calcium (2 mM) Ringers solution plus leupeptin (~0.05mg/mL) until use.

Solutions All chemicals were from Sigma (St. Louis, MO) unless otherwise noted. Intracellular solution (in mM): 30 KCl, 110 K-gluconate, 10 NaCl, HEPES, 1 $MgCl_2$, 0.0023 $CaCl_2$. Ringers was (in mM): 145 NaCl, 3 KCl, 20 HEPES (acid free), 1 MgCl, 1.5 CaCl, 5 glucose (pH 7.2 with NaOH). For Fura-2 calcium imaging the Ringers solution was slightly modified by decreasing HEPES to 10mM and increasing glucose to 10mM (pH 7.3). High K Ringers (in mM): 65 KCl, 83 NaCl, 10 HEPES (acid free), 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose (pH 7.3). Chemicals: 1 mM or 500μM 3-isobutyl-1methybcanthine (IBMX) was made up into a 500mM stock with DMSO and stored at -80°C until use when it was diluted to a final concentration of 1mM for electrophysiology experiments or 500μM for calcium imaging experiments. The cell permeable analog of Forskolin (Calbiochem, Gibbstown, NJ), 7-deacetyl-7-[O-(N-methylpiperazino)-gamma-butyryl]-,Dihydrochloride (Fsk) was diluted in Ringers solution to a final concentration of 30μM.

Perforated Patch Clamp Electrodes with a resistance of 8-14 MΩ were used. Only cells with a seal > than 1 GΩ, and with voltage activated currents were included in the data. All recordings were done in voltage clamp where the cell was held at -60 mV and stepped from -100 mV to +100 mV in 20 mV steps. Electrodes were backfilled with an intracellular solution with 0.73 mg/mL gramicidin. Gramicidin

free intracellular solution was used to fill the tips of the electrodes. Most cells were also tested with 65 mM KCl Ringers to confirm the cells were responsive. Recordings were done on a Nikon TE20000-S or TE300 inverted microscope. Stimulus application was done using a Warner fast-step profusion system. Data were collected using Digidata 1322A and a Multiclamp 700A or 700B with the software Clampex 8.2. Traces were recorded with a Bessel filter at 2 kHz and sampled at 10kHz.

Calcium Imaging Cells were isolated as described above and plated on Concanavalin A (Sigma, St. Louis MO) coated cover slips fitted into a recording chamber. Fura-2 AM (Invitrogen, Molecular Probes, Eugene OR) and Pluronic acid (Invitrogen) made up to 0.01% in DMSO, final concentration of 5μM Fura. Cells were loaded for 20 minutes before washing in Ringers solution for at least 15 minutes prior to the start of recording. Cells were maintained at room temperature (21-23˚ C) from the time they were dissociated to the end of the experiment. Each cell was stimulated with 500μM IBMX/30μM Fsk for 6 or 8 sec (depending on the experiment) and allowed to return to baseline. Capture rate was 1 sec during stimuli, and 5-8 sec between stimuli unless otherwise noted. Calcium imaging data were recorded using Simple PCI V6 software with a Nikon Eclipse TE-20000U inverted fluorescence microscope, using a Hamamatsu CCD camera (computer controlled) with a Xenon lamp. Cells were excited at 340 and 380 nm and emission recorded at 510 nm. Solutions were applied using a Warner fast-step profusion system.

Statistics Statistical analysis was done using Graph Pad Prism V5. Decay kinetics were measured using a non-linear one-phase curve fit (decay rate constant, K). Cells were included provided they were above a 0.05 relative change in fluorescent intensity with a goodness of fit > 95% ($r^2 \geq 0.95$). Two-way ANOVA for comparison between strain ($OMP^{-/-}$ and C57BL/6) and odor-environment (standard and enriched) was followed by unpaired *t*-tests unless otherwise noted. An alpha level of 0.05 was considered significant.

Results

Odor-environment alters calcium recovery after activation of the odor-mediated cAMP signaling transduction pathway.

If the odor transduction pathway is altered by odor-environment, a probable point of regulation is through Ca dependent signaling. Ca has multiple roles in odor transduction, acting either to enhance or suppress the odor response in OSNs (review, Matthews and Reisert, 2003). To examine calcium responses in odor signaling we used the radiometric calcium dye Fura-2AM. We first examined the properties of calcium recovery following increased intracellular cAMP via stimulation with the general phosphodiesterase (PDE) inhibitor IBMX combined with a cell permeable analog of forskolin (FSK) to stimulate adenylyl cyclase. These stimuli bypass the olfactory receptor protein and increase intracellular cAMP ($[cAMP]_i$) directly, eliminating the need for a matching odorant. Reproducible calcium transients in the dendritic knob (DK) and in the soma were obtained with 6-8 sec application of IBMX/FSK on isolated OSNs (**Figure 1A**). Responses to IBMX/FSK were due to activation of the signaling pathway in the cilia since de-ciliated cells did not respond (data not shown) as has been shown by others (Delay and Restrepo, 2004). The calcium increases in response to IBMX/FSK stimulation began in the dendritic knob (DK) followed by the soma, as was previously described by Saidu et al. (2009). Due to limitations of the capture rate (1 second maximal speed) time to peak could not be definitively measured. Instead we measured the

recovery time from the peak of the response to the return to baseline. Since peak $[Ca]_i$ varied from cell to cell, the responses in the knobs were then normalized and fitted to a one-exponential non-linear regression curve to measure and compare the rate constant, K (**Figure 1B**) as described by Saidu et al., (2009). As shown in **Figure 1C**, OSNs from odor-enriched mice showed significantly slower recovery to IBMX/FSK stimulation than standard OSNs ($p<0.05$; unpaired *t*-test).

PKA inhibition does not significantly alter the calcium transient properties

The increase in cAMP produced in response to odor stimulation can lead to protein kinase A (PKA) activation which is known to alter OSN responses through phosphorylation of voltage-gated channels thereby decreasing Na and Ca currents (Wetzel et al., 2001). To investigate if PKA activity was altered by the odor-environment, we stimulated OSNs with IBMX/FSK then allowed them to recover to within 10% from the starting baseline. They were then treated with the PKA inhibitor H-89 for 3 minutes and re-stimulated (**Figure 2A**). Although several cells showed a change in recovery, they were in the minority, and the direction was not unidirectional, while the majority of cells showed little change (**Figure 2B**). The rate constants were not significantly different between untreated and H-89 treated responses for either group of mice (paired *t*-test, $p>0.05$).

Odor-enriched OSNs show similar calcium rate constants when depolarized with high K ringers

Calcium transients are the combined effect of the net calcium influx and the calcium extrusion mechanisms. **Figure 3** shows a hypothetical model of this transient. Increased $[cAMP]_i$ opens CNG channels initiating the calcium influx in the cilia. This signal is amplified by the calcium dependent chloride channels which lead to depolarization and opening of voltage gated calcium channels (VGCC) (Boccaccio and Menini 2007). These two sources provide the calcium influx into the cilia and knob (review Schild and Restrepo 1998). Throughout this process, calcium is removed through multiple mechanisms. In the knob, Saidu et al., (2009) showed that the plasma membrane ATPases (PMCAs), sodium calcium exchanger (NCX) and sarco/endoplasmic reticulum (SERCA) all contribute to calcium removal. In order to clarify which part(s) of this transient were responsible for the difference in the recovery rate during stimulation of the signal transduction pathway, we measured the recovery rates of calcium transients produced by stimulation with high K. Due to the apparent lack of VGCC in the cilia, this should eliminate the contribution of the cilia to the calcium transient (Leinders-Zufall et al., 1997; Bradley et al., 2001). Calcium transients in response to an 8 sec IBMX/FSK stimulus or to a 3 sec high K solution are shown in **Figure 4A.** The calcium clearance rates are shown in **Figure 4B.** In OSNs from the odor-enriched and standard odor-environments the clearance rates were similar (M=0.07, SD=0.03 and M=0.07, SD=0.04 respectively; unpaired *t*-test; p=0.62). This suggests that the differences seen in calcium recovery after activation of the odor signal transduction pathway might be due to events occurring in the cilia rather than the knob.

Progressive slowing of the calcium recovery rate corresponds to increased amount of time in an odor-enriched environment.

If odor exposure changes the properties of the cilia through calcium regulation or signaling, then these effects may require changes in protein regulation or expression. In order to determine if odors alter the calcium response through a long-term effect of odors in the environment we moved 6 mice born in the standard environment to the odor-enriched condition and then sacrificed at either 8-10 days, or 6 weeks (**Figure 5**). The mice housed for 6-8 days were slightly, but not significantly, slower than the OSNs from mice in the standard environment (Mean=0.09, SD=0.03; Mean=0.1, SD=0.04 respectively). OSNs from mice housed for 6 weeks in the odor-enriched environment had slower calcium recovery rates more closely resembling the OSNs from mice born in the odor-enriched condition. These results suggest that odor-environments may alter calcium signaling in OSNs through a long-term change.

$OMP^{-/-}$ OSNs produce slower calcium transients in response to increased depolarization with high K solution

Since $OMP^{-/-}$ mice have altered responses to odors (Buiakova et al., 1996; Reisert et al., 2007) we tested if the calcium clearance rates from $OMP^{-/-}$ OSNs were effected by odor environment. First we stimulated the cells with high K and measured the calcium recovery rates of cells from both odor-environments. Consistent with the findings of Kwon et al., (2009), we found that calcium transients

in OMP$^{-/-}$ OSNs depolarized with high K had slower recover rates than their wild type counterparts in both odor-environments (two-way ANOVA followed by unpaired *t*-tests; significant effect of mouse type, $p<0.05$, but not odor-environment or interaction); results are shown grouped by mouse strain (**Figure 6A)**.

This slower calcium recovery in OMP$^{-/-}$ OSNs could be due to reduced NCX activity (Kwon et al., 2009). To measure overall activity of NCX in cells from C57 odor-enriched and OMP$^{-/-}$ standard OSNs we did an experiment similar to Kwon et al., (2009) where we reversed NCX with a 30 second bath application of a low sodium (24 mM NaCl) solution. This causes the exchanger to run in reverse thereby increasing $[Ca]_i$. We measured the time to recover once the cells were returned to regular Ringers, (**Figure 6B**). The calcium transients did not fit an exponential line within the required 0.95 confidence interval therefore we measured the time (sec) from peak response to recovery. No difference between recovery speeds from NCX reversal was found (unpaired *t*-test $p>0.05$; **Figure 6C**). Although these results do not directly test NCX activity after IBMX/FSK stimulation they provide a general measure for overall NCX activity in the cell. Thus, in our recording conditions, the general activity of NCX does not appear to be significantly different between OMP$^{-/-}$ and C57BL/6 OSNs.

Stimulation of the cAMP pathway produces faster calcium transients in OMP$^{-/-}$ OSNs from mice in standard-odor housing

Next we examined the IBMX/FSK stimulated calcium recovery properties of $OMP^{-/-}$ OSNs from standard and odor-enriched groups. Responses were normalized as described before, shown here in **Figure 7A**. Unlike the responses to high K (**Figure 6A**), when we stimulated the odor-transduction pathway with IBMX/FSK, $OMP^{-/-}$ OSNs were significantly faster than odor-enriched C57 OSNs (unpaired *t*-test, $p<0.001$) (**Figure 7B**). The odor-enriched $OMP^{-/-}$ cells were comparable in recovery time to those of OSNS from C47 mice housed in the standard C57s. $OMP^{-/-}$ OSNs from mice in the standard housing environment were significantly faster than both groups (not shown together but compare to **Figure 1C**; $p<0.05$). Thus, OSNs from $OMP^{-/-}$ animals in a standard environment had significantly faster calcium recovery after stimulation of the odor-mediated cAMP-signaling pathway than any other condition.

The rate of recovery in response to increased $[cAMP]_i$ is not dependent on $[Ca]_i$

Fast calcium recovery after activation of the cAMP pathway in $OMP^{-/-}$ OSNs must be due to either a lack of continued calcium influx or faster calcium removal (Figure 3A). Calcium (usually with CaM) regulates many steps in the signal transduction pathway (review, Menini, 1999), as well as calcium extrusion mechanisms (review, Di Leva et al., 2008; Blaustein and Lederer, 1999). It was therefore necessary to determine if 1) there were differences in the resting calcium concentrations between groups, and 2) if the calcium concentration reached during peak stimulation correlated with the speed of recovery. To test these possibilities

calcium calibration was done (see Methods) and we measured the resting $[Ca]_i$ before IBMX/FSK stimulation, and the peak response in dendritic knobs and somas after IBMX/FSK stimulation. An example of the change in florescence (Ca) during stimulation is cell shown in **Figure 8B,C.** Peak calcium concentrations in the dendritic knobs of $OMP^{-/-}$ OSNs were slightly lower than those of the C57s although the difference was not statistically significant (two-way ANOVA, mouse type p=0.09; odor-environment p=64; interaction p=0.39; **Figure 8C.**. These differences were not due to $[Ca]_i$ resting differences; both the soma and dendritic knob baseline calcium concentrations were not significantly different (ANOVA followed by unpaired t-tests p>0.3; data not shown). Next we looked for a correlation between the peak calcium concentrations after IBMX/FSK stimulation in the knobs against the rate constant. None of the groups, separately or combined showed a significant correlation between the concentration of calcium and the recovery rate (Table 1).

Perforated patch clamp recordings showed a response deficit in standard housed $OMP^{-/-}$ OSNs

The calcium dependent chloride current makes up ~90% of the current response to increased $[cAMP]_i$ (Leinders-Zufall et al. 1997; Boccaccio and Menini, 2007). To test if the faster calcium transients altered the chloride currents we used gramicidin perforated patch clamp recordings from isolated OSNs. This technique has the advantage of maintaining an intact intracellular environment without disrupting the intracellular Cl concentration or signaling molecules such as cAMP

and ATP. All cells were repetitively stimulated at least 3 times which should potentiate the response by recruiting more calcium activated chloride channels (Zhang and Delay, 2006). An example of a cell under these recording conditions is shown in **Figure 9A**. IBMX/FSK stimulation elicited a small inward current at negative potentials and an outward current at more positive potentials. The amplitude of currents elecited by increased $[cAMP]_i$ were highly variable and no trend was seen (data not shown). Both C57BL/6 groups and the odor-enriched $OMP^{-/-}$ OSNs responded ~50% of the time, while only 2/25 cells from the standard $OMP^{-/-}$ OSNs responded (8%) (**Figure 9B**). Thus, when looking at the number of cells that responded to IBMX/FSK it was clear that most of the $OMP^{-/-}$ OSNs from animals in standard housing did not show a response. Note that the duration of the stimulation in perforated patch recordings is much shorter than in the calcium imaging experiments (~2.5 sec/holding step compared with 8 sec stimulation). These results suggest that the calcium activated chloride current is not as readily activated in the standard $OMP^{-/-}$ OSNs as in wild type or odor-enriched conditions.

$OMP^{-/-}$ OSNs were less responsive to AC stimulation

The results from the IBMX/FSK stimulation compared with the high K stimulation suggest that the events responsible for the differences in the calcium recovery rates are occurring in the cilia. However it was unclear if this was due to differences in the signaling cascade response or due to differences in calcium removal. In order to help elucidate the mechanism we altered our stimulation

method so that cells were stimulated with IBMX and FSK separately. Thus far, our signal transduction stimuli have been a combination of FSK and IBMX thereby increasing AC activity while inhibiting PDEs. If the absence of OMP significantly alters the activity of calcium removal mechanisms in the cilia such as the 3 PMCA isoforms present there (Weerarantne et al., 2006) either stimuli would show similar results since both stimuli will increase intracellular calcium. On the other hand, if PDE or AC activity were altered, the two stimuli presented separately should show different response properties.

The properties of IBMX and FSK differ in their activation time. IBMX rapidly increases cAMP by inhibiting PDEs (Leinders-Zufall et al., 1997) whereas the stimulatory effect of FSK on AC is quite slow (Hartzell and Budnitz 1991). Therefore, we used IBMX alone or FSK alone to stimulate the OSNs. In order to get measureable responses we had to increase our stimulus time to 12 sec for IBMX and FSK. Indeed, when we initially measured the time to peak for IBMX and for FSK stimulation in our cells (data not shown), we found that FSK peaked nearly 10 seconds slower than IBMX (M=26.4s, SD = 6.4s; M=17.8s, SD = 5.8s respectively; n=24).

To determine what, if any, the differences between IBMX and FSK stimulation were we measured the amplitude of the responses and the recovery kinetics as done with IBMX/FSK together. An example of an OSN responding to IBMX and to FSK separately is shown in **Figure 10A**. Analysis of IBMX responses showed that the speed of recovery was similar to the results of the IBMX/FSK combination data with both standard groups and both $OMP^{-/-}$ groups being faster than the odor-enriched

C57 OSNs (**Figure 10B**). The standard OMP$^{-/-}$ OSNs were again the most rapid to recover (standard OMP$^{-/-}$: Mean=0.12, SD=0.02; odor-enriched C57: Mean=0.08, SD=0.02, unpaired *t*-test <0.01). While both the odor-enriched OMP$^{-/-}$ and standard C57s were similar in recovery speed, they were not quite significantly different from the C57-enriched OSNs (unpaired *t*-tests p=0.06). The amplitude of IBMX alone was not significantly different between groups (**Figure 10C**). Responses to FSK alone showed very different results. Analysis of the recovery speeds for FSK showed no significant difference between the recovery rates of any of the four groups (**Figure 10D**). However, the amplitude of the FSK response was significantly reduced in the OMP$^{-/-}$ OSNs (Two-way ANOVA, strain: p=0.02; **Figure 10E**). These results suggest that OMP$^{-/-}$ OSNs in either odor condition have less capacity for more cAMP production. The amplitude of the standard C57s was slightly higher than the C57 enriched OSNs, but was not significant (unpaired *t*-test, p=0.08).

In addition to the differences in recovery speed and amplitude of the responses, cells that responded to IBMX did not always response to FSK and vise-versa (**Figure 11A,B**). Responsiveness between C57 and OMP$^{-/-}$ OSNs were noticeably different (**Figure 11C**). OMP$^{-/-}$ OSNs were 2-3 times less likely to respond in general, and responses to FSK alone only occurred in ~4% of cells (1/21) for the standard OMP$^{-/-}$ OSNs and only ~6% (1/16) cells for the odor-enriched OMP$^{-/-}$ OSNs compared with ~21% and ~26% of standard C57s and odor-enriched C57s, respectively. These data in combination with the low amplitude responses from FSK

alone in the OMP$^{-/-}$ OSNs lead us to conclude that ACIII activity is limited in OMP$^{-/-}$ OSNs.

Discussion

The calcium transient

The calcium transients measured in OSNs are the net result of calcium influx and calcium clearance from the cilia and the knob of OSNs. The duration of the calcium influx and the speed of the calcium clearance combine to produce the recovery rate we used to measure the recovery speed after stimulation (**Figure 3**). When odor molecules bind to ORs on the cilia, or when $[cAMP]_i$ is increased by IBMX or FSK as was done in these studies, the CNG channel opens and sodium and calcium influx (review, Schild and Restrepo 1998). This influx is terminated by Ca/CaM feedback acting on the subunits CNGB1b that close the channel, and CNGA4 which desensitizes the channel to additional cAMP (Bradley et al., 2004; Song et al., 2008; Munger et al., 2001; Waldeck et al., 2009). The calcium influx opens the calcium dependent chloride channels that are expressed ~8X higher then the CNG channels and act as low noise (conductance = ~0.5-1.5pS) amplifiers to depolarize the membrane (Reisert et al., 2003; Kleene and Gesteland, 1991). Calcium dependent chloride channels account for about 90% of the odor-mediated current in OSNs (Boccaccio and Menini 2007). This depolarization activates voltage-gated channels, of which the voltage gated calcium channels (VGCCs) are likely the largest component of the calcium response seen in our calcium imaging experiments. Both low voltage activated (LVA) and high voltage activated (HVA) currents are present in OSNs (review Schild and Restrepo 1998; Gautam et al., 2007; Wetzel et al., 2001; Trombley and Westbrook, 1991). However there is no evidence that VGCC are

present in the cilia (Leinders-Zufall et al., 1998; Bradley et al., 2001; Gautam et al., 2007).

Calcium levels in OSNs are regulated by the degree of activity of multiple calcium clearance mechanisms. These calcium extrusion mechanisms that contribute to calcium removal include all four plasma membrane ATPases (PMCAs), SR/ER Ca ATPase (SERCA), and the Na/Ca exchanger (NCX) (Weeraratne et al., 2006; Castillo et al., 2007; Saidu et al., 2009; Noe et al., 1996). Calcium/calmodulin (Ca/CaM) increases the affinity of PMCAs for calcium from a Kd of ~10μM to ~0.2μM although this can go even lower depending on the splice variant and the lipid composition of the membrane (reviews Di Leva et al., 2008; Blaustein et al., 2002; Strehler et al., 2007). NCX has a much greater turnover of calcium than PMCAs, however it is dependent on the driving force of the Na/Ca gradient so the direction, and the amount of transport can change with a shift in the concentration of calcium or sodium (review, Blaustein and Lederer 1999). In general, PMCA regulates lower levels of calcium while NCX handles larger loads of calcium more quickly (review, Noble and Herchuelz, 2007). SERCA has been best characterized in skeletal and cardiac muscle however like the PMCAs and NCX affinity and transport depends, among other things, on the concentration of calcium and the isoform expressed (reviews, Inesi et al., 2007; Periasamy and Kalyanasundaram 2008). However, smooth ER is not present in the cilia and can only be present in the knob. To remove the calcium increase that occurs with odor stimulation, some or all of

these clearance mechanisms may be activated depending on the location and intracellular calcium concentration reached.

Odor environment

The role of chronic odor exposure in odor detection and signaling is not well understood. In the bulb, odor exposure is known to have a profound effect, generally enhancing cell proliferation and survival in both the granule and glomerular layers (Rochefort and Lledo, 2005; Woo et al., 2006). In the olfactory epithelium, naris occlusion has been commonly used to study odor-deprivation, however it is an extreme form of deprivation. Here we show for the first time that moderate differences in odor exposure are sufficient to produce differences in calcium transients in OSNs when stimulating the canonical odor transduction pathway.

The results of our odor-exposure experiments indicate that daily odor exposure lengthens the duration of calcium transients elicited in response to activating the cAMP pathway (**Figure 1**). Odor-exposure had the same effect on OSNs from wild type (C57BL/6) and $OMP^{-/-}$ mice (**Figure 7**). Since inhibiting PKA has been shown to increase the calcium response to odors in rat OSNs (Gomez et al., 2000) we tested if PKA activity was altered between odor environments. Inhibiting PKA with H-89 did not significantly change the calcium transient recovery rate in the majority of cells from either of the C57BL/6 odor groups (**Figure 2**) or in $OMP^{-/-}$ OSNs from the standard condition (n=12; data not shown). These data seem to suggest that the basal level of PKA activity was not greatly altered between odor-conditions, although without direct activity measurements this is not certain.

In order to determine if the primary change in the calcium transients was due to calcium regulation in the knob or in the cilia we tested the recovery rate with high K depolarization to activate voltage gated calcium channels to raise $[Ca]_i$ in the knob without increasing it in the cilia (Leinders-Zufall et al., 1998; Bradley et al., 2001; Gautam et al., 2007). In both wild type and $OMP^{-/-}$ OSNs depolarization with high K had similar calcium recovery rates between odor-environments, suggesting that the change in calcium handling was occurring in the cilia when IBMX/FSK was used.

Most of the mice used in our experiments were born in their respective odor environments. To determine if the cAMP mediated calcium transient duration was due to differences occurring during development or occurred with brief odor exposure, we transferred mice from the standard housing condition to the odor-enriched condition. Although the numbers are low, the data indicate that greater time in odor-enrichment progressively slowed the average calcium recovery rates in response to cAMP stimulation (**Figure 5**). We were unable to determine if this effect went in both directions due to animal housing restrictions, however that too needs to be addressed. Thus the effect of odor-enrichment on OSNs appears to take time to develop.

As illustrated in **Figure 3** the calcium transient is made up of multiple components. Eliminating contributions from the VGCC and short-term effects of PKA activity we are left with the possibility that a change in regulation is occurring on the signal transduction pathway itself or on the calcium extrusion mechanisms

present in the cilia. SERCA can be eliminated from this since the endoplasmic reticulum does not extend into the cilia (McClintock and Sammeta 2003). Possible points of regulation include PMCA isoforms 1, 2, and 4 and an unspecified NCX all of which have been detected in the cilia of mouse and rat OSNs respectively (Weeraratne et al., 2006; Noe et al., 1996). PDE and ACIII expression has been shown to be up regulated in the naris-occluded side of mouse OEs (Coppola et al., 2006) offering another possible point of regulation.

What is clear from our odor-enrichment experiments is the importance of housing conditions on research animals. The odor-enriched condition used here more closely resembles what, several years ago, was our standard housing condition. Many institutions have shifted to the HEPA-filtered cages with high throughput air systems. Mice (and humans) in the wild are exposed to a barrage of odors on an hour-to-hour basis. The condition we refer to as "odor-enriched" compared to our now standard condition is odor limited if contrasted to mice in the wild. Even so, the modest change in the amount of odor exposure mice received in our experiments was sufficient to produce significant changes in calcium handling.

The differences between the recovery rates seen in the calcium transients between all of our experimental conditions (mouse type and odor environment) could be differences in points of calcium influx, calcium removal, or multiple points within both categories. The resting calcium concentrations of the cells we recorded from were all below 100nM (data not shown). This is the same range reported by others who have shown the resting calcium in the cilia and knobs to be around

40nM (Leinders-Zufall et al., 1997; Leinders-Zufall et al., 1998; Restrepo et al., 1993; Saidu et al., 2009). To determine if the peak calcium concentration reached after IBMX/FSK stimulation correlated with the speed of the calcium transient recovery we looked for correlation between the rate constant and the peak calcium concentration (**Table 1**). No significant correlation occurred, and there was no difference between the calcium concentrations at resting levels (not shown) or at peak levels (**Figure 8**) between any of the groups (although the average concentrations for $OMP^{-/-}$ were slightly lower). It was not possible under these conditions to measure the calcium concentration within the cilia of OSNs. Calcium dependent chloride channels have a $K_{1/2}$ for calcium of 1-5μM, however the peak calcium concentrations measured in the cilia are ~300nM. This discrepancy is accounted for by the surface to volume difference in cilia and the probability of microdomains that reach much higher calcium concentrations (Reisert et al., 2003; Kleen and Gestelad, 1991; Leinders-Zufall et al., 1998). In our own recordings, we cannot eliminate the possibility that differences in peak calcium concentration are occurring in the cilia that are altering the termination and clearance of the cAMP response. Indeed, an appealing possibility is that the regulation of calcium clearance is being changed with prolonged odor-exposure thus altering the duration of the calcium transient. Future work blocking PMCA, NCX, and SERCA preferably on cells with and without cilia would help elucidate the mechanism(s) at work.

$OMP^{-/-}$ signal transduction

In addition to the studying the effect of odor-enrichment on wild type OSNs, we also examined mice lacking the hallmark of mature olfactory cells, OMP. We found that $OMP^{-/-}$ OSNs cleared calcium more slowly in the knobs than their wild type counterparts when stimulated with high K (**Figure 6**). However, $OMP^{-/-}$ OSNs had faster calcium transients than OSNs from C57BL/6 mice when IBMX/FSK was used (**Figure 7**). The reason for the faster recovery with increased $[cAMP]_i$ appears to be the reduced response to the AC activator, FSK. Unlike C57BL/6 OSNs, $OMP^{-/-}$ cells had smaller responses to FSK alone (**Figure 10**) and were less likely to respond (**Figure 11**) when FSK was used to increase AC activity. The smaller responses and response rate to FSK in the $OMP^{-/-}$ is likely responsible for the slightly (but not significantly) lower average peak responses seen when IBMX/FSK was used together (**Figure 8**). The properties of IBMX and FSK suggest that, when used together, IBMX produces an initial fast response and FSK produces a secondary slower response (illustrated in **Figure 12**). This is clear from the differences we saw in time to peak when used separately (**Figure 10,11**), and from the known slow properties of FSK (Laurenza et al., 1987; Hartzell and Budnitz, 1991) compared with the fast responses seen with IBMX (Reisert et al., 2007). Additionally, the calcium transients from $OMP^{-/-}$ OSNs from the standard odor condition were faster than the responses from the odor-enriched $OMP^{-/-}$ OSNs as was seen in the C57BL/6 mice suggesting a similar effect of odor-enrichment on $OMP^{-/-}$ OSNs. It is likely that the faster recovery seen in $OMP^{-/-}$ OSN population was due to fewer cells responding to FSK, or having a smaller contribution of FSK to the calcium transient (**Figure 12B**).

Recently, Kwon et al., (2009) reported that $OMP^{-/-}$ mice showed slower calcium clearance that was attributed to reduced NCX activity. Furthermore the authors proposed a role for OMP in odor signal transduction and calcium signaling through the interaction of OMP and Bex, which may directly bind to CaM (Buiakova et al., 1994; Behrens et al., 2002; Koo et al., 2007; Kwon et al., 2009). Unlike Kwon et al., (2009) reversing NCX with a low-Na solution and measuring the time to recovery was not different between $OMP^{+/+}$ and $OMP^{-/-}$ OSNs in our study (**Figure 6B**). This conclusion assumes that the activity of NCX reversed is similar to that in to "forward" (calcium extruding) direction, but that is not certain. At this time it is premature to disagree with Kwon et al., (2009) and claim OMP does not act on NCX. Rather, it seems likely that if OMP is acting indirectly on CaM, as appears likely, then the primary effect seen in $OMP^{-/-}$ OSNs will vary with the experimental conditions. Indeed, our work supports that of Kwon et al., (2009) when we bypassed the signal transduction pathway and increased $[Ca]_i$ by high K stimulation. In that case the $OMP^{-/-}$ OSN dendritic knobs showed significantly prolonged recovery (**Figure 6**) unlike the IBMX/FSK stimulation.

An important distinction between the methods we used to study $OMP^{-/-}$ OSNs compared to others work, is the longer stimulus application time. Previous work with $OMP^{-/-}$ mice has consistently used short (2 sec or less) stimulation and these studies have found that $OMP^{-/-}$ mice have slower electroolfactogram (field potential) responses and isolated cell current or calcium responses to odor stimulation (Buiakova et al., 1996; Ivic et al., 2000; Reisert et al., 2007; Kwon et al., 2009). In our

work, the stimulus time was >6 seconds for the calcium imaging studies. This suggests to us that prolonged stimulation is required to detect the lack of AC stimulation. Indeed, in the studies just mentioned, IBMX and odorants were used but FSK or forskolin was not. Further the work done by Reisert et al., (2007) found that a 2 sec IBMX stimulation had a higher response rate in $OMP^{-/-}$ OSNs that wild types (45% versus 24%). However, they reported that there was no difference in AC mRNA levels. Our $OMP^{-/-}$ OSNs responded at roughly equivalent rates to IBMX stimulation but responded less to FSK stimulation (**Figure 7,8, 11**). Since both IBMX and FSK increase $[cAMP]_i$ the number of OSNs that respond to each stimulus should be the same. Given that the $OMP^{-/-}$ OSNs have smaller and less frequent responses to FSK, but not to IBMX, one possibility is that the responses seen to IBMX in $OMP^{-/-}$ OSNs is primarily due to increased cGMP rather than cAMP. IBMX inhibits all PDEs, and the ciliary PDE1C is has a high affinity for both cAMP and cGMP (reviews, Soderling and Beavo, 2000; Goraya and Cooper, 2004; Omori and Kotera, 2007). OSNs produce a slow increase in cGMP in response to odors, and the rise of cGMP contributes to odor adaptation (Kroner et al., 1996; Zufall and Leinders-Zufall 1997). Olfactory CNG channels have a higher affinity for cGMP than cAMP (Nakamura and Gold, 1987; Crary et al., 2000). Thus inhibition of PDEs with IBMX increases both cAMP and cGMP whereas FSK increases cAMP alone. Therefore it is possible that $OMP^{-/-}$ OSNs produce calcium transients as CNG channels are opened when IBMX inhibits the break down of the basal levels of cAMP and cGMP, but lack

the additional cAMP increase from AC stimulation that comes more slowly due to the properties of FSK.

OMP and odor environment

Work with OMP$^{-/-}$ mice and odor-exposure has not been done before to our knowledge, yet OMP-null mice clearly handle odors in a different way than wild type mice. OMP-null mice have been shown to have altered odor response properties across the olfactory epithelium and have odor perceptual differences in behavioral studies (Youngentob et al. 2001; 2003). OMP has also been shown to be up regulated in response to odor-deprivation (Waguespack et al., 2005). These studies led us to ask if OMP$^{-/-}$ OSNs could be more sensitive to odor environment. However, we did not find an indication of a strong interaction between odor environment and OMP. Although the OMP$^{-/-}$ OSNs from the standard housing condition have much faster calcium recovery rates then any of the other groups (**Figure 7**) both the OMP$^{-/-}$ and C57BL/6 mice showed increased calcium transient recovery rates when the mice were housed in an odor-enriched environment (**Figure 1, 7**). The OMP$^{-/-}$ OSNs from the standard housing environment had much lower response rates when perforated patch clamp recordings were used to measure current responses of the entire cell (**Figure 9**). Since the calcium transients were recorded from the knob in the OMP$^{-/-}$ OSNs from the standard housing environment, when electrophysiology recordings were done, the lower response rate may reflect an inability to sufficiently increase $[Ca]_i$ to the levels necessary to see a response in the soma. Thus, the large

difference in effects seen between the OMP$^{-/-}$ OSNs from the standard environment and the odor-enriched C57BL/6 OSNs appears to be due to the cumulative effects. The faster responses of the OMP$^{-/-}$ due to reduced AC stimulation, and the as yet unknown cause of faster calcium recovery that occurs without odor-enrichment (**Figure 1**). None of our experiments indicated a separate effect occurring in either group of the OMP$^{-/-}$ OSNs that could not be explained by the lack of OMP or the odor-condition alone. Thus, our results suggest that odor-environment and the loss of OMP alters calcium recovery rates without having an interacting additional effect.

Conclusions

Collectively, our data demonstrate that 1) odor-exposure alters calcium transients produced when the cAMP-mediated odor transduction pathway is stimulated, and 2) in OMP$^{-/-}$ OSNs AC activation was greatly reduced. This failure to respond to FSK explains the faster transients seen in OMP$^{-/-}$ OSNs. Although the mechanism for how odor-enrichment alters the calcium response remains unclear, there is no evidence to suggest that odor-enrichment and OMP have an interacting effect. Rather our data seem to suggest that two independent mechanisms are at work.

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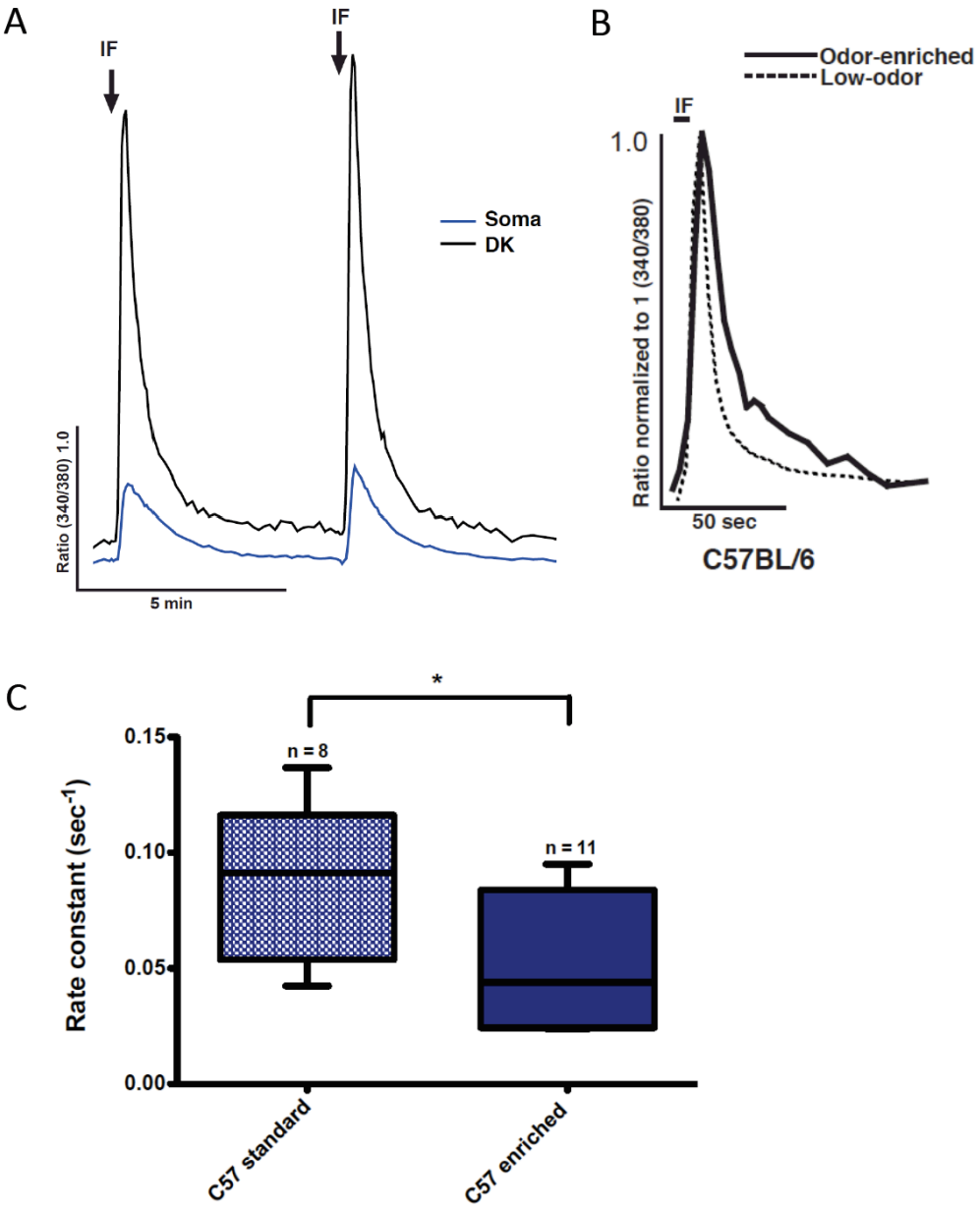
A
IF
IF
Soma
DK
Ratio (340/380) 1.0
5 min
B
Odor-enriched
Low-odor
IF
1.0
Ratio normalized to 1 (340/380)
50 sec
C57BL/6
C
*
n = 8
n = 11
0.15
0.10
0.05
0.00
Rate constant (sec-1)
C57 standard
C57 enriched

Figure 1.

Figure 1. Odor environment alters the recovery rates of calcium transients elicited in response to stimulation of the cAMP pathway. A) Increasing $[cAMP]_i$ with a 6-8 second IBMX/FSK stimulation produces an increase in $[Ca]_i$ of the dendritic knobs (DK) and somas of isolated OSNs in a reproducible manner. B) To analyze the recovery rate $[Ca]_i$ responses were normalized to 1. Odor-enriched (solid line) are slower to recover than the responses of OSNs from the standard housing environment (dashed line) in the dendritic knob. C) Calcium imaging recordings show that IBMX/FSK stimulation results in slower calcium recovery in the dendritic knobs of odor-enriched environment (n=11) than the standard environment (n=8) unpaired t-test, $*p<0.05$. Error bars = min-to-max.

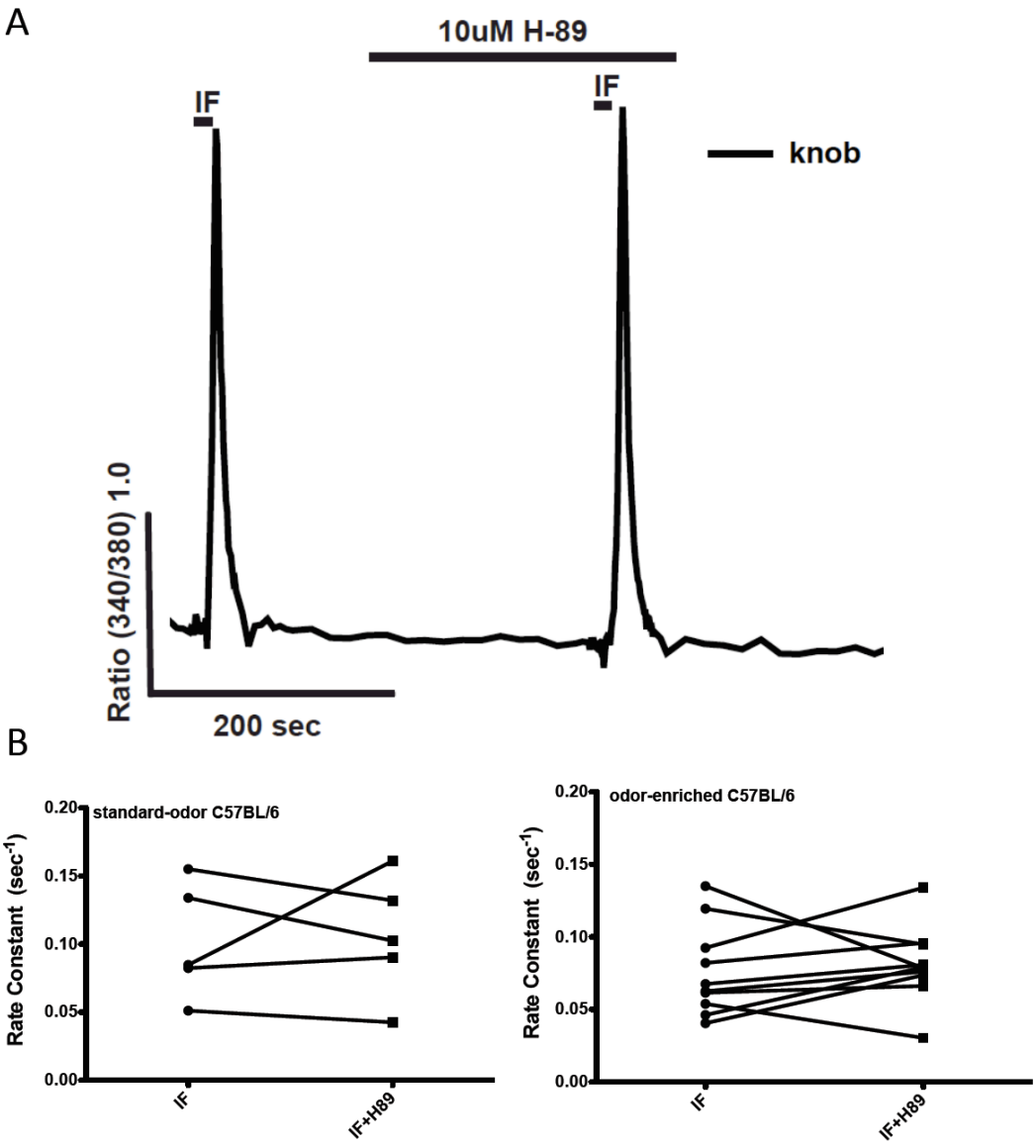
A
10uM H-89
IF
IF
knob
Ratio (340/380) 1.0
200 sec
B
standard-odor C57BL/6
odor-enriched C57BL/6
Rate Constant (sec-1)
0.20
0.15
0.10
0.05
0.00
IF
IF+H89

Figure 2.

Figure 2. Treatment with the PKA inhibitor, H-89, did not systematically alter $[Ca]_i$ recovery rate. A) IBMX/FSK (8 sec application) elicited calcium responses in the dendritc knob before and after inhibition of PKA with H-89. B and C) Calcium recovery 10μM H89 did not significantly alter calcium recovery speed for either standard (n=5) or odor-enriched OSNs (n=9) responses.

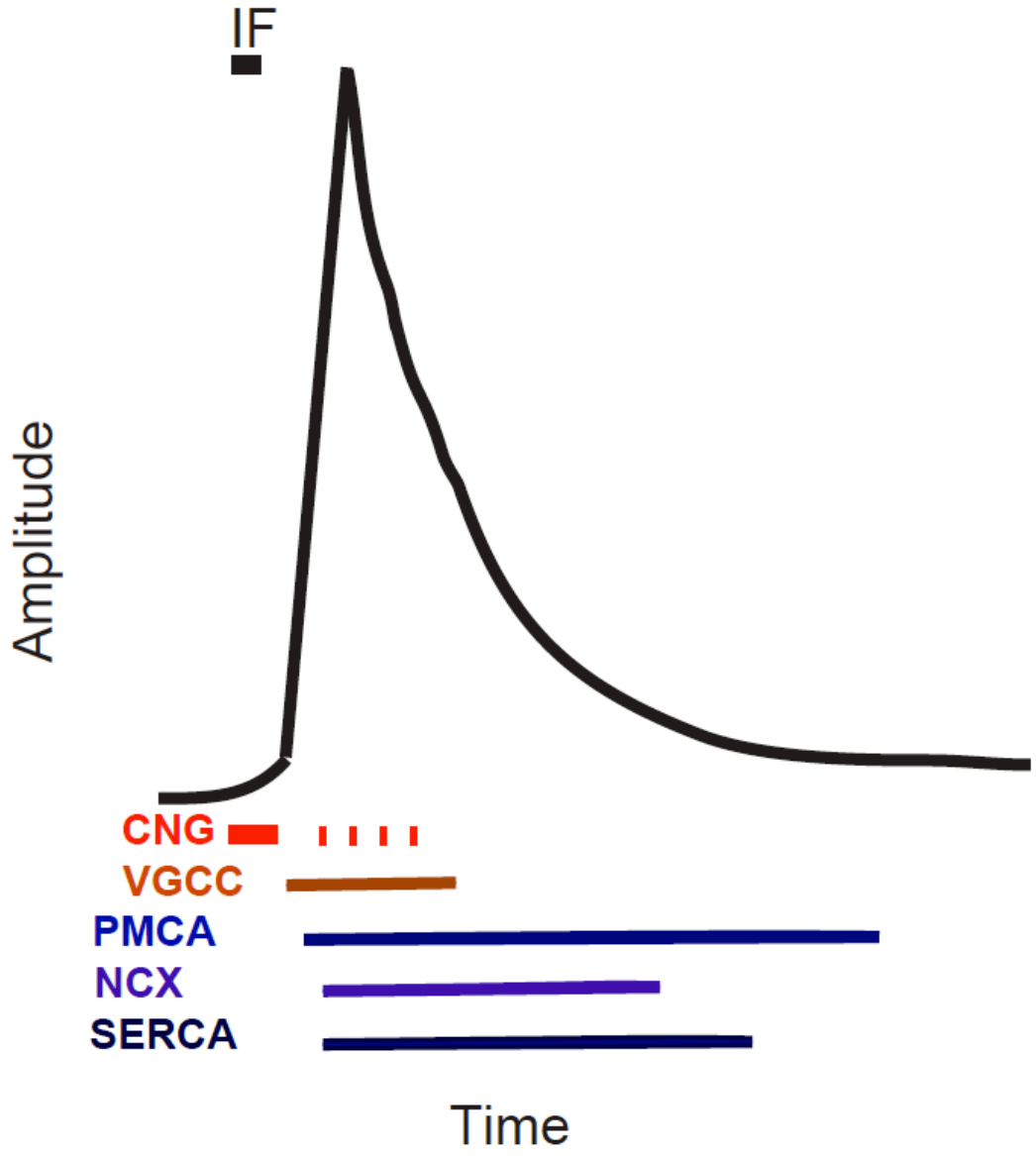

Figure 3.

Figure3. The calcium transient is the net effect of the calcium influx and calcium removal. Activation of ACIII and inhibition of PDEs leads to increased $[cAMP]_i$ and calcium influx through the CNG channels. Calcium dependent chloride channels amplify this signal, thereby depolarizing the cell that leads to the opening of voltage-gated channels including the voltage gated calcium channels (VGCC) that contributes to the calcium transient. Plasma membrane ATPases (PMCA), sodium calcium exchanger (NCX) and sarco/endoplasmic reticulum calcium ATPase (SERCA) remove calcium from the cytoplasm to return calcium to resting levels.

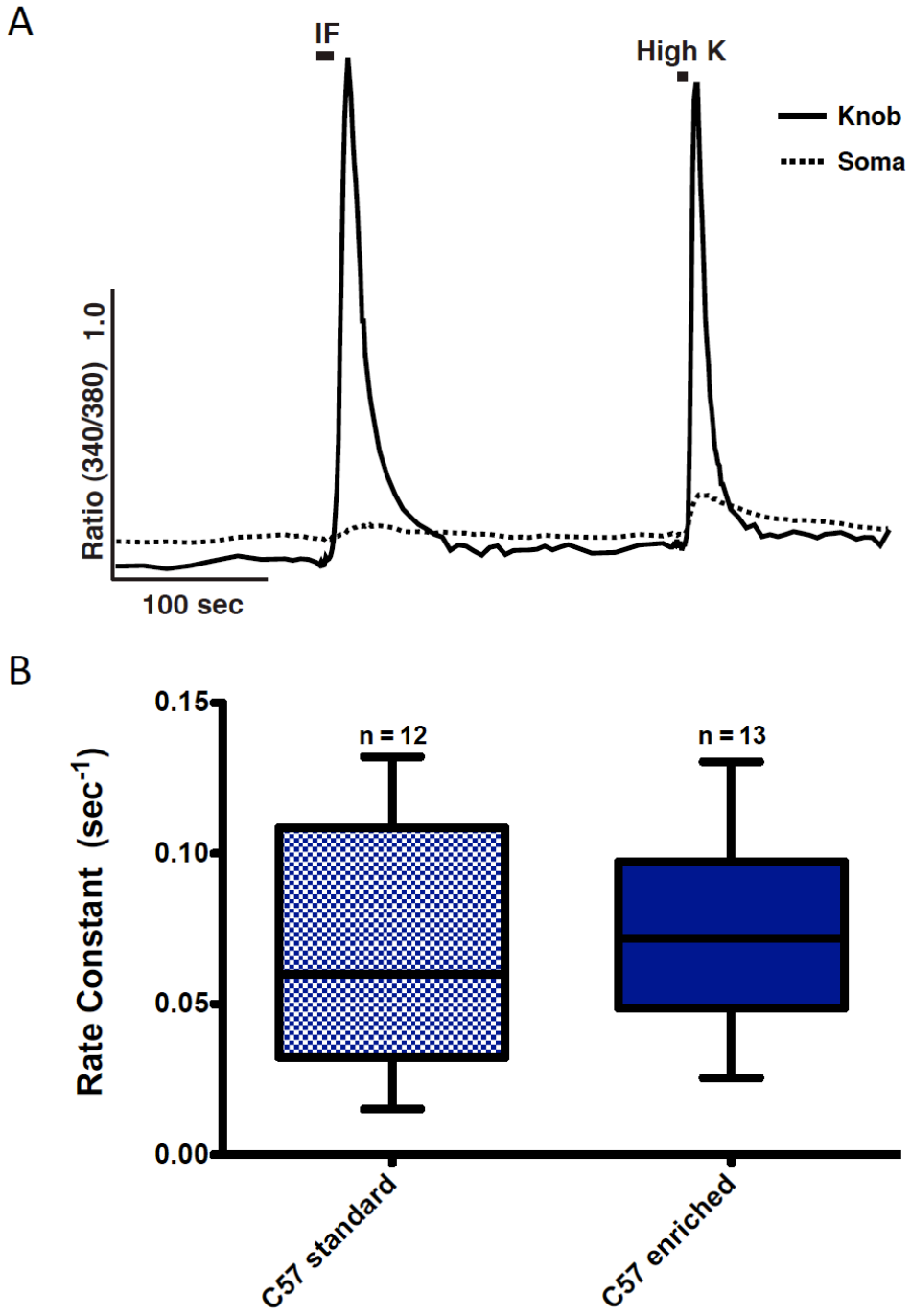
A
IF
High K
Knob
Soma
Ratio (340/380) 1.0
100 sec
B
0.15
0.10
0.05
0.00
Rate Constant (sec-1)
n = 12
n = 13
C57 standard
C57 enriched

Figure 4.

Figure 4. Housing environment does not alter calcium recovery rates with high K stimulation. A) Depolarization with IBMX/FSK (IF) or 65mM KCl Ringers (high K) produces responses in the knob and soma. High K stimulation consistently had larger responses in the soma (dashed line) that IF stimulation. B) Calcium transients with 3 sec application of high K showed little difference between standard odor environment and odor-enriched OSNs (standard: n= 12; enriched: n=13). Error bars = min-to-max.

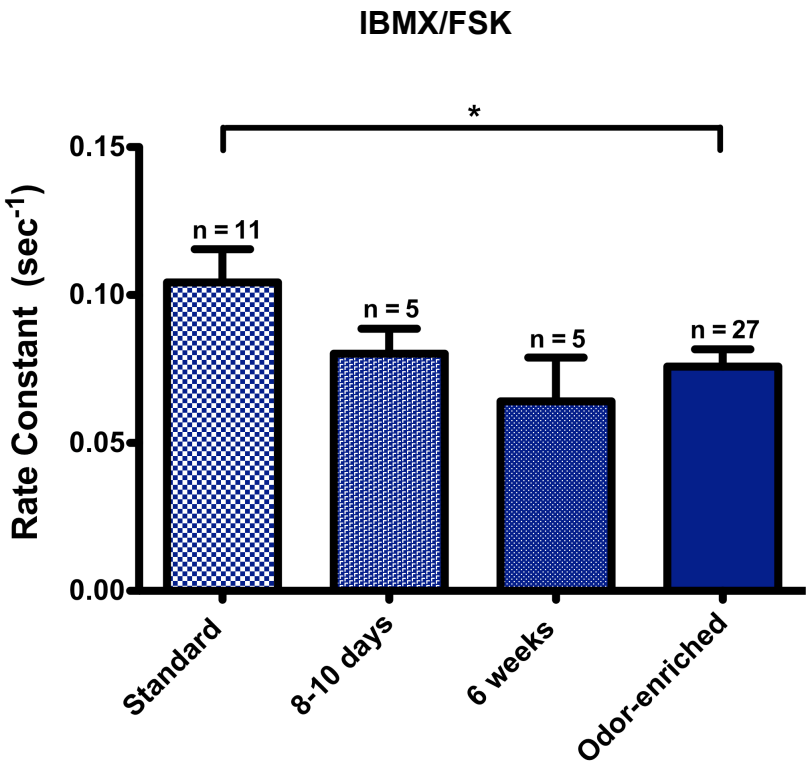

Figure 5.

Figure 5. Calcium recovery rates became slower after weeks of exposure to a more complex odor environment (*p<0.05; error bars = SEM). The mice housed for 6-8 days (Mean=0.09, SD=0.03) in the standard environment (Mean=0.1, SD=0.04). OSNs from mice housed for 6 weeks in the odor-enriched environment had slower calcium recovery rates with IBMX/FSK stimulation (Mean=0.06, SD=0.03) more closely resembled the OSNs from mice born in the odor-enriched condition (Mean=0.08, SD=0.03). The OSNs from six-week odor-enriched mice were not significantly different than the OSNs from the standard condition although they approached significance (unpaired *t*-test, p=0.06).

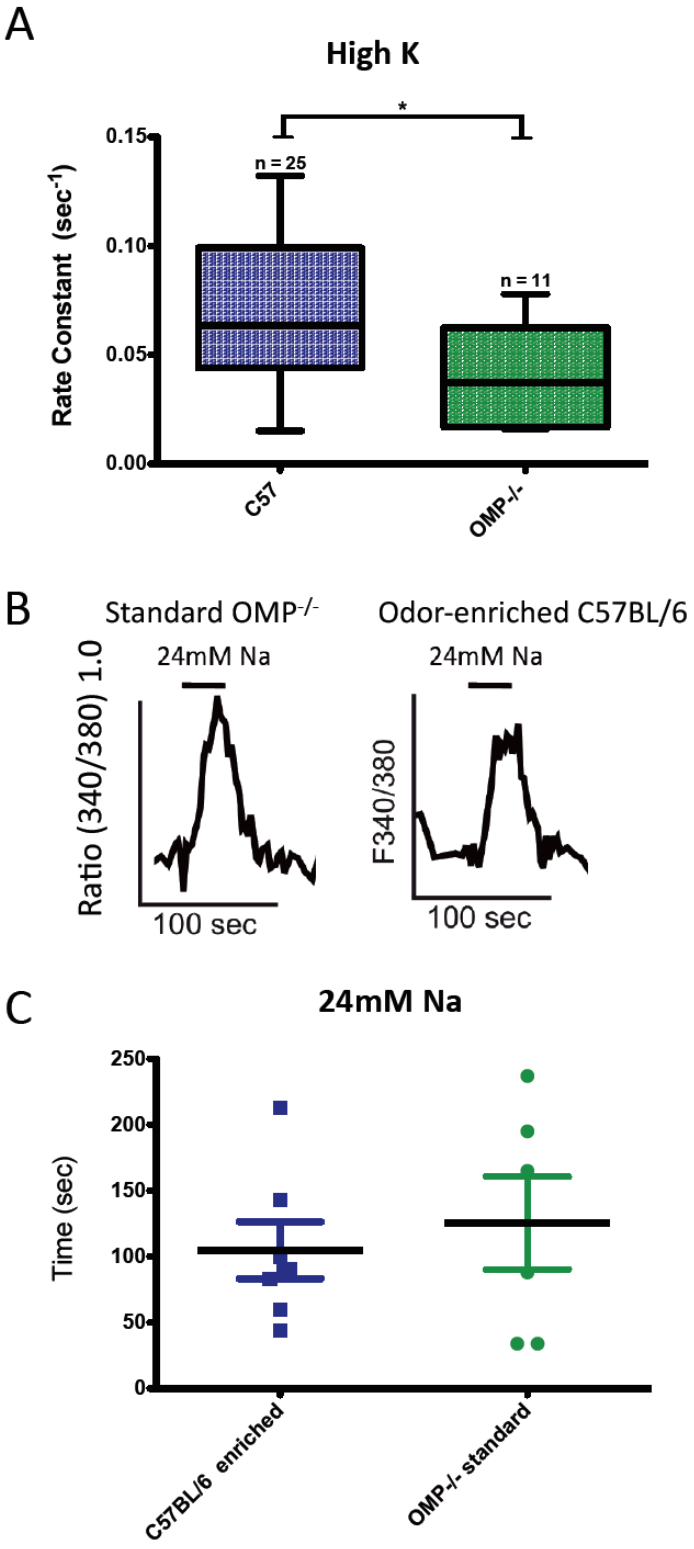
A
High K
*
0.15
0.10
0.05
0.00
n = 25
n = 11
Rate Constant (sec-1)
C57
OMP-/-
B
Standard OMP-/-
Odor-enriched C57BL/6
24mM Na
24mM Na
Ratio (340/380) 1.0
F340/380
100 sec
100 sec
C
24mM Na
250
200
150
100
50
0
Time (sec)
C57BL/6 enriched
OMP-/- standard

Figure 6.

Figure 6. OMP$^{-/-}$ OSNs showed a slower recovery rate when stimulated with high K than wild type (C57BL/6) OSNs. A) Calcium transients after stimulation with high K in C57BL/6 (C57) and OMP$^{-/-}$ OSNs are shown grouped (a mix of odor-enriched and standard), error bars = min to max. Calcium recovery from high K stimulation was much slower in OMP$^{-/-}$ (n=11) OSNs than C57 (n=25)(two-way ANOVA *=p<0.05 for strain). No difference between recovery rate to high K stimuli was found between odor environments (two-way ANOVA, p>0.05), therefore data are shown here grouped by strain (OMP$^{-/-}$ and C57), error bars = min-to-max. B and C) A 30 sec low sodium (24mM NaCl) Ringers solution was used to reverse NCX activity in order to test if general NCX activity was responsible for the calcium transient recovery rate differences between strains. B) Examples of $[Ca]_i$ increases with 24mM NaCl Ringers application from a dendritic knob of an OMP$^{-/-}$ OSNs (left) and a response of a C57 OSN (right). C) No differences were seen between the recovery speed (measured in seconds) between the odor-enriched C57 (n=7) and standard OMP$^{-/-}$ mice (n=6), (unpaired t-test, p>0.05).

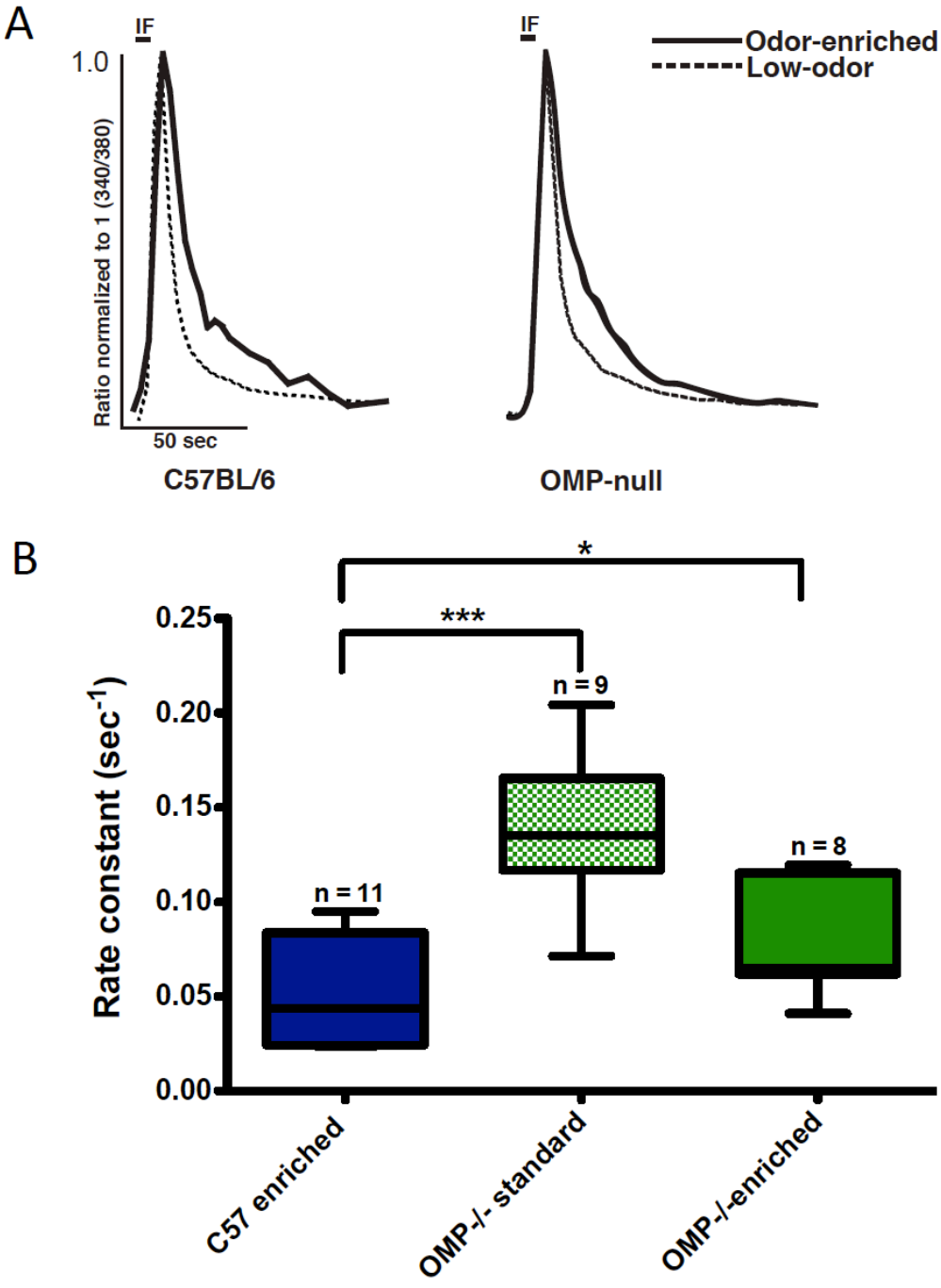
A
IF
1.0
Ratio normalized to 1 (340/380)
50 sec
C57BL/6
IF
OMP-null
Odor-enriched
Low-odor
B
*
***
n = 9
n = 8
n = 11
0.25
0.20
0.15
0.10
0.05
0.00
Rate constant (sec-1)
C57 enriched
OMP-/- standard
OMP-/-enriched

Figure 7.

Figure 7. A comparison of calcium transient recovery rates in $OMP^{-/-}$ and C57 OSNs when the odor-mediated cAMP pathway is stimulated with IBMX/FSK in standard and enriched odor-environments. A) Responses were normalized to = 1 for comparison of curve fit of the recovery rate. Normalized responses for rate constants: Odor enriched (solid line) from C57BL/6 (left) and $OMP^{-/-}$ (right) OSNs were consistently faster to recover than the OSNs from the mice in the standard housing environment (dashed line). B) Calcium recovery in the dendritic knobs of $OMP^{-/-}$ OSNs (standard: n=9, enriched: n=8) was faster than odor-enriched C57 responses (n=11), with the greatest difference between the standard $OMP^{-/-}$ and odor-enriched C57 cells (unpaired *t*-test, $***p<0.001$, $*p<0.05$).

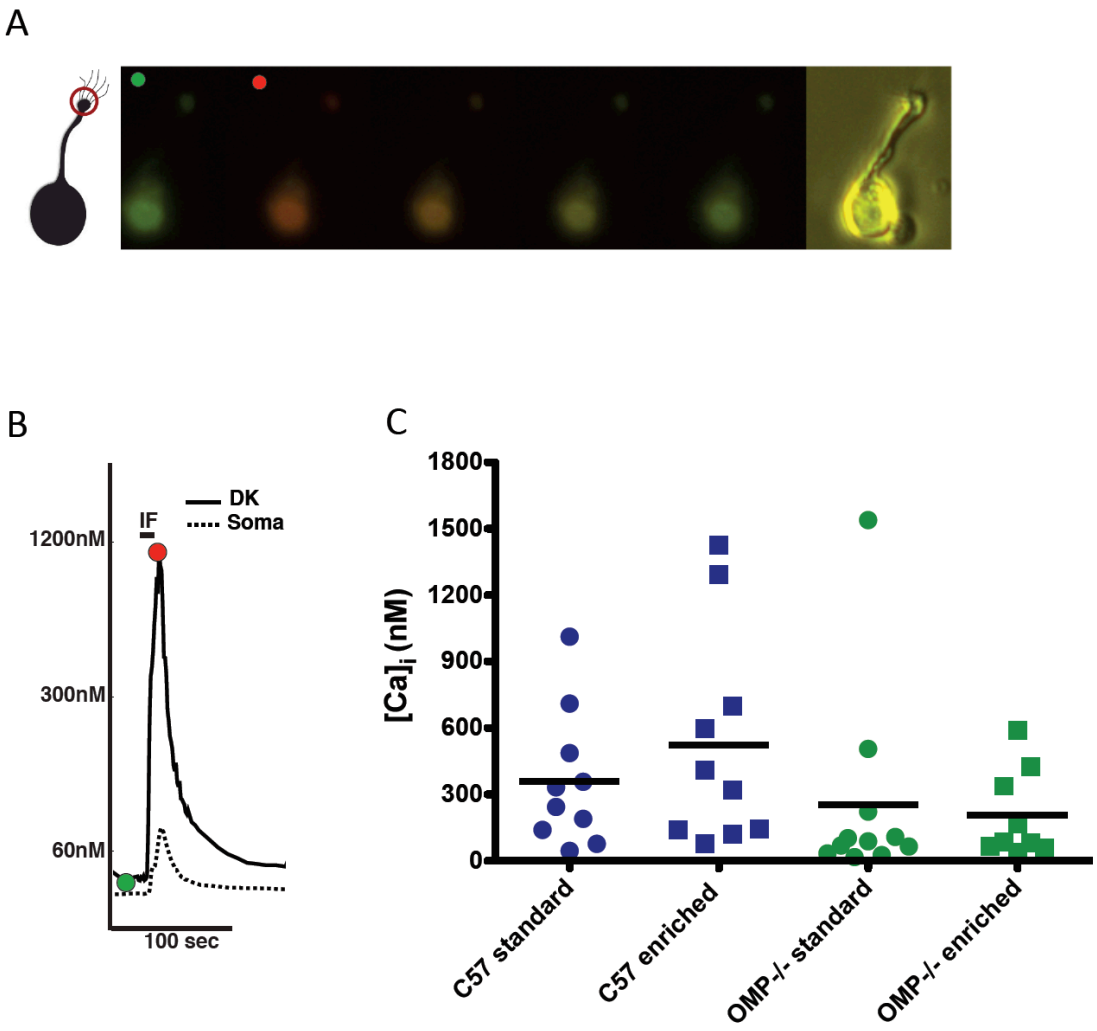
A
B
1200nM
300nM
60nM
IF
DK
Soma
100 sec
C
[Ca]i (nM)
1800
1500
1200
900
600
300
0
C57 standard
C57 enriched
OMP-/- standard
OMP-/- enriched

Figure 8.

Figure 8. Determination of the calcium concentrations in OMP$^{-/-}$ and C57BL/6 OSNs from both odor environments. A) Top: Select five time frames from a cell stimulated with IBMX/FSK for calcium calibration. B) Green and red dots indicate baseline and peak responses respectively from an odor-enriched C57 cell. C) Analysis of peak calcium concentration in the dendritic knobs from all four odor/strain combinations. Two-way ANOVA showed no significant difference between odor-environment, interaction, or strain.

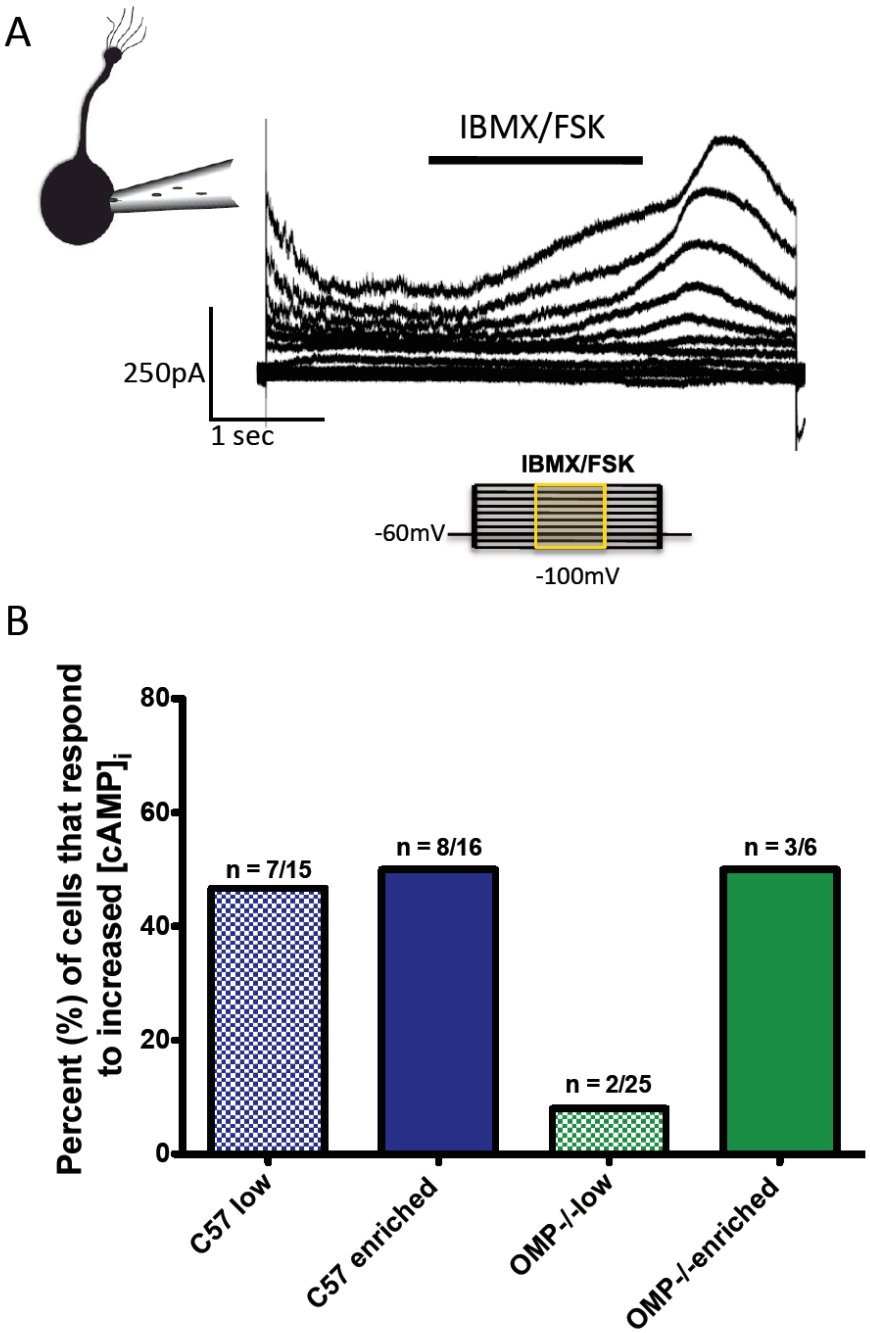
A
IBMX/FSK
250pA
1 sec
IBMX/FSK
-60mV
-100mV
B
Percent (%) of cells that respond to increased [cAMP]i
80
60
40
20
0
n = 7/15
n = 8/16
n = 2/25
n = 3/6
C57 low
C57 enriched
OMP-/-low
OMP-/-enriched

Figure 9.

Figure 9. Gramicidin perforated patch clamp recordings from isolated OSNs (illustration cartoon) showed a decreased response rate from standard $OMP^{-/-}$ OSNs. A) Example of C57 cell responding to IBMX/FSK stimulation (application time = bar). Lower inset shows the protocol used for electrophysiology recordings, cells held at -60mV and stepped from -100mV to 100mV in 20mV increments. B) The number of cells responding to IBMX/FSK (recordings repeated 3 times). Standard $OMP^{-/-}$ OSNs were severely reduced in their response frequency.

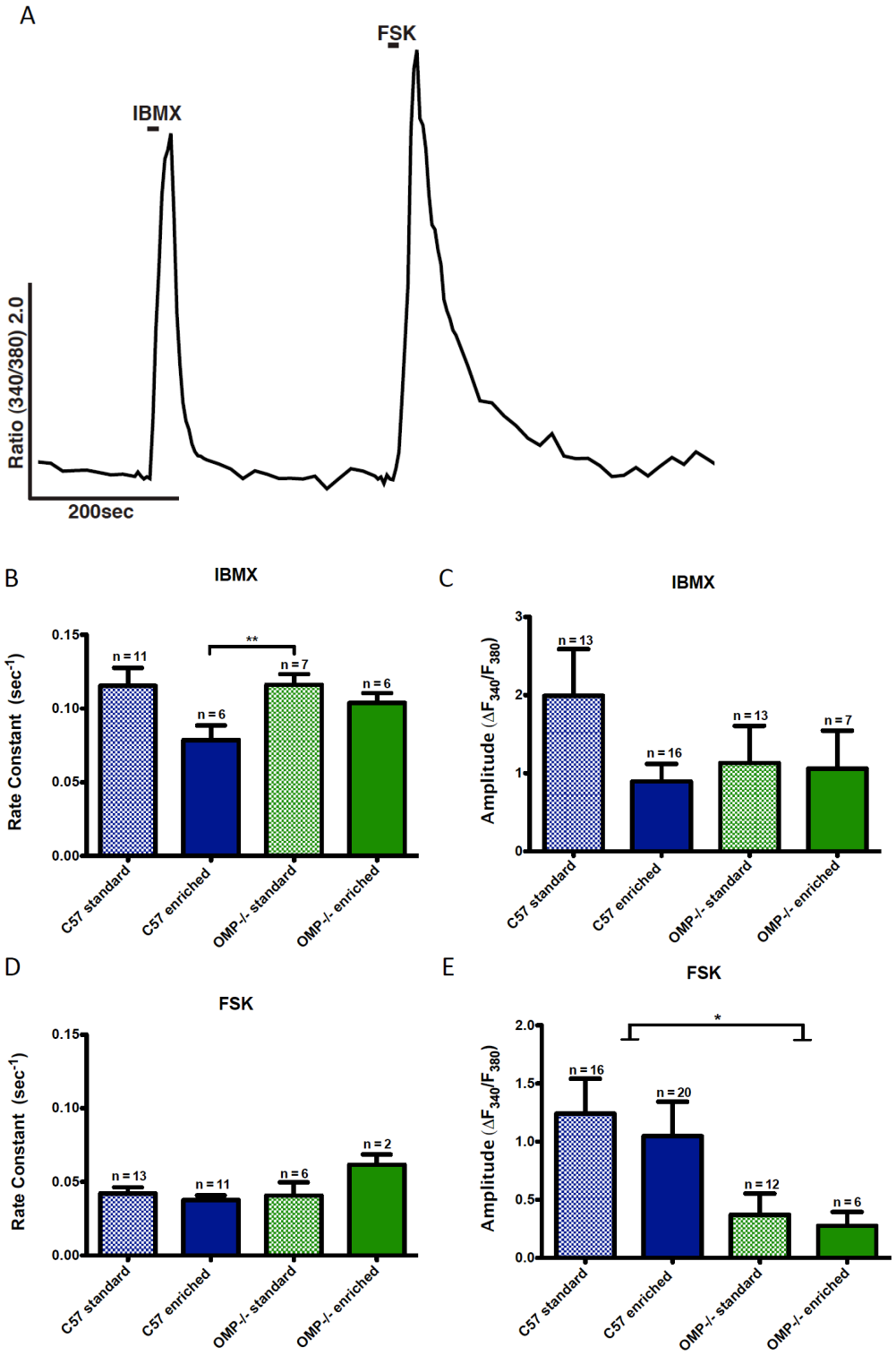
A
FSK
IBMX
Ratio (340/380) 2.0
200sec
B
IBMX
Rate Constant (sec-1)
0.15
0.10
0.05
0.00
n = 11
n = 6
n = 7
n = 6
**
C57 standard
C57 enriched
OMP-/- standard
OMP-/- enriched
C
IBMX
Amplitude (ΔF340/F380)
3
2
1
0
n = 13
n = 16
n = 13
n = 7
C57 standard
C57 enriched
OMP-/- standard
OMP-/- enriched
D
FSK
Rate Constant (sec-1)
0.15
0.10
0.05
0.00
n = 13
n = 11
n = 6
n = 2
C57 standard
C57 enriched
OMP-/- standard
OMP-/- enriched
E
FSK
Amplitude (ΔF340/F380)
2.0
1.5
1.0
0.5
0.0
n = 16
n = 20
n = 12
n = 6
*
C57 standard
C57 enriched
OMP-/- standard
OMP-/- enriched

Figure 10.

Figure 10. IBMX and FSK used separately produced calcium transients with very different recovery rates and amplitudes. A) An example of the response in the knob of a cell stimulated with IBMX and FSK separately showing the characteristic faster offset of IBMX versus the slower FSK response. B and D) Recovery rates for IBMX. B) Responses to IBMX recovered faster in both standard groups than the odor-enriched C57 group (unpaired *t*-test, **=$p < 0.01$). D) Recovery rate to FSK stimulation was significantly slower than IBXM (not shown) but no difference between groups occurred. C and E) Amplitude of the responses (change in florescent intensity from baseline). C) IBMX amplitudes were roughly equivalent between all groups whereas E) FSK amplitudes were significantly lower in OMP-null cells (two-way ANOVA, *=$p < 0.05$). All error bars = SEM.

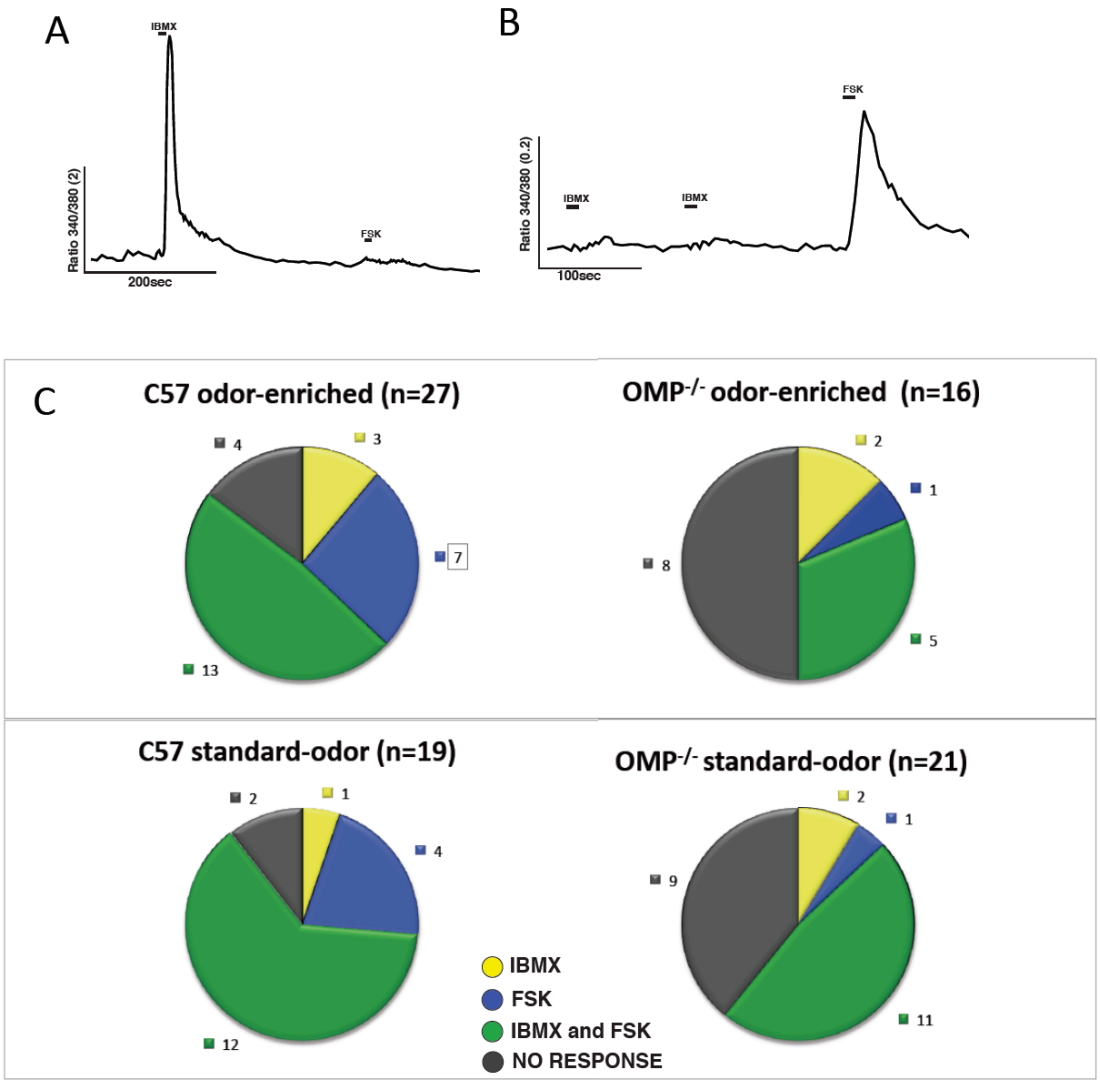
A
IBMX
FSK
Ratio 340/380 (2)
200sec
B
FSK
IBMX
IBMX
Ratio 340/380 (0.2)
100sec
C
C57 odor-enriched (n=27)
4
3
7
13
OMP-/- odor-enriched (n=16)
2
1
8
5
C57 standard-odor (n=19)
2
1
4
12
OMP-/- standard-odor (n=21)
2
1
9
11
IBMX
FSK
IBMX and FSK
NO RESPONSE

Figure 11.

Figure 11. Responses to IBMX and FSK when used separately showed different response rates in C57BL/6 and OMP$^{-/-}$ OSNs from both odor environments. A) An example of a cell responding to IBMX but not FSK. B) An example of a cell responding to FSK but not IBMX. C) The number of responsive OSNs differed across groups with FSK, IBMX, and FSK as well as IBMX (note that IBMX and FSK were never applied at the same time in this experiment, green represents cells that responded to IBMX and FSK individually). Total response rates in C57 and OMP cells respectively. Odor-enriched (top) and standard (bottom) within groups was similar. Differences between response rates for OMP-null were ~20% lower that C57 cells, and responses to FSK alone were less then 6% in OMP-null cells compared with at least 21% of C57 cells.

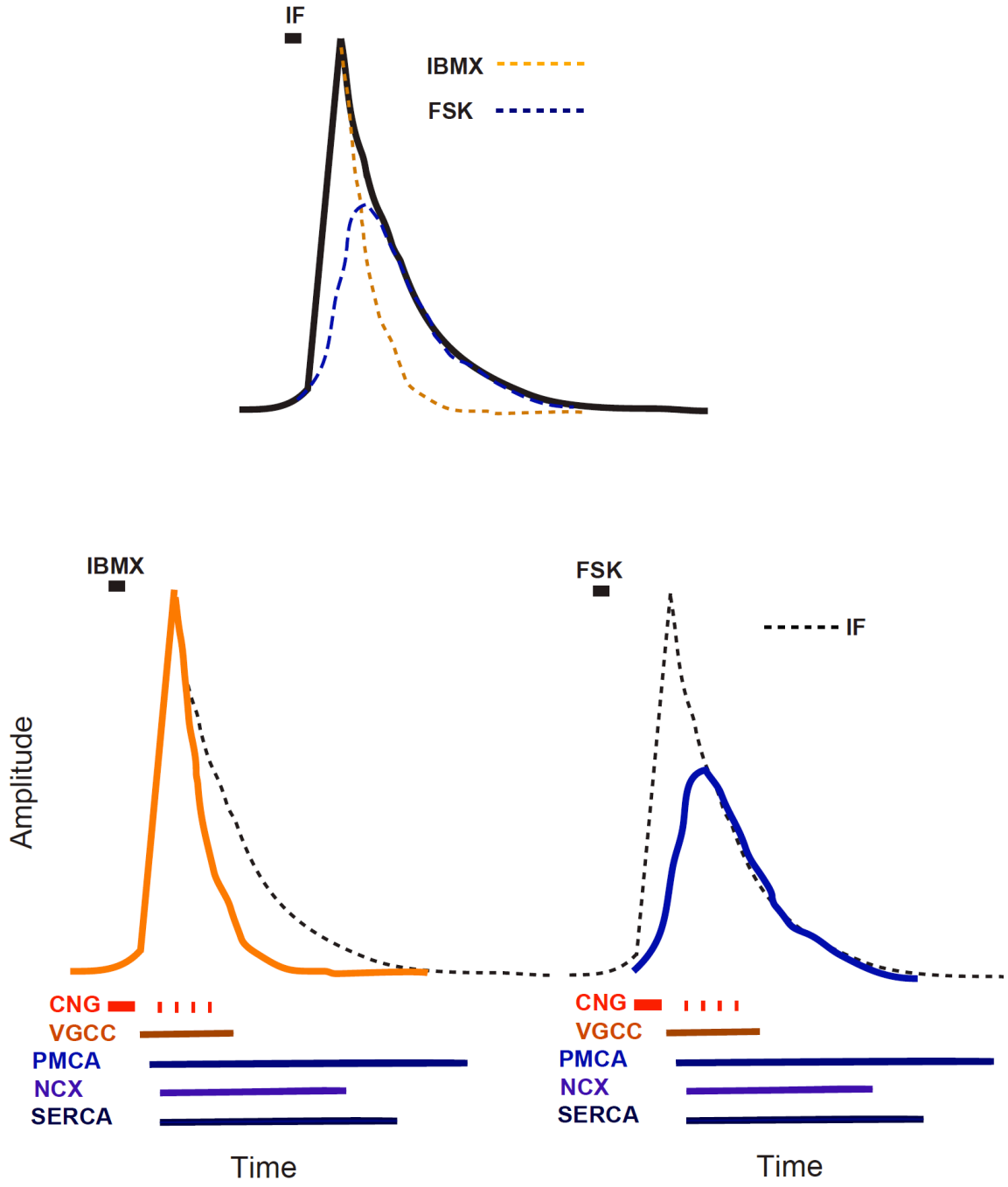
IF
IBMX
FSK
IBMX
FSK
IF
Amplitude
CNG
VGCC
PMCA
NCX
SERCA
Time
CNG
VGCC
PMCA
NCX
SERCA
Time

Figure 12.

Figure 12. The elements shaping a calcium transient in OSNs. Top) The calcium transient is a combination of PDE inhibition with IBMX and AC stimulation with FSK. Left) IBMX is the faster component of the IBMX/FSK response with a fast onset and fast offset, whereas right) FSK activates more slowly and recovers more slowly. These two components make up the calcium transient to IBMX/FSK stimulation. Reduced AC stimulation by FSK would produce faster responses looking more like the IBMX response alone.

Table 1. Correlation of $[Ca]_i$ and rate constant (K) at the peak calcium concentration following an 8 sec IBMX/FSK stimulation. Correlation was done on individual groups and combined by odor condition or by $OMP^{-/-}$ versus wild type strain.

Condition	**r**	**n**	**p *value***
Combined (all groups)	-0.06	28	0.77
Individual groups			
Standard $OMP^{-/-}$	-0.31	7	0.5
Standard C57BL/6	0.68	6	0.14
Odor-enriched $OMP^{-/-}$	0.67	6	0.15
Odor-enriched C57BL/6	-0.15	9	0.7

CHAPTER 3

Olfactory detection and perception in different strains of mice

Abstract

Behavioral testing in order to determine the sensitivity and perceptual qualities of odorants to animals is an important part of understanding the olfactory system. The accuracy of these measurements is somewhat complicated by the extreme sensitivity and breadth of odor perception that mice and rats have compared to their human experimenters. This is illustrated both by the much higher number of functional olfactory receptors present in mice, and from observations that mice seem to readily detect odors at concentrations humans can not.. We used a modified descending method of limits test in a simple signal detection task to determine the detection threshold for C57BL/6 and CD1 mice for the odorant geraniol. Male and female C57BL/6 mice as well as female CD1 mice all showed a detection threshold to geraniol between 0.00005% and 0.0001% geraniol on the Knosys olfactometer. This information is useful for further olfactory studies since geraniol is commonly used in olfactory research and is known to be detected through the cAMP mediated odor detection signaling transduction pathway. Unfortunately because the actual differences in detection ability of mice may be less than the half log concentrations tested here, threshold detection may be impractical for olfactory function assessment in cases where there is not a significant olfactory impairment expected. Due to the extreme sensitivity of mice to odor detection, threshold estimations were deemed useful, but impractical, for detecting subtle differences in olfactory function. Therefore we expanded our studies to find an olfactory test that would be sensitive to differences in odor perceptual or odor detection in cases where only very subtle differences may occur. We looked at strain differences in healthy male adult mice that should not have any impairment in olfactory function. Both of these strains were inbred strains (C57BL/6, Balb/c). An odor-masking test where 2-heptanone was used to mask the presence of amyl acetate was found to be sufficiently sensitive to detect differences between these two strains of mice. We discuss the advantages of this test and future uses.

Introduction

Human and rodent olfactory ability differ in sensitivity and the range of odors that are detectible. Mice have ~1000 specific olfactory receptors while humans only have several hundred (Buck and Axel, 1991; review, Hasin-Brumshtein et al., 2009; Spehr and Munger, 2009). Not surprisingly olfactory sensitivity differs greatly between species (Joshi et al., 2006). Many olfactory tests are used to test olfactory ability; however two common problems encountered in behavioral testing are the lack of studies doing threshold estimations, and secondly, the lack of simple tests that are sensitive enough to detect differences in rodent odor perception.

Having odor threshold estimates for commonly used odors in olfactory research is essential for several reasons. Each receptor may respond to several odorants with different sensitivity. In any stimulus test, perceived intensity (how strong the odor is) can be used as a detection cue rather than the qualities of the substance itself. Increasing the concentration of the odor can cause changes in the perception of that odor (Arctander, 1994; Johnson et al., 2009). This makes it imperative to have estimations of the threshold so that comparisons between odors can be appropriately controlled. From an olfactory processing perspective it suggests that as more olfactory sensory neurons are recruited to respond to the odor, the overall activity pattern of the olfactory epithelium alters the perceptual qualities of the odor. For example an odor perceived at a low concentration as "fruity" may be perceived as similar to "flowery" odors at a high concentration. Thus, in order to make meaningful comparisons among odors it is necessary to have

the minimum concentration for detection so that odors at appropriate, and controlled concentrations can be compared. When comparing two or more odors on the perceptual quality of the odor, knowing the threshold of the two odors allows you to more closely match the perceptual intensity as well. For example, odor A could have a threshold of 0.001% whereas odor B could have a threshold of 0.1%. In order to compare the two odors in terms of their perceptual similarities it is necessary to know what their thresholds are and then use a log scale to pick several concentrations above threshold to test. In this example, being more sensitive to odor A, concentrations of 0.01% and 0.05% could be compared to odor B concentrations at 1% and 5%. Currently many studies use odors at concentrations well over threshold to study odor function and impairment (Bodyak and Slotnick, 1999; Youngentob, 2005; Slotnick, 2007). While important in their own right, this makes comparisons across studies more difficult.

The focus of our study was two fold; first to determine a detection threshold for the odorant geraniol in two commonly used strains of mice, the inbred strain C57BL/6 and the out-bred strain, CD1. Two methods are shown, the first was a standard descending method of limits task done with the emphasis on using it for rapid assessment. The second, longer protocol, to test geraniol provided a reliable means of determining detection threshold. The second purpose of our study was to utilize the olfactometer for detecting differences in odor detection or perception where only very subtle differences may occur. As mentioned already, threshold estimates are needed for research purposes, however as a means of comparing odor

impairment between mouse models, transgenic mice, or mouse strains, they may not be sensitive enough to detect odor perceptual differences. Therefore, we used an odor-masking task that uses one odor at a substantially higher concentration to "mask" the second odor. This method was used to examine differences between two inbred mouse strains, C57BL/6 and Balb/c. A similar method has been used in rats (Sokolic et al., 2007) and provides a valuable means of examining odor perception and detection that will be informative for understanding how individual odors can be detected and identified in the presence of complex mixtures or other strong odors (Sokolic et al., 2007; Goyert et al., 2007; review, Su et al., 2009). This method may also be useful in determining which odors are perceptually similar. The masking test has an advantage over a simple two-odorant discrimination test because very subtle differences in intensity of the odorants could be used as a cue (Bodyak and Slotnick, 1999; Gamble and Smith, 2009). We choose two inbred mouse strains that have been shown to differ in their anxiety levels (Balb/c > C57BL/6), and maternal behavioral differences (Michalikova et al., 2010; Shoji and Kato, 2006). Our results showed that Balb/c mice were significantly poorer that C57BL/6 mice at detecting the odorant amyl acetate in the presence of a strong concentration of 2-heptanone. Additionally we suggest that the masking test is a more sensitive and quicker means of assessing olfactory function than odor threshold detection or discrimination tests. By comparison, threshold detection studies are greatly needed for estimations about odor detection ranges, however the acuity of mice to detect

odors may make these tests too simple for the mice and therefore a less useful measure of olfactory function except in the most extreme cases.

Material and Methods

Subjects

Two inbred strains of mice, C57BL/6 (n=12) and Balb/c mice (n=10), and one out bred strain (CD1, n=4) were used in the behavioral experiments. CD1s were compared to C57BL/6 in geraniol threshold detection, and C57BL/6 and Balb/c were compared in an odor-masking test for amyl acetate detection. All mice were between the ages of 3 and 6 months. Mice were originally obtained from Charles River, Canada, but bred in-house. Mice were backcrossed to their original strain approximately every 3-4 generations. All mice were housed in a temperature and humidity controlled room with a 12hr light/dark schedule and food provided *ad libitum*. The water restriction schedule began 10-14 days before training, and consisted of 1 hr free access to water. Mice were weighed daily throughout the experiments, and were maintained at 80-85% body weight during training and testing. In general mice were able to obtain most of their water during behavioral testing, but were given additional water 20 minutes after the end of a session if they failed to obtain enough water from reinforcements (e.g. poor performance). Mice were run once a day, 7 days a week for 45-50 minutes/session. Geraniol threshold detection studies lasted up to 3 months/group of mice, whereas the odor masking experiments each lasted 7-9 days/group of mice.

Odorants and chemicals

All odors (Amyl acetate, 2-heptanone, geraniol) were obtained from Sigma Aldrich (St. Louis, MO) and were the highest purity available. Mineral oil

(McKensson, San Francisco, CA) was used to dilute geraniol experiments and as the "no odor" condition. Both 2-heptanone and amyl acetate were mixed in distilled water with a long period of sonication (~1hr).

Apparatus

Olfactory tests were conducted on a Knosys LD-8-1 olfactometer (Knosys Ltd., Lutz, FL). This equipment and the general training and operating procedure are described at length in Bodyak and Slotnick (1999). In brief, mice were contained in a small Plexiglas box with a glass chamber at one end that the mouse was trained to stick its head into, and an outward directed fan at the other. The odor-chamber consisted of pressurized air entering the head-space that contained either clean air or an odor into the odor-chamber from the bottom of the chamber. The top of the chamber contained an exhaust tube so that air entering the head space was sucked out from vacuum suction. At either side of the headspace was a photo beam. A trial was initiated when beam was broken by the mouse inserting its head into the chamber. In the glass chamber was a lick spout attached to a syringe filled with water. When the mouse licked the spout an electrical circuit was completed and recorded by the KNOSYS DOS program on a Windows '98 ThinkPad computer. Odors were contained in PET plastic bottles attached to C-flex tubing. The tubing was attached to a vacuum pump and computer-controlled valves controlled airflow. Each session used either 4 or 6 bottles (geraniol and 2-heptanone respectively) where half of the bottles contained the odor (S+) and half contained the no-odor

condition (S-). Each bottle was randomly presented an equal number of times with no one bottle presented more than 3 consecutive trials.

All sessions were run in a dimly lit room, and white noise was on at all times. Additionally, the final valve acts as a masking noise for the odor valves. Tubing and bottles were new for each concentration, and rinsed with EtOH, deionized water, and let dry before use. Air pressure was calibrated daily, and water reinforcement was calibrated every 2-3 days (sessions).

Mouse Training

Training followed the protocol described in Bodyak and Slotnick (1999). Briefly, water restricted mice were initially trained to lick from a waterspout using a training program that taught the mice to lick in order to receive a water reinforcement. Mice were trained to continue licking for 1-1.5 seconds before receiving the reinforcement (which generally took 2-3 sessions). Once trained, the mice began a simple discrimination task between an odor that was paired with water reinforcement (S+) and a no-odor condition that was not reinforced for licking (S-). After initial training an incorrect response to an S- trial resulted in a loud buzzer sounding followed by a 10-15 second time out (time before the next trial could start). Trials were separated by a 5 sec inter-trial-interval. Initiation of a trial was dependent on the mouse putting its head into the odor sampling port, breaking a photo beam. This activated a three-way valve (the final valve, FV) that directed the odor to an exhaust vent for 1.5 seconds. At and end of the 1.5 FV period the air stream (with or without the odor, depending on the trial) a 0.5sec odor

sample period occurred followed by a 2 sec response time. The trial was aborted if the mouse removed its head any time before the first 0.1 sec of the odor sample period (see **Figure 1A**). The 2 sec response period was broken down into 0.2-second intervals. If the mouse licked for 1.4 seconds of the 2 sec response period that counted as the mouse had "licked"; less than 1.4 seconds was counted as "not licked". On S+ trials a "licking" response was a correct response ("hit") and on S- not licking was the correct response ("correct rejection"). Alternatively, the opposite response was a "miss" or "false alarm" respectively (**Figure 1B**).

Geraniol threshold detection with C57BL/6 mice

In order to examine different approaches of measuring olfactory thresholds to the odorant geraniol we used two protocols. The first approach was the "short protocol". The "short protocol" was used to see if a rapid decrease in odor-concentration would accurately measure threshold with comparable results to the long protocol. Mice were initially trained on a high concentration (0.005% geraniol) just as they had been for the long protocol. Then each mouse was given 2-3 blocks (30-45 trials/concentration + 30-45 trials no odor). If the mouse responded at >85% correct for the first two blocks (30 S+ trials), the next concentration was decreased to the next half log unit lower (e.g. 0.0005 to 0.0001%).

The second, "long protocol", consisted of 4-6 sessions per odor-concentration, and the best two sessions were averaged for that concentration. Each session consisted of two concentrations of geraniol. One concentration was held constant (0.005% geraniol) as a "perceptual anchor". While the second

concentration “test concentration” was lowered after 4-6 sessions (4 sessions if mice were above 85% correct responding, 6 sessions if they were not).

Odor-Masking test: 2-Heptanone trials and Amyl Acetate detection in C57BL/6 and Balb/c mice

Mice were trained to discriminate between 0.1% 2-Heptanone (S+) and clean air (water). Following a minimum of 3 consecutive blocks (60 total trials/block) at >85% correct detection mice were tested the following session. On test-day all 6 odor bottles contained 0.1% 2-heptanone (2hep). S- (not-reinforced) also contained 0.001% amyl acetate (AA). Mice were given one session to discriminate between 2hep and 2hep+AA.

Statistical analysis

All analyses were done on Prism Graph Pad (V5). For comparisons between strain or sex mixed ANOVAs [2(sex/strain) x 7(concentration)] were done, or similarly, strain difference by block number (2x number of blocks). Bonferroni post-tests were done for each block/concentration. An unpaired *t*-test was used for mean trial analysis between C57BL/6 and Balb/c in Figure 4A. An alpha level of <0.05 was reported as significant.

Results

Short block tests of threshold detection with a descending method of limits

Threshold detection was tested in two ways. We did a rapid descending method of limits test ("short-protocol") with 2 C57BL/6 and 2 GFP-OMP mice (GFP-OMP mice not shown). As the concentration is decreased the detection should become more difficult, and the correct responses begin to drop. Instead, we saw initial high correct responses in the beginning followed by a drop off during the first few lower concentrations, and then recovery to correct detection again before dropping off as the concentration actually dropped below detection levels. The results for two of the C57BL/6 mice are shown in **Figure 2A**. As can be seen in mouse 2, the mouse was became better at detecting the odor at 1.5 log units lower than the highest (starting) concentration. This suggests a learning effect, as the mouse becomes better at paying attention to the odor cue. An example of this change in attention was visible when the mice would learn to reach their heads toward the air vent (where the odor came in) and sniff.

As exemplified by the variability of the results shown in **Figure 2A** the short protocol was not sensitive enough to provide an accurate measure of threshold. The reasons for this are multifold. First, the rapid drop in concentration caused all mice to initially treat the S+ trial as an S-. Second, motivational state changes from the start of the session until the end of the session as the mouse obtained more water. The first block was discounted for each session so as not to include initial over-motivation error. However, by the end of a session lack of motivation appeared to

affect the responses of the last concentration tested during the session. Third, with only 10-15 trials/odor bottle for each concentration it was not always possible to determine if a mouse was picking up a non-specific cue from one bottle before the protocol changed to test the next concentration. Re-testing all of the mice on concentrations at 0.0001% and above showed that they were easily able to make the detection (data not shown). Thus we concluded that longer sessions with repeated testing was necessary to accurately obtain thresholds.

Threshold for the odorant geraniol is similar between male and female C57BL/6 mice and between female CD1mice and C57Bl/6 mice

To determine a behavioral threshold for the odor geraniol we next tested a longer modified descending method of limits protocol. Geraniol was chosen due to a clear deficit in detection ability in mice lacking an essential component of the cAMP odor signaling cascade, the CNG channel subunit CNGA2 (Clevenger and Restrepo, 2006). We ran 8 C57BL/6 mice (4 male/4 female) and 4 CD1 (female) mice. In order to ensure that mice did not lose motivation at lower concentrations we ran one high concentration (0.005%) along with each descending concentration. Thus, during each session 50% of the trials were with mineral oil (S-; no odor), 25% of the trials with the high concentration of geraniol, and 25% of the trials with each consecutively lower concentration of geraniol. Both geraniol concentrations were S+ trials (water reinforced). Therefore, at low concentrations mice could reliably

receive water reinforcement 25% of the time and therefore were motivated to continue to make the discrimination.

Results for the C57BL/6 threshold detection of geraniol are presented in **Figure 2B**. Mice readily detected geraniol above 0.0005%. Threshold was defined as 65% correct detection (dashed line). For C57BL/6 mice, threshold was between 0.00005% and 0.0001% geraniol. To determine if there was a difference between the sexes we compared male versus female thresholds. Although there was a trend for suggesting female mice may be better able to detect geraniol (threshold at 0.00005% for females compared to 0.00005-0.0001% for males) the differences between the C57BL/6 male and female mice were not significantly different (mixed ANOVA for concentration x sex, $p>0.1$ with Bonferroni posttests) (**Figure 2C**).

We next compared the threshold detection for C57BL/6 and CD1 female mice. Geraniol threshold for the female CD1 mice was nearly identical to those of the C57BL/6 female mice (**Figure 2D**). Threshold was very close to the 0.00005% concentration of geraniol. These data provide the first determination of the behavioral threshold for geraniol in mice

C57BL/6 and Balb/c learn a simple discrimination task at equivalent rates.

Training data were analyzed for the time to learn a simple discrimination either between mineral oil and geraniol (**Figure 3A**), or between water and 2-heptanone (**Figure 3B**) where the S+ was the odor in both cases. There was no significant difference observed in the time to learn the discrimination between strains (presented as % correct by block). There was a slight tendency for Balb/c

mice to learn more slowly over the first two sessions but it was not significant (mixed ANOVA for block x strain, $p>0.1$ with Bonferroni posttests). All mice reached criteria within 17 blocks (~3 sessions; data not shown).

Odor detection in the presence of a masking odor: strain differences between C57BL/6 and Balb/c mice.

The clear sensitivity of mice to odorants suggested that threshold detection experiments of this nature might be insufficiently sensitive to detect olfactory differences between strains. Therefore we designed an odor masking test to asses olfactory function. Using olfactory sensory neurons (OSNs) from physiology experiments using C57BL/6 and Balb/c conducted in our lab we observed that isolated OSNs were larger in Balb/c mice, cilia were more abundant, and that the turbinates of the olfactory epithelium were further apart suggesting greater air space within the nasal cavity. We therefore hypothesized that Balb/cs may be better at odor tasks.

In rats, a masking odor makes it more difficult to detect aldehydes (elevated threshold) (Sokolic et al., 2007). We used a somewhat different approach to create a more difficult detection test for mice that had no known olfactory deficits, and are therefore highly sensitive to odor detection. The odorant 2-heptanone (2hep) was used as the S+ (water reinforced) condition during training, then on testing day S+ and S- trials had equal concentrations of 2hep, but the S- also contained amyl acetate (AA). Thus, 2hep was the masking odor and mice were required to detect the AA in order to avoid the consequence of an incorrect S- trial (**Figure 1B**). These

odors were selected based on their perceptual similarity to humans (review, Wise et al., 2000) and their ability to elicit similar activity patterns in the main olfactory bulb of mice (Xu et al., 2005). Mice were trained with a high concentration of 2hep (0.1%). This concentration was selected based on the study by Clevenger and Restrepo (2006) showing that 0.1% was the lowest concentration mice were able to detect the 2hep correctly 85% of the time on the same type of olfactometer. After all mice successfully completed at least 3 blocks (90 trials S+ and S-) at >85% accuracy, they were tested to determine their ability to detect 0.005% AA in the presence of 0.1% 2hep. Mice were tested on how quickly they were able to recognize AA and avoid it in order to avoid a loud buzzer and 10 second time out (thus reducing how quickly they could start the next trial where they might get water). Testing results were analyzed two ways. In order to see the initial detection ability the first 120 trials were averaged by strain and compared for overall accuracy (**Figure 4A**). C57BL/6 were significantly better at detecting AA than the Balb/c mice (unpaired *t*-test, $p<0.05$). Analysis was also examined block by block (**Figure 4B**) for the first 6 blocks. A significant strain difference was found (Mixed ANOVA, block # x strain, strain = $p<0.05$; no interaction). Bonferroni posttests by block showed a significant difference (*) at the 6th block (**Figure 4B**). All four C57BL/6 mice had reached criteria (>85% for two consecutive blocks) by the end of 8 blocks whereas all four Balb/c mice failed to reach criteria by the end of 8 blocks. Only the first 6 blocks are shown since two of the C57BL/6 mice had met criteria and stopped drinking before 8 blocks (end of the session) were completed. Since mice were already trained to

discriminate between S+ and S- trials their acquisition time to discriminate between a new S- condition should be faster than their original training to learn discrimination in general. Therefore we compared their original training acquisition time (by block) of 2hep versus water to the discrimination time between 2hep and 2hep+AA (**Figure 4C**). As expected, C57BL/6 mice were significantly better at the second discrimination even though S+ and S- odors were much more alike (**Figure 4C left**). Interestingly, there was no difference between the improvement in detection between the original discrimination and the 2hep versus 2hep+AA discrimination for Balb/c mice (**Figure 4C right**) further supporting the difficulty of this task for the Balb/c mice. Thus, our initial hypothesis that Balb/c mice would be better at odor detection in general was not correct, at least with this set of odorants.

Discussion

The present study has established threshold estimates for the odor geranial and to use an odor-masking task as a more sensitive, and faster method of examining olfactory differences in mouse olfactory capabilities (Bodyak and Slotnick, 1999; Sokolic et al., 2007; Abraham et al., 2007).

Geraniol threshold detection

In our initial work with threshold detection we use a test that took ~8 sessions to complete, and was designed to use short exposure (30-45 trials) for each decreasing concentration of the odor geraniol. The results were highly variable (**Figure 2A**). Thus our preliminary data led us to conclude that this method was unsuitable, using the standard olfactometer, due to changes in 1) motivation from the start to the end of a session, 2) problems adjusting from a high concentration to a lower concentration with no training time, and 3) lack of enough trials to determine when additional odor cues, or non-odor cues, were being used rather than the test odorant. The first of these, motivation, is problematic when ~3 concentrations are tested within a single session. Satiation from the water reinforced trials leads to decreased attention to the task, and reduced motivation to respond accurately (review, Davidson, 1993). The motivation changed from the start to the end of a session, and therefore was different between first and last concentrations of the odorant. While we tried to control for this by making the first odor concentration the high concentration to act as a re-training block (using a

concentration the mice were able to easily detect) motivation at the end of the session remained problematic. The rapid drop in concentrations appeared to produce problems for the mice in the perception of the odor (S+ trials). This could be due to either a change in perception due to concentration (Arctander, 1994; Johnson et al., 2009) or simply because the high concentration was perceived as much more intense and therefore the half-log drop in concentration was not initially noticeable as the same odor. This drop is clearly visible in mouse 1 (**Figure 2A**) between the concentrations of 0.001 and 0.0001%. The decrease in accuracy continued until 0.00005% geraniol at which point the mouse was again able to recognize the odor as the reinforced condition. Mouse 2 showed a less extreme version of this between the first two concentrations. Retesting both mice after they had completed the task showed that they could easily detect the odors (data not shown), thus it was not likely a difference in detection, but a difference in perception that caused the drop in the number of correct responses to the initial odor concentrations.

The third problem mentioned above is a general difficulty in olfactory research with animals. Odor cues other than the test odor can confound the experiment since mice are extremely sensitive to odors (Sorwell et al., 2008; Gamble and Smith 2009). During our pilot experiments using the odorant eugenol we discovered that most of the mice were able to discriminate between mineral oil taken from a container that had been opened for several days and a newly opened container (data not shown). Mineral oil has been described in the literature as

"odorless" (Bodyak and Slotnick, 1999; Slotnick, 2007), and humans do not appear to be able to smell it (personal observation). However recently Gamble and Smith (2009) reported that mice were able to discriminate between different brands of mineral oil. Based on our experience we therefore made up each odorant concentration with matching mineral oil so that the same bottle of mineral oil was used for both the S- and S+. This emphasizes the fact that mice are likely aware of a much greater spectrum of odors than humans. Running the geraniol threshold study we observed that the humans conducting the study were not able to accurately detect geraniol under the 0.0001% concentration. Because of the dilution factor in the olfactometer as the odor reaches the headspace where the mouse sniffs, the concentration that the mice receive is ~40 fold lower than the concentration of the solution (Bodyak and Slotnick, 1999). This further emphasizes that mice are much more sensitive to odors than humans. Thus, being able to demonstrate that all other odors have been eliminated is extremely important for olfactory threshold detection studies.

In order to obtain an accurate measurement for threshold detection for geraniol we used a long protocol that allowed for much better control over extrinsic variables that could alter the accuracy of our results. We established that C57BL/6 mice and CD1 female mice have a threshold between 0.00005% and 0.0001% geraniol (+the dilution factor). This method was advantageous for the amount of control that could be maintained. First, since we used male and female C57BL/6 and female CD1 mice it was important to control for the estrous cycle of female mice

(Sorwell et al., 2008; Meziane et al., 2007). Therefore we used a minimum of four sessions (four days) of testing per odor concentration so that female mice were tested in each day of their four-day cycle for every concentration. Second, we ran regular mineral oil tests where both S+ and S- trials were mineral oil to ensure that mice were not picking up on any other cues. Third, as mentioned already, each odor concentration was mixed using the same bottle of mineral oil so that mice could not increase their accuracy based on the mineral oil alone. Fourth, learning effects improve discrimination performance (Witte and Kipke, 2005) however the long duration of the task ensured that mice were all well trained before any of the concentrations that were not easily detected were reached.

An additional strength, and important one for accurate threshold estimation, was the presence of the high concentration (0.005%) of the odor for 25% of the trials for every odor concentration. This guarantied that the mice continued to have intermittent reinforcement, since these trials were easy to detect. Intermittent reinforcement is a well-characterized behavioral method that insures that the mice continue to respond when the difficulty of the task could otherwise lead to extinction of the response (Purdy et al., 2001).

Although we were able to establish a clear response curve and determine the behavioral threshold with our long protocol, this process was both time and labor intensive. Running one group of mice with initial training, mineral oil tests between concentrations, and periodic equipment problems, mice took nearly 3 months to run. Thus, while an accurate method to obtain threshold, it is not necessarily

practical. Using the Knosys olfactometer with a different computer control program may solve this problem. Clevenger and Restrepo (2006) were successful in obtaining threshold detection of estimates for the odors 2-heptanone, ethyl acetate, octaldehyde, and isoamyl acetate between wild type and CNG2A knock out mice using the same olfactometer used here but modified by using an alternate program to better control stimuli presentation. Their threshold detection testing program was designed to automatically calculate and change the concentration each time threshold is reached (the "maximaum likelihood parameter estimation by sequential testing" or MLPEST). Furthermore, they compared a descending method of limits test (similar to that used here) to the thresholds determined by the MLPEST to the odor ethyl acetate. Like us, they found the descending method of limits require much more effort to obtain a threshold estimation, however their MLPEST test estimated the threshold of ethyl acetate to be 3 orders of magnitudes higher than the descending method of limits even though they were using the same olfactometer (10^{-3} and 10^{-6}0% respectively). Although they propose that the MLPEST test may be actually be the more accurate threshold because of increased sensitivity to odors with training (Salcedo et al., 2005), our own work offers a slightly different interpretation. The MLPEST test randomly tested S- trials with an S+ of the high concentration and (importantly) randomly any of the lower concentrations (randomly presented not descending). However, the concentration used as the high odor was 1% ethyl acetate, whereas their descending method of determined threshold to be 10^{-6} % ethyl acetate. The results from our "short-protocol"

descending method suggest that mice may find it much more difficult either to detect, or respond to low concentrations of an odor immediately after a much higher concentration (**Figure 2A**). However, this could easily be solved by using a much lower concentration of the odor as the training concentration and would not require as much of a perceptual leap to recognize the lower concentrations as the same odor (and therefore S+) trial. In general we believe that the MLPEST test used by Clevenger and Restrepo (2006) is probably the best method for rapid assessment of odor detection thresholds with the caveat that much lower initial training concentration should be used.

Female C57BL/6 and Balb/c mouse strains had similar threshold detection for geraniol

Threshold detection in female C57BL/6 mice for the odor geranial was not significantly different than the female CD1 mice, an outbred strain (**Figure 2D**) This may be due to the fact that strain differences may only differ between some odors, or the detection differences are so small that they were not detectible in the half log unit concentrations measured here. Our results did provide a useful estimation of odor threshold for this odorant however.

Odor-masking as a fast assessment of olfactory differences in mice with a Knosys olfactometer

The sensitivity of mice to odors makes it difficult to find tasks that are sensitive enough to test differences between mouse strains, genetically modified mice, or disease models. New and creative ways of approaching this problem may prove very useful. One of the more sophisticated methods of measuring odor detection utilizes 2-deoxyglucose uptake in the olfactory bulb to examine activity, in aresponse to different odors and odor concentrations (Johnson et al., 2009). The authors' results show that different concentrations of the same odor can produce very different activity patterns. Another promising approach is the response matrix used by Youngentob et al., (2001) where multiple (5) odors are presented and mice are trained to respond in a different way to each odor. In this way, multiple odors are compared simultaneously, and differences in the errors made depending on the odor presentation order can help detect differences in perception. Difficulties with both of these approaches however are the high degree of training or technical requirements to do the experiments. A need for a simpler, but sensitive, olfactory assessment test is apparent.

One of the most important features of olfaction is the ability to recognize a specific odor within a complex odor environment (Goyert et al., 2007). Each olfactory receptor is activated by multiple odorants with different affinities (Kajiya et al., 2001). Thus, the activation pattern of the olfactory epithelium is dependent on mixture of odorants and their respective concentrations in order to recognize a distinct odor perception. This process is thought to be at least partially controlled at the cortical level (Zou and Buck, 2006; Kadohisa and Wilson 2006) although the

olfactory epithelium and olfactory bulb contribute to encoding the overall sensory input as well (Johnson et al., 2009; review, Su et al., 2009). The odor-masking test used here provides an interesting tool for analyzing odor properties. The odors we chose were both known to activate the adenylyl cyclase/cAMP pathway in the main olfactory epithelium (review, Wang et al., 2007; Song et al., 2008). Rather than a simple discrimination between odors that may be easily detected on the basis of intensity alone, using a masking task may help identify odors that are perceptually similar based on the inability to differentiate them in the presence of the other odorant. Moreover, because of the cortical aspect of identifying mixed odors (Zou and Buck, 2006; Kadohisa and Wilson 2006) this task may be useful in looking at mouse models of diseases where olfactory deficit is due to problems in the olfactory bulb or brain (Fleming et al., 2008; Wesson et al., 2010) where more complex olfactory processes occur (Zou and Buck, 2006; Kadohisa and Wilson 2006; Johnson et al., 2009; review, Su et al., 2009). The olfactometer is ideally set up for this simpler, and shorter task. One of the advantages of this method is that mice have to *avoid* the odor that is being masked (S- rather than S+). This ensures that any loss in motivation is immediately apparent if mice incorrectly respond to S+ trials since mice under these conditions, will be more likely to make false alarm errors, than misses. We have demonstrated that this behavioral test is easily used in mice, in addition to rats, and provides a useful behavioral test for detecting differences in mice that show similar ability to learn odor/no odor discriminations (**Figure 3**).

Using a alternative test to measure differences between C57BL/6 and Balb/c strains we found a significant effect of strain in detecting the odor amyl acetate when masked by 2-heptenone (**Figure 4**). There was no difference in the time to learn to discriminate between an odor (either geraniol or 2-heptanone) and their no-odor condition (mineral oil or water respectively) (**Figure 3**). A great deal of work has been done looking at differences between mouse strains on various aspects of behavior. Reports of strain differences have included differences in maternal behavior, and estrous cycle effect on behavior (Shoji and Kato, 2006; Meziane et al., 2007). The effect of the estrous cycle was more pronounced in Balb/c mice whereas C57BL/6 were less affected in the test battery used. Anxiety differences between strains suggest that Balb/c are generally more anxious than C57BL/6 mice (Kim et al., 2002; Michalikova et al., 2010), whereas C57BL/6 show less exploratory behavior and less aggression (Parmigiani et al., 1999). An important finding was reported by Lee et al., (2003) showing that out of three mouse strains (including C57BL/6 and Balb/c) Balb/c were the most sensitive to odors in a simple novel odor sniff test, whereas C57BL/6 were the least sensitive. Interestingly the authors correlated these findings with decreased neuroblast migration to the olfactory bulb of the C57BL/6. Their findings emphasize the need for careful analysis of olfactory function between strains since, as they pointed out, genetically modified mice could have altered behavior due to strain effects rather than the genetic modification.

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Figure 1.

A


5 sec ITI
FV
0.5sec
sample odor
2 sec response area

B

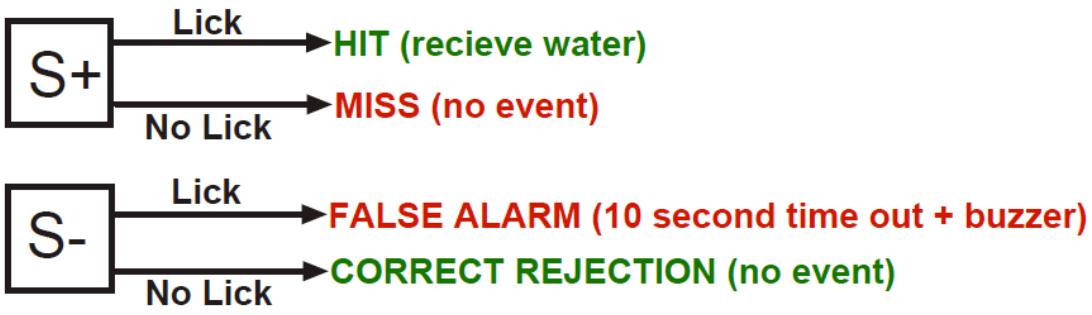
S+
Lick
HIT (recieve water)
No Lick
MISS (no event)
S-
Lick
FALSE ALARM (10 second time out + buzzer)
No Lick
CORRECT REJECTION (no event)

Figure 1. Illustration of the trial and response options for the olfactometer. A) A trial from start to finish. Inter-trial-interval (ITI) shown and the final valve (FV) that redirects odor from the exhaust port to the headspace are shown in black. The final valve is followed by the odor-sample time where the mouse must decide if the odor is present or absent, and then the response area were licking is recorded. B) Correct responses are in green, incorrect responses are in red. Each S+ trial is rewarded (water reinforcement) if a correct response is made (hit) or no event if the incorrect response is made (miss). For the S- trials an incorrect response (false alarm) is punished (10 sec time out + buzzer) if the mouse responds correctly (correct rejection) the mouse avoids the time out and can start the next trial.

Figure 2.

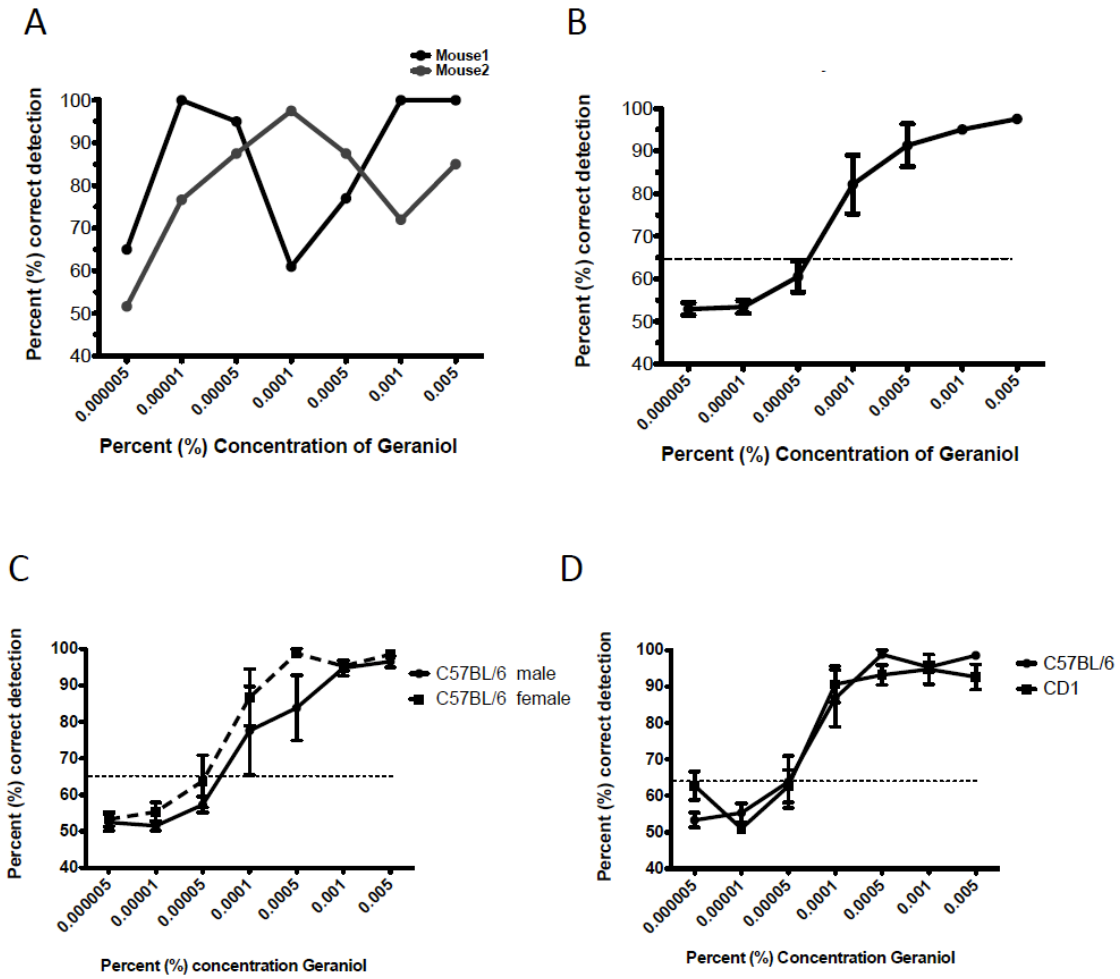
A
Mouse1
Mouse2
Percent (%) correct detection
100
90
80
70
60
50
40
0.000005
0.00001
0.00005
0.0001
0.0005
0.001
0.005
Percent (%) Concentration of Geraniol
B
Percent (%) correct detection
Percent (%) Concentration of Geraniol
C
Percent (%) correct detection
C57BL/6 male
C57BL/6 female
Percent (%) concentration Geraniol
D
Percent (%) correct detection
C57BL/6
CD1
Percent (%) Concentration Geraniol

Figure 2. Geraniol threshold detection in C57BL/6 male and female mice and CD1 female mice. A) Short protocol threshold in two C57BL/6 mice showed difficulties with doing a rapid descending test (mouse 1, black trace, and mouse 2, grey trace, showed individually). B) Geraniol detection in C57BL/6 mice (n=8) using multiple sessions with one concentration held constant provided a well-defined detection curve and showed that mice were able to detect geraniol at low concentrations (threshold detection was between 0.00005% and 0.0001%). C) Comparison female and male mice (n=4 each, from B) were not significantly different although female mice had a slightly lower threshold (dashed line). D) Female C57BL/6 (n=4, from B) were compared with female CD1 mice (n=4) detection for geraniol. Both strains showed highly similar response curves and nearly identical thresholds (dashed line). All error bars = SEM.

Figure 3

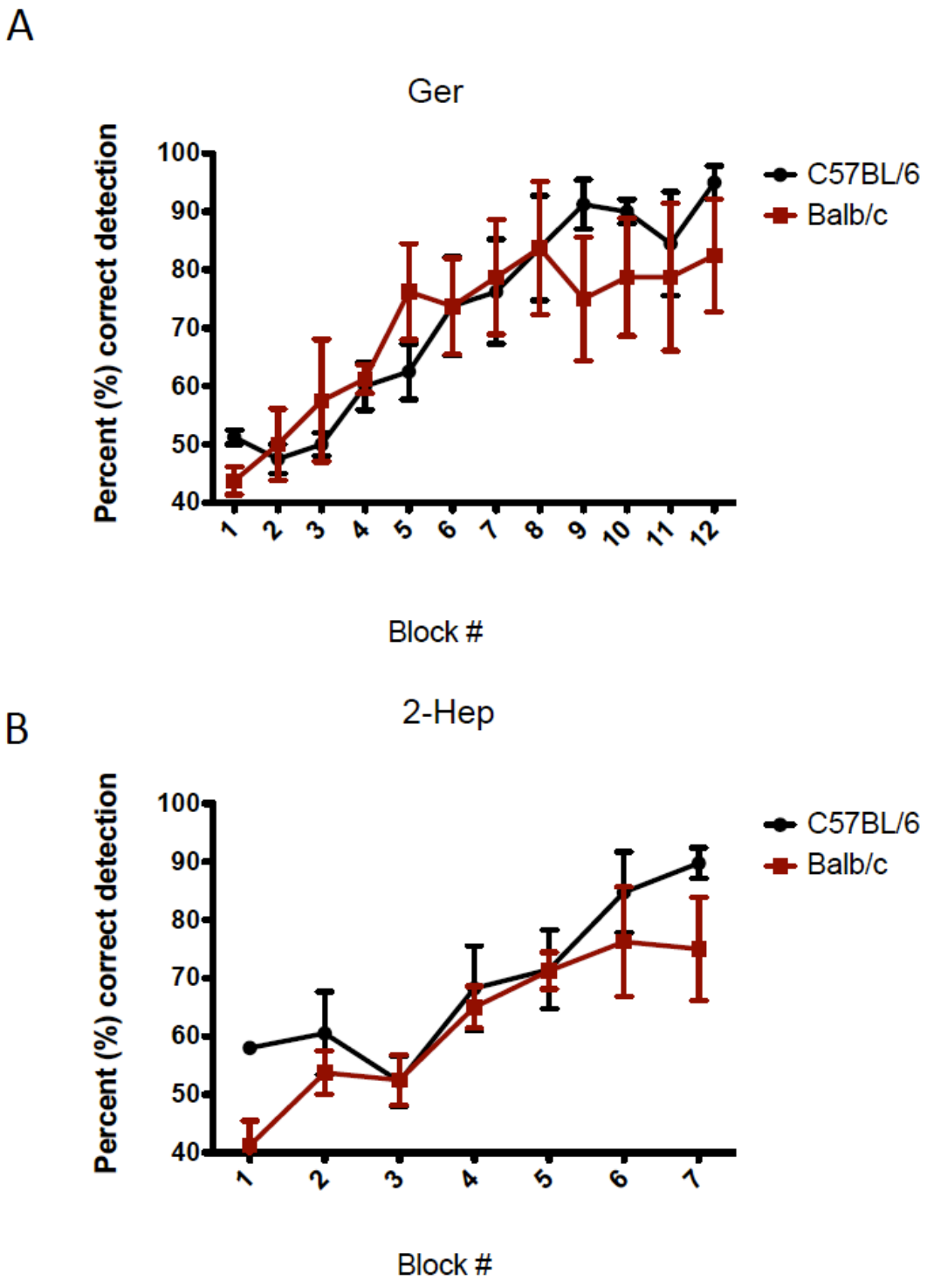
A
Ger
Percent (%) correct detection
100
90
80
70
60
50
40
1
2
3
4
5
6
7
8
9
10
11
12
Block #
C57BL/6
Balb/c
B
2-Hep
Percent (%) correct detection
100
90
80
70
60
50
40
1
2
3
4
5
6
7
Block #
C57BL/6
Balb/c

Figure 3. Training times between 4 male C57BL/6 and 4 male Balb/c mice trained on a simple odor (S+) versus no odor (S-) discrimination task. A) Training sessions for discrimination between geraniol and mineral oil for the first 12 blocks. B) Training for 2-heptanone discrimination (first 7 blocks shown) between male C57BL/6 (n=4) and male Balb/c (n=4); mice in A and B are different mice (all naïve). All error bars = SEM.

Figure 4.

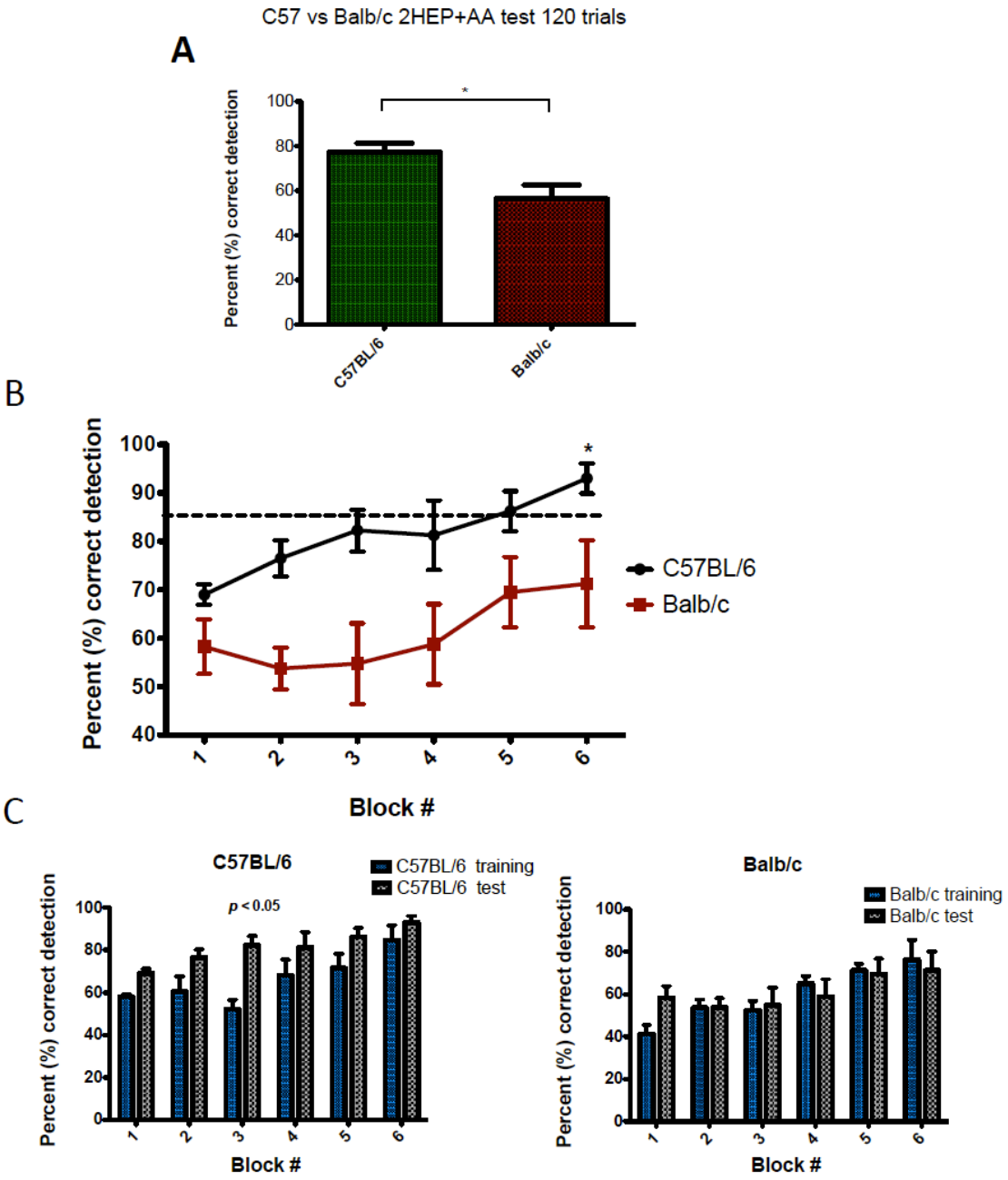
C57 vs Balb/c 2HEP+AA test 120 trials
A
Percent (%) correct detection
100
80
60
40
20
0
*
C57BL/6
Balb/c
B
Percent (%) correct detection
100
90
80
70
60
50
40
*
1
2
3
4
5
6
Block #
C57BL/6
Balb/c
C
C57BL/6
C57BL/6 training
C57BL/6 test
p < 0.05
Percent (%) correct detection
Block #
Balb/c
Balb/c training
Balb/c test
Percent (%) correct detection
Block #

Figure 4. C57BL/6 (n=4) mice detected amyl acetate in the presence of 2-heptanone sooner than Balb/c (n=4) mice. A) The average of the first 120 trials (S+ and S-) of the test for AA detection in the presence of 2-hep. B) Analysis of AA detection in the presence of 2-hep over the first 6 blocks of the test day session. Strain was significantly different (Mixed ANOVA, $p<0.05$) analysis by block showed a difference at the 6th block (*Bonferroni posttest, $p<0.05$). C) A comparison of the detection accuracy by block # between the initial 2hep vs. water discrimination ("training") and the test day discrimination between 2hep vs. 2hep + AA ("test") for C57BL/6 (left) and Balb/c (right) mice. Dashed line shows 85% correct detection. All error bars = SEM

CHAPTER 4: GENERAL DISCUSSION AND FUTURE DIRECTIONS

1*. Odor detection and human health*

The olfactory system is unique from the other sensory systems in several ways. Regeneration of neurons occurs throughout life not only in the olfactory epithelium, but also in the olfactory bulb, making it a complex system from a developmental and ongoing standpoint (review, Whitman and Greer, 2009). The direct input from the bulb into cortical regions makes the most direct connection to the brain of any sensory modality (reviews, Benignus and Prah, 1982; Haberly, 2001). With second order synapses going to the amygdala, olfaction is tied into emotional processing and this is thought to explain the emotional strength retained by odor memory (reviews, Benignus and Prah, 1982; Slotnick, 2001). Instead of small organized receptive fields, the olfactory epithelium contains OSNs expressing specific olfactory receptors distributed in broad zones across the turbinates and septum. Each receptor can respond in varying degree to multiple odors, which makes odor encoding a complex spatial phenomenon with activation patterns sorted and refined by the olfactory bulb, and includes feedback from the cortex (Haberly, 2001).

Olfaction is not as utilized in humans as it is in some species such as rodents, cats, dogs or insects (review, Ma, 2007; Nakagawa and Vosshall, 2009). However, growing evidence shows a clear connection between olfactory impairment and neurological disorders such as Parkinson's diseases and Schizophrenia (reviews, Barone, 2009; Turetsky, et al., 2009). This connection to neurological disorders has

increased the interest in utilizing olfactory acuity with tests to detect odor impairment that may be early signs of neurological diseases (Wattendorf et al., 2009). In order to accurately discern when odor impairment is due to a disease it is necessary to rule out other possible reasons for lack of sensitivity to odor testing such as work environment or smoking habits (review, Gabba, 2006). The change in odor detection may be due to changes in the bulb or cortex, which might be occurring in Parkinson's disease (Wattendorf et al., 2009). In contrast, in Schizophrenia the olfactory epithelium appears to be altered (Turetsky, et al., 2009). The implication for disease detection makes olfactory processing and odor detection research pertinent for humans in addition to increasing our understanding of its role in animal behavior.

2. OMP and odor exposure alter calcium responses of the olfactory cAMP transduction pathway.

2.1 Odor exposure alters the calcium transient elicited by odor stimulation

The effect of increased odor exposure in the environment on signal detection in the olfactory sensory neurons (OSNs) has not previously been studied. Our findings have show that first, OSNs from odor enriched mice have longer recovery times for calcium transients produced by stimulating the cAMP pathway (Chapter 2, Figure 1). Second, this alteration in the calcium transient is selective for the response elicited by the cAMP pathway and not for responses elicited by depolarization (Chapter 2, Figure 4). This implies that the events occurring within

the cilia are determining the rate of calcium recovery rather than throughout the plasma membrane. Further, odor exposure needed weeks to increase the duration of the calcium transient. The results indicate that the effect of odor-exposure may take weeks to occur (Chapter 2, Figure 2D). Last, we used $OMP^{-/-}$ OSNs from both environmental conditions to see if odor environment altered their responses as well. Like the C57BL/6 mice, we found that $OMP^{-/-}$ OSNs also had slower calcium transients when the mice had lived in a more complex odor environment.

Since the majority of odor responses in mice are mediated by increased $[cAMP]_i$ we used a PDE inhibitor, IBMX, and an AC activator, FSK, to bypass the odor receptor and increase $[cAMP]_i$. To clarify the response profile we tested these stimuli together (IBMX/FSK), or individually (IBMX or FSK) and examined the type of response produced. In C57BL/6 and in $OMP^{-/-}$ OSNs from the standard housing environment, IBMX/FSK and IBMX alone produced faster calcium transients than their odor-enriched counterparts. When high K solution was used to depolarize the membrane, bypassing the signal transduction pathway in the cilia, no difference in calcium recovery between odor environments for either strain occurred (Chapter 2, Figure 4 and 6). Due to the apparent lack of voltage gated calcium channels in the cilia (Leinders-Zufall et al., 1998; Bradley et al., 2001; Lagostena and Menini 2003) these results indicate to us that odor-enrichment is altering the signal within the cilia. This could be occurring either through the signal transduction pathway, or through the calcium clearance mechanisms that regulate calcium levels within the cilia.

Future studies need to address two points: first, calcium calibration should be done to precisely compare calcium concentrations under different stimulation conditions. Although the amplitude differences tended to vary greatly with our stimuli, it is possible that the lack of an effect seen when high K was used is due to a overall $[Ca]_i$ concentration difference (thus potentially changing calcium mediated regulation of calcium extrusion). For example, NCX generally has a lower affinity for calcium than the PMCAs, and PMCAs can be stimulated by Ca/CaM to increase their activity (Di Leva et al., 2008). Thus, calcium removal rates could be altered by the increase in $[Ca]_i$. We find this unlikely, since the differences in recovery rates between odor-environments were maintained when IBMX and FSK were used separately (Chapter 2, Figure 10), and there was no significant difference between the peak calcium concentrations of IBMX/FSK stimulation (Chapter 2, Figure 8). However, this needs to be tested in more rigorous experimental conditions.

The second question raised is what factor(s) in the cilia is responsible for this alteration of the calcium transient? To answer this question, calcium pumps and exchangers should be systematically inhibited to determine if they are mediating the calcium response in the cilia. Although it is still unclear if the PMCAs or one or more forms of NCX are primarily responsible for calcium removal in the cilia, it is clear that they are present (Weerantne et al., 2006; Noe et al., 1996). Examination of stimuli that increased intracellular cAMP through different mechanisms (IBMX and FSK) showed that the PDE inhibitor, IBMX, produced the faster calcium recovery responses in OSNs from animals in the standard environment, whereas FSK, the AC

activator, did not. The slower response time of FSK, seen by us (Chapter 2, text) and characterized by others (Laurenza et al., 1987; Hartzell and Budnitz, 1992) may reduce differences between recovery rates. This could be due to the slower increase in cAMP that causes changes in adaptation occurring at the CNG channel, or because the difference in time to the peak response alters the rate of calcium removal. Alternatively, these data hint at a difference between AC or PDE activity. ACIII has unique characteristics, it has a low basal activity and is regulated by CaMKII (review, Willoughby and Cooper, 2007). PDE1C, (the form enriched in the olfactory cilia) is known to be stimulated by calcium/CaM, but inhibited by PKA phosphorylation (Goraya and Cooper 2005). Thus, the spatial and temporal dynamics in the olfactory signaling transduction pathway may greatly alter the overall response that reaches the knob. Micro-domains where ACs, PDEs, CaM, and CNG channels are thought to co-localize make it more challenging to separate which protein(s) are most involved (review, Willoughby and Cooper, 2007). The signal is amplified by the calcium dependent chloride channels, which remain open provided sufficient calcium is present ($K_{1/2}$ ~2μM) (Reisert et al., 2003; Kleen and Gesland 1991; Kleen 1993). Therefore, it seems that the calcium removal mechanisms are better candidates for modification by changes in the odor environment. Ideally, measuring the calcium levels in the cilia at the same time as the knob, and then blocking the chloride component either with one of the chloride channel blockers DIDS and niflumic acid would answer this question.

Although an effect is clearly present between odor-environments in C57BL/6 OSNs, the underlying cause is unclear. In our work we found that mice moved from a standard housing environment to an odor enriched environment needed at least six weeks for the calcium transients to resemble those the OSNs from mice raised in an odor-enriched environment (Chapter 2, Figure 5). Odor stimulation is known to activate the CREB/CRE/ERK gene transcription pathway, which could be a long-term regulatory mechanism (Watt and Storm, 2001). This may indicate that the change in calcium recovery is due to events that regulate protein expression. Others have found a change in expression of proteins in the signal transduction pathway when mice are deprived of odor (Coppola et al., 2006). It is possible that odor exposure also regulates the expression or regulation of a variety of proteins. An attractive possibility is that the difference in the frequency and duration of odor exposure may change the expression of the splice variant of calcium pumps and exchangers. While this is purely speculation, analysis of the PMCA forms present in the cilia would be a starting point to answer this question. The PMCAs have two major splice sites (A and C) which give rise to over 20 different known isoforms among the four PMCA genes (1-4) (review, Di Leva et al., 2008). The distribution of specific splice variants appears to correlate with functional differences (Brurette et al., 2009; Polimeni et al., 2007). Thus these are an attractive mechanism for regulating odor responses.

Another possibility is that the differences seen between low and enriched odor environments are actually due to a difference in the ages of the olfactory

sensory neurons. Suh et al., (2006) showed that naris occlusion increases apoptosis and decreases proliferation, and Waguespack et al., (2005) found that OMP levels increased in naris occluded olfactory epithelia. OMP expression is highest in young mice or in olfactory epithelial that are regenerating after injury (Kream and Margolis, 1984). These data raise the possibility that odor exposure or odor deprivation changes the survival and turnover of OSNs. By extension we hypothesize that this may contribute to the differences we see in our odor environments. Older OSNs may handle calcium removal differently than young OSNs. In order to determine if odor environment changes the overall age of the OSN population we propose to use bromodeoxyuridine (BrdU) labeling to quantify the number of new OSNs in the olfactory epithelium from each environment. Additionally, looking for the expression of OMP (the hallmark of mature OSNs) and Gap43 (for immature neurons) in the olfactory epithelium of both odor environments will also give an estimation of how much turnover is occurring (Iwema and Schwob 2003).

2.2 OMP$^{-/-}$ OSNs

Olfactory marker protein (OMP) has eluded functional categorization in OSN function for over 30 years. Our results show that OMP$^{-/-}$ OSNs have increased calcium recovery rates when simulating the cAMP odor transduction pathway (Chapter 2, Figure 7). Further, when FSK was used alone these OSNs showed pronounced deficits in the number of responses (Chapter 2, Figure 11) and the response amplitudes (Chapter 2, Figure 10). Like the wild type C57BL/6 mice, when

OMP$^{-/-}$ mice are housed in an odor enriched condition the calcium recovery rate slows down, although it is still faster than the C57BL/6 OSNs (Chapter 2, Figure 7). Since OMP expression increases in naris occlusion (Coppola et al., 2006) it led us to hypothesize that mice without OMP may be more sensitive to odor environment. Our results showed that OMP$^{-/-}$ OSNs from the standard environment had a more noticeable effect (reduced responsiveness, Chapter 2, Figure 9). Further our results suggest that the lack of OMP changed the ability of AC to respond to stimuli regardless of the odor environment (Chapter 2, Figure 10, 11, and 12).

Signaling differences between OMP$^{-/-}$ OSNs and C57BL/6 OSNs were clearly visible when comparing activation of AC to inhibition of PDEs. In the OMP$^{-/-}$ OSNs there was a lack of response to the AC activator, FSK, and the responses that were obtained were significantly smaller in amplitude than the C57BL/6 responses to FSK alone (Chapter 2, Figure 10 and Figure 11). This suggests that one of the following has happened: 1) ACIII is down regulated or inhibited, and therefore stimulation has very little effect, 2) that ACIII activity is already high and therefore further stimulation has very little effect, or 3) that the FSK site activation is somehow altered in OMP$^{-/-}$ OSNs. If ACIII expression has been decreased, quantitative western blots for protein expression of ACIII could be used on OMP$^{-/-}$ olfactory epithelia. Immunostaining of the olfactory epithelia should also be done. If the results of these experiments fail to show a decrease in expression of ACIII, the next possibility is that ACIII is inhibited. CaMKII inactivates ACIII by phosphorylation. Therefore, one of the CaMKII inhibitors, CaMKIINtide or AIP

(autocamtide-2-related inhibitory peptide) could be used to see if ACIII is constitutively inhibited (Wei et al., 1998; Leinders-Zufall et al., 1999).

In our experiments, IBMX and FSK did not always produce calcium transients in the same cells (Chapter 2, Figure 11). Given that IBMX inhibits most PDEs and that PDE1C breaks down cAMP and cGMP in the cilia (reviews, Soderling and Beavo, 2000; Goraya and Cooper, 2005; Omori and Kotera, 2007) then cells that respond only to IBMX may have a deficit in AC altogether. Activation of the CNG channels would still occur from cGMP (Nakamura and Gold, 1987; Crary et al., 2000). OSNs have soluble GC that produces a slow response to odors apparently aiding in adaptation (Kroner et al., 1996; Zufall and Leinders-Zufall 1997). To test this, IBMX and FSK can be used separately in the presence of GC inhibitor ODQ (Sigma Aldrich). In OSNs that responded to IBMX but not FSK using the GC inhibitor should block the IBMX response if cGMP is mediating the effect. This would be followed by inhibition of AC with SQ 22,536 (Sigma Aldrich) doing the same experiment. In the presense of the AC inhibitor, FSK should not produce any responses, but IBMX should still produce be able to elicit a response. Also, the PDE1 specific inhibitors, 8-MM-IBMX and vinpocetine (Ortiz-Capisano et al., 2009), should be used to inhibit the ciliary PDE without altering the cAMP specific PDE4A. In this case, if cGMP is the primary second messenger, then calcium transients from both $OMP^{-/-}$ and wild type OSNs should be equivalent.

One surprising observation that comes from our results is the fact $OMP^{-/-}$ OSNs showed faster recovery rates than wild type OSNs. This is in contrast to

others' findings using field potential recordings, suction electrode recordings, and one calcium imaging study that showed slower odor recovery in $OMP^{-/-}$ mice compared to wild type controls (Buiakova et al., 1996; Reisert et al., 2007; Kwon et al., 2009). The primary difference between our methods and those of the studies just mentioned, is the stimulation time. Our stimuli were applied for 6-12 seconds depending on the experiment, whereas the other studies used stimuli for under 2 seconds. This difference is not trivial, and may be quite important for understanding what OMP does in odor signal transduction. If a stimulus is brief, the regulatory mechanisms (namely through calcium) will not be activated in the way that they are if the stimulus has a longer duration. Reisert et al., (2007), recording from the somas of $OMP^{-/-}$ OSNs with suction electrodes, did not see slower responses when they used IBMX alone but did see a slow offset when odors were used. Odors activate ACIII via the GPCR whereas IBMX simply inhibits PDE without any effect on ACIII. This is interesting in respect to our own work where we found no real difference in the response time when we stimulated ACIII with FSK treatment. To test the temporal aspect of this OMP effect we propose to use suction electrode recordings like those done by Reiser et al., (2007) but varying the time of the stimulus. Furthermore, we would use IBMX and FSK separately to characterize which part of the odor-mediated signaling cascade was affected.

Although much research has been done examining the changes that occur when OMP is not present, the molecular interactions of OMP are still poorly understood. Kwon et al., (2009) proposed that OMP acts as a regulator of CaM

indirectly through the brain expressed X-linked protein (Bex), another cytosolic protein heavily expressed in restricted regions of the brain as well as the olfactory epithelium (Koo et al., 2005). This theory is based on research showing OMP can dimerize with Bex, and that Bex is able to interact with CaM (Koo et al., 2005; Behrens et al., 2003; Koo et al., 2007). This model is attractive given the role of calcium in many cellular functions, and is supported by the reports indicating OMP has developmental, regenerative, and axon targeting roles in cell function (Graziadei et al., 1977; Kream and Margolis, 1984; Carr et al., 1997; Youngentob et al., 2003; St. John and Key, 2005; Reisert et al., 2007; Kwon et al., 2009). However the question remains, what does OMP do in OSNs? Why is it only present in the olfactory neurons? The unique properties of olfaction may be a clue to answering this question. The olfactory epithelium must respond to an immense array of odors, and individual OSNs expressing a single odor receptor will respond with different properties depending on the odor (Delay and Restrepo, 2004; review Su et al., 2009). With the overlap of sensory stimuli and tuning to particular odorants, odor responses are likely very highly regulated. If OMP is able to alter Ca/CaM function then perhaps OMP helps refine the calcium response both for the odor stimuli and for the lifespan of the cell. However, all speculation is no more than that until a clear interaction with Ca/CaM or another binding partner for OMP is found.

3. Behavioral testing, odor detection and discrimination

3.1 Behavioral testing to understand odor-perception

With a commonly used olfactometer our study examined the efficiency of threshold detection with a signal detection/modified descending method of limits method, and described a method for rapid assessment of olfactory perception in mice using an odor-masking test. To help define the sensitivity of the tests we looked for sex differences in C57BL/6 mice, and strain differences between female C57BL/6 and CD1 mice. In the odor-masking test we found strain differences between males of two inbred strains, C57BL/6 and the albino strain, Balb/c.

The general conclusion from our work with the Knosys olfactometer was that there are limitations of the programming and design that, given the remarkable sensitivity of mice to odors, makes it less than ideal for threshold detection studies. Rather, we suggest Clevenger and Restrepo's (2006) model of threshold detection testing using the same olfactometer but with a modified program would be better. The Knosys olfactometer's usefulness is demonstrated by an odor-masking task similar to that previously described with rats (Sokolic et al., 2007). This odor test has several advantages in detecting odor perception/detection differences in mice than a comparison of detection thresholds appears to.

One of the problems with threshold detection studies is that it is very difficult to determine when the mouse is able to detect the odor based on just odor cues, and when it might be responding to trigeminal stimuli. The trigeminal nerve innervates the nasal passages and many volatile odorants can stimulate it as well as the olfactory system (Doty, 1975). This may be evidenced by the fact that in Clevenger and Restrepo's (2006) study mice lacking the CNG2A subunit of the CNG channel

(and therefore lacking a functional CNG channel) had similar thresholds for 2-heptanone, octaldehyde, ethyl acetate and isoamyl acetate, all of which probably elicit trigeminal responses in mice as they do in humans (personal observation). These same mice were unable to detect geraniol even at extremely high concentrations (1% compared to the 0.005% we used for our "high" concentration for threshold work). Geraniol, even at a concentration of 1%, does not produce the same burning sensation as the other odorants tested. Without blocking the trigeminal nerve it is hard to get past this variable, so that comparisons of mouse olfactory function can be confounded by possible compensation from trigeminal stimulation. We bypassed this problem by using a masking experiment with a fairly high concentration of 2-heptanone so that any detection of amyl acetate should have been purely olfactory if the presence of 2-heptanone was already stimulating the trigeminal system. Indeed, this method enabled us to detect a significant effect between C57BL/6 and Balb/c odor perception, which given the sensitivity of mice to odors, is likely a very small difference in general. This masking test can help identify perceptual differences by mixing and matching odors to see which odors are most difficult to detect in the presence of another. Thus, this is a test of perception not just detection, and that requires higher cortical function as well. This method may be a powerful means of assessing olfactory impairment or perceptual differences, especially in mouse models of neurodegenerative diseases that are expected to gradually show olfactory deficits.

3.2 Behavioral future directions

The sensitivity of the odor masking test makes it highly applicable to studies where only subtle changes in olfactory perception are expected. The lack of differences between geraniol threshold detection in sex differences between C57BL/6 mice, and the lack of difference between CD1 and C57Bl/6 female mice would be interesting to investigate with the masking odor. Using the geraniol threshold concentrations obtained from our studies we would use two concentrations of geraniol, one just above threshold (0.0001%) and a more easily detected concentration (0.005%) and mask the odor with the odor geranyl acetate, which has similar perceptual qualities as geraniol (rose, fruity, floral; Flavors & Fragrances, 2001). Using these similar odorants the masking task would be carried out as before, but with geranyl acetate as the masking odorant. Thus, we could test 1) if threshold detection for the odorant was more difficult when in the presense of a similar odorant (threshold increased), and 2) if the mice that appeared to have the same general thresholds during threshold detection were actually equally sensitive, or if one sex or strain was actually more sensitive.

Of great interest to olfactory researchers is the link between olfactory deficits and diseases. Given that the perceptual aspects of olfactory function are higher order processes, this makes perceptual testing an important method for detecting olfactory loss in mouse models of diseases such as Parkinson's disease and Alzheimer's disease. Using a battery of odorants as test odorants against the same background odor, perceptual differences between diseased models and wild type

mice could be compared for olfactory perceptual differences. Additionally, over time, disease model strains should steadily worsen. By retraining the mice on a new S+ odor and then a masking test, we can look for which impairments are due to learning deficits that may also come with age compared to age matched wild type controls. Because of the short training time (~3 days) and simple 1 session testing day it is possible to run multiple tests on the same group of mice fairly easily. Thus we suggest that this test is extremely useful for further olfactory testing with mice with much less effort or time than the threshold detection tasks.

4. Closing remarks

It is no longer disputed that genetics and environment together shape physiology and behavior. Although this is fundamental to our understanding of science, researchers ignore how subtle differences in the genetics or environment of the organisms used may impact the results obtained. Although most people keep in mind potential differences due to sex, and control for sex accordingly. Over the years more and more research has looked at how strains of mice vary the results obtained, to the point of selection for preferred traits or physiology (Charles River Laboratories: http://www.criver.com/en-US/ProdServ/ByType/ResModOver/diseasemodels/Pages/diseasemodelsbyindication.aspx). Our work with mouse strains has added to this understanding and demonstrates that inbred mouse strains can have different abilities to process odor cues. Additionally, we also demonstrate that changes in housing conditions of mice

are sufficient to alter odor signaling within individual sensory neurons. These results collectively remind us that our data are dependent on the organism we use, and that an awareness of the living conditions, as well as the sex and strain of the animal, alter the outcome of our results.

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