ENCODING AND PROCESSING OF ACCESSORY OLFACTORY SYSTEM ODORANTS

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DEDICATION

I would like to thank my mentor, Dr. Julian Meeks, for his unwavering support and kindness. Every day, Dr. Meeks has shown how to be a thoughtful and dedicated scientist. I would also like to thank all the members of my Graduate Committee for their helpful comments and guidance. I thank all the members of the Meeks' lab for their support, conversations, and technical assistance. I would also like to extend a very warm thank you to my collaborators Jordan Dinser and Ian Riddington for their assistance. I wish to thank all of my friends, especially Charles Fermaintt, Jason Miller, and Sofya Perelman, for their support. Thank you to my parents, John and Sheila Doyle, for their love, support, and for first teaching me how to think like a scientist. Finally, and most importantly, I thank my wife Monica Doyle. Your love, support, and patience have made all of this possible.

ENCODING AND PROCESSING OF ACCESSORY OLFACTORY SYSTEM ODORANTS

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY
The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2017

ENCODING AND PROCESSING OF ACCESSORY OLFACTORY SYSTEM ODORANTS

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The accessory olfactory system is a mouse olfactory subsystem dedicated to the processing of nonvolatile odors originating from the excretions of other animals. Accessory olfactory system activity drives behaviors critical for mouse survival such as mating, aggression, and predator avoidance. Odorants are detected by sensory neurons in a specialized structure called the vomeronasal organ, and these neurons project to the accessory olfactory bulb. The accessory olfactory bulb is the first site of information integration and processing within the accessory olfactory system, and this processing can be regulated by noradrenaline and other neuromodulators. Despite years of targeted research,

there are only a few known ligands for this system limiting our ability to understand how odorants are processed.

Part of my thesis has focused on the discovery of novel ligands for this system. I discovered that feces are a novel and potent source of accessory olfactory system odorants. Fecal-driven responses in the accessory olfactory bulb are unique from other known odorant sources and exhibit sex-selectivity, indicating that feces are a potentially rich source of information for the mouse. I determined that bile acids, which vary in identity and concentration by both sex and species, are one class of fecal chemosignals. Accessory olfactory bulb responses to bile acids show patterns of overlapping and specific activity, supportive of a bile acid combinatorial code.

During my thesis I have also studied the role of noradrenaline on stimulus processing in the accessory olfactory bulb. I found that noradrenaline has different effects on spontaneous and stimulus-evoked activity. In a small subset of cells noradrenaline increases spontaneous activity, while the majority of cells show suppressed stimulus-evoked activity. This suppression is not consistent across stimulus responses. Some responses are immediately suppressed; some require multiple exposures to stimuli, while others are completely resistant to noradrenaline. Theses results indicate that noradrenaline can have heterogeneous and nuanced actions on accessory olfactory bulb activity.

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PRIOR PUBLICATIONS

Doyle WI, Meeks JP (2017) Heterogeneous effects of noradrenaline on spontaneous and stimulus-driven activity in the male accessory olfactory bulb. J Neurophys. 117(3): 1342-1351

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LIST OF ABBREVIATIONS

AOS - accessory olfactory system

AOB - accessory olfactory bulb

A6940 - epitestosterone-17-sulfate

CA - cholic acid

CDCA - chenodeoxycholic acid

d' - discriminability index

DCA - deoxycholic acid

ECL - external cellular layer

EGC - external granule cell

EIC - extracted ion chromatogram

ESP - exocrine secreting peptide

E0893 - 17α-estradiol-3-sulfate

GC - granule cell

GL - glomerular layer

GPCR - G-protein coupled receptor

ICL - internal cellular layer

IGC - internal granule cell

LC- locus coeruleus

LC-MS - liquid chromatography-mass spectrometry

LCA - lithocholic acid

MC - mitral cell

MCA - muricholic acid

MHC - major histocompatibility complex

MOB - main olfactory bulb

MOE - main olfactory epithelium

MOS - main olfactory system

MUP - major urinary protein

NA - noradrenaline

PGC - periglomerular cell

Q1570 - corticosterone 21-sulfate

Q3910 - hydrocortisone 21-sulfate

VNO - vomeronasal organ

VSN - vomeronasal sensory neuron

V1R - vomeronasal type I receptor

V2R - vomeronasal type II receptor

ΔR - change in firing rate

CHAPTER ONE

INTRODUCTION

Fundamental goals in neuroscience are to understand how the brain is able to detect, process, and respond to environmental cues. During my thesis work I have approached these questions by studying mouse olfaction. Unlike humans, mice rely heavily on their sense of olfaction in order to find food and respond to other animals in their environment. In fact, mice have developed four unique olfactory sensory tissues to assist in these tasks: the septal organ, the Grueneberg ganglion, the main olfactory epithelium (MOE), and the vomeronasal organ (VNO) (Munger, Leinders-Zufall, & Zufall, 2009; Ferrero and Liberles, 2010; Liberles, 2014). The Grueneberg ganglion is one of the first olfactory tissues to develop and responds to cues important to pups, such as cold temperature and alarm cues (Brechbühl, Klaey, & Broillet, 2008; Schmid et al., 2010). The MOE detects volatile odorants and is associated with the detection of food and other animals (Munger et al., 2009; Liberles, 2014). The septal organ also detects volatile odorants although its function is not well understood compared to the MOE (Tian & Ma, 2004). The VNO houses the sensory epithelium for the accessory olfactory system (AOS), an olfactory subsystem that guides animal-related behaviors like mating and aggression. Sensory neurons in the VNO detect non-volatile odorants, typically present in the liquid excretions of other animals (Dulac & Torello, 2003; Chamero, Leinders-Zufall, & Zufall, 2012; Liberles, 2014; Stowers, 2015). Although the AOS has been lost in Old World primates like humans, this system is a useful and

ethologically important model for studying the encoding of sensory information in the mouse (Dulac & Torello, 2003; Chamero et al., 2012).

Accessory Olfactory System Anatomy

The mouse VNO is a blind-ended tube that opens anteriorly into the nasal cavity (Fig. 1.1a). Odors dissolved in the aqueous nasal mucus are pulled into the VNO through the active pumping of a blood vessel lying adjacent to the sensory epithelium (Meredith, 1994; Dulac & Torello, 2003). This pumping allows for odors to enter the VNO during active olfactory investigation, as well as during self and social grooming (Wysocki, Beauchamp, Reidinger, & Wellington, 1984). Odorants bind to receptors expressed in vomeronasal sensory neurons (VSNs). Vomeronasal receptors primarily belong to one of three major families: vomeronasal type I receptors (V1Rs), vomeronasal type II receptors (V2Rs), and formyl peptide receptors (Dulac & Axel, 1995; Herrada & Dulac, 1997; Matsunami & Buck, 1997; Ryba & Tirindelli, 1997; Pantages & Dulac, 2000; Rodriguez, Del Punta, Rothman, Ishii, & Mombaerts, 2000; Liberles, 2009; Rivière, Challet, Fluegge, Spehr & Rodriguez, 2009). Also expressed in the VNO are a handful of olfactory receptors and a MHC class I molecule that acts as a co-receptor for V2Rs (Ishii & Mombaerts, 2008; Leinders-Zufall, 2014; Nakahara et al., 2016).

VSN cell bodies are located within two layers of the VNO sensory epithelium, a separation that leads to both anatomical and functional divisions (Fig, 1.1a-b). V1Rs, a family of G-protein coupled receptors (GPCRs) related to bitter taste receptors, are expressed by VSNs with their cell bodies in the apical layer of the sensory epithelium. V2Rs, a GPCR

family related to metabotropic glutamate receptors, are expressed by VSNs located in the basal layer of the sensory epithelium (Dulac & Wagner, 2006; Liberles, 2014). V1Rs, which couple to the G-protein $G\alpha_{i2}$, are hypothesized to bind to small molecules while V2Rs, which couple to the G protein $G\alpha_{o}$, are believed to bind to large molecules like peptides (Del Punta et al., 2002b; Leinders-Zufall et al., 2004; Kimoto, Haga, Sato, Touhara, 2005; Stowers, 2015). Even though V1Rs and V2Rs couple to different G-proteins, they are both believed to signal through a common signaling pathway involving phospholipase C and a cation channel called TRPC2 (Stowers, Holy, Meister, Dulac, & Koentges, 2002; Liberles, 2014).

VSNs project from the VNO to the accessory olfactory bulb (AOB), a small brain region located posterior to the main olfactory bulb (MOB) (Fig. 1.1a). VSN axons synapse with the apical dendrites of excitatory mitral cells (MCs) in specialized structures called glomeruli on the surface of the AOB (Fig. 1.1c) (Chamero et al., 2006; Larriva-Sahd, 2008; Munger et al., 2009). VSNs that express V1Rs project to the anterior region of the AOB, while V2R-expressing cells project to the posterior region of the bulb (Rodriguez, Feinstein, & Mombaerts, 1999; Belluscio, Koentges, Axel, & Dulac, 1999). Surprisingly, VSNs expressing the same receptor project to multiple glomeruli and MCs make synapses with VSNs expressing different receptors (Del Punta, Puche, Adams, Rodriguez, & Mombaerts, 2002a; Wagner, Gresser, Torello, & Dulac, 2006). This wiring allows for responses to be integrated from multiple sensory neurons, producing a unique combinatorial code of responses at the level of the AOB (Meeks, Arnson, & Holy, 2010). This integration is distinct from the main olfactory system (MOS), which uses a labeled line paradigm in which sensory neurons expressing the same receptor project to the same glomeruli without integration

(Dulac & Wagner, 2006; Baum & Kelliher, 2009; Uchida, Poo, & Haddad, 2014). The AOS, therefore, likely evolved to combine information from multiple sources to guide behavior. MCs send their axons to higher brain regions including the bed nucleus of the stria terminalis, the nucleus of the accessory olfactory tract, and the medial amygdala (Dulac & Wagner, 2003; Baum & Kelliher, 2009; Chamero et al., 2012).

In addition to MCs, the AOB contains a number of local inhibitory interneurons, including periglomerular (PGCs) and granule (GCs) cells (Fig. 1.1c). These interneurons release the inhibitory neurotransmitter GABA in response to sensory stimulation and are hypothesized to play roles in modulating sensory tuning and memory formation in the AOB (Brennan & Keverne, 1997; Matsuoka, Kaba, Mori, & Ichikawa, 1997; Matsuoka et al., 2004; Brennan, 2009; Hendrickson, Krauthamer, Essenberg, & Holy, 2008; Larriva-Sahd, 2008; Oboti et al., 2011). All inhibitory interneurons in the bulb are thought to make reciprocal dendrodendritic synapses with MCs, in which a synapse forms between the dendrites of a MC and the dendrites of an interneuron (Fig. 1.1d) (Matsuoka et al., 1997; Matsuoka et al., 2004, Larriva-Sahd, 2008; Yokosuka, 2012). PGCs are located between glomeruli in the glomerular layer (GL) of the bulb, and may suppress sensory input into the AOB (Larriva-Sahd, 2008; Eckmeier & Shea, 2014). External granule cell (EGC) bodies are located in the external cellular layer (ECL) of the bulb, and their role in bulbar processing is not particularly well understood (Larriva-Sahd, 2008). Internal granule cell (IGC) bodies are located in the internal cellular layer (ICL) and they send their dendrites into the ECL where they synapse with MC apical dendrites (Larriva-Sahd, 2008). IGC activity is thought to result in self-inhibition (inhibition back onto the same mitral cell) and lateral inhibition (inhibition

between mitral cells) (Brennan & Keverne, 1997; Hendrickson, 2008; Larriva-Sahd, 2008). These inhibitory interneurons are the target of a number of centrifugal projections, including noradrenergic (NAergic) fibers from the locus coeruleus (LC) and cholinergic fibers from the horizontal limb of the diagonal band of Broca (Fallon & Moore, 1978; Araneda & Firestein, 2006; Smith, Weitz, & Araneda, 2009; Smith & Araneda, 2010; Smith et al., 2015). These neuromodulators are likely to change interneuron activity in response to environmental and internal state changes.

Accessory Olfactory System Ligands

The AOS detects and processes odors to guide animal-related behaviors, such as mating and avoidance. These odors are typically categorized, based on the their source and function in relation to the detector, as pheromones, kairomones, or allomones. Pheromones are cues released from conspecifics with no established benefit for either the donor or recipient. The prototypical pheromone is an odor that encourages mating behaviors. Kairomones are released from heterospecific animals and benefit the animal detecting the odorant, such as a predator odor that causes prey avoidance. Allomones are similar to kairomones in that heterospecific animals also release them, but they instead benefit the releaser rather than the detector. This class of cues has been described in plants and insects, but not in mice (Ferrero & Liberles, 2010; Liberles, 2014). Despite decades of research there are only a handful of known ligands (Table 1), severely limiting our understanding of how odors are encoded and processed within the AOS.

All known AOS cues have been discovered in the excretions of animals. Urine is the major known source of AOS activity, with 30-40% of VSNs responsive to dilute mouse urine (Holy, Dulac, & Meister, 2000; Nodari et al., 2008). Urinary cue processing contributes to all known mouse-to-mouse behaviors, including mating and territorial aggression (Brennan, 2009; Chamero, 2007; Chamero et al., 2011; Haga-Yamanaka et al., 2014; Boillat et al., 2015; Fu et al., 2015). As a major driver of AOS activity, researchers have dedicated decades of targeted research into isolating urinary ligands. Identified compounds include both low- and high-molecular weight compounds that can drive behaviors either alone or in combination (Nodari et al., 2008; Chamero et al., 2007; Haga-Yamanaka et al., 2014). The next best-studied sources of odorants are mouse tears, which contain peptides that influence mating selection and male-male aggression (Kimoto et al., 2005; Haga et al., 2010; Ferrero et al., 2013; Hattori et al., 2016). Saliva, both conspecific and heterospecific, has recently been identified as an activator of the AOS (Papes, Logan, & Stowers, 2010; Kahan & Ben-Shaul, 2016). Finally, vaginal secretions have also been shown to produce activity but neither the behavioral relevance or actual odorants have been identified (Kahan & Ben-Shaul, 2016). Surprisingly, one of the other major animal excretions, feces, has not received any attention in the field as a potential source of AOS odorants.

Many low-molecular weight activators of the AOS have been identified, all of which have been discovered in mouse urine. These small compounds mainly activate V1R-expressing VSNs that project to the anterior AOB, allowing for the functional and anatomical separation of responses to low- and high-molecular weight compounds at the level of the AOB (Jie & Halpern, 1996; Rodriguez et al., 1999; Liberles, 2014). The first identified

urinary ligands were small, non-polar chemicals belonging to the thiazole, ketone, pyrazine, and farnesene classes (Novotny, Harvey, Jemiolo, & Alberts, 1985; Novotny, Jemiolo, Harvey, Wiesler, & Marchlewska-Koj, 1986; Leinders-Zufall et al., 2000). These chemicals have been implicated in a number of hormonal effects, but the exact method of detection is still unclear. One hypothesis in the field is that they require carrier proteins in order to reach VSNs within the aqueous mucus of the VNO, although responses to these compounds have been observed without protein binding (Leinders-Zufall et al., 2000; Sharrow, Vaughn, Žídek, Novotny, & Stone, 2002).

The predominant low-molecular weight ligands in urine are sterols with polar moieties, which allow them to be dissolved in the VNO mucus without protein co-factors. The first identified sterol activators were sulfated steroids (estrogens, androgens, glucocorticoids, and pregnanolones) that contain one to two attached sulfate groups (Nodari et al., 2008; Hsu et al., 2008). These sterols are potent activators of the AOS; a battery of just 12 sulfated steroids was fond to activate approximately a quarter of all VSNs (Meeks et al., 2010). Recently, a novel class of steroids with a carboxylic acid moiety has been found to drive significant amounts of activity in the VNO (Fu et al., 2015).

This reliance of sterols as major olfactory ligands is present not only in mice, but can be found across the animal kingdom (Fig. 1.2). Sulfated sterols are not only potent olfactory cues in mice but can also serve as ligands in fish and amphibians. A sulfated pregnanolone is a mating pheromone in goldfish, while frogs can detect the same sulfated estrogens and pregnanolones that activate the mouse AOS (Sorensen, Scott, Stacey, & Bowdin, 1995; Poling, Fraser, & Sorensen, 2001; Sansone et al., 2015). Bile acids, a different class of polar

sterols, are potent olfactory ligands for many fish species (Buchinger, Li, & Johnson, 2014). Fish detect unconjugated and conjugated bile acids, as seen most clearly in the sea lamprey. Sulfated bile acids are potent mating and migration cues in the sea lamprey, guiding movements over miles (Doving, Selset, & Thommesen, 1980; Sola & Tosi, 1993; Li et al., 2002; Johnson, Yun, Thompson, Brant, & Li, 2008; Zhang & Hara, 2009; Huertas et al., 2010; Li, Brant, Siefkes, Kruckman, & Li, 2013). Unconjugated sex hormones can also act as pheromones, indicating that polar moieties are not necessary for olfaction. For example, unconjugated androgens guide both aggression in goldfish and mating in pigs (Poling et al., 2001; Sorensen, Pinillos, & Scott, 2005; Ferrero & Liberles, 2012). For many of the sterol activators it is unknown if the detecting receptors are V1Rs, V2Rs, or main olfactory receptors.

In addition to small chemicals, peptides and proteins are potent activators of the AOS. The first identified protein cues were major urinary proteins (MUPs), a family of androgen-regulated lipocalins released into male mouse urine (Chamero et al., 2007; Roberts et al., 2010; Kaur et al., 2014). Lipocalins are a large family of structurally homologous proteins that contain an internal binding pocket for small compounds (Sharrow et al., 2002). These proteins are hypothesized to act as cues on their own and as carriers for small chemical cues, such as thiazoles (Dulac & Torello, 2003; Ferrero & Liberles, 2010; Kaur et al., 2014). The quintessential lipocalin pheromones are MUPs, which account for more than 99% of the peptide mass in male mouse urine (Chamero et al., 2007). Further supporting their role as a chemosignal, the complement of MUPs released into urine can vary among strains providing a potential mechanism for strain identification (Kaur et al., 2014). Mice can also detect and

avoid a heterospecific kairomone, released into cat saliva, called Feld4 (Papes et al., 2010). Other proteins called exocrine secreting peptides (ESPs), released from facial glands, are able to activate the AOS. Both lipocalins and ESPs are detected by V2R-expressing neurons that project to the posterior AOB (Chamero et al., 2007; Haga et al., 2010).

Surprisingly, proteins typically associated with the immune system also act as olfactory cues in the mouse AOS. Peptides bound to the major histocompatibility complex (MHC) are potent activators of V2R-expressing VSNs (Leinders-Zufall et al., 2004; Leinders-Zufall, Ishii, Mombaerts, Zufall, & Boehm, 2009). MHC detecting VSNs also express MHC Ib peptide receptors, although these receptors do not appear to act as MHC peptide detectors in the VNO. The MHC receptor instead acts as a co-receptor to enhance V2R responses (Ishii & Mombaerts, 2008; Leinders-Zufall et al., 2014). The other known protein cues for this system are bacterial peptides, which activate a unique subset of VSNs that express formyl peptide receptors instead of the classical V1Rs and V2Rs (Liberles, 2009; Rivière et al., 2009). Unlike the other peptide cues, VSNs activated by these ligands are known to project to the anterior AOB and may project to the posterior AOB as well (Dietschi, Assens, Challet, Carleton, & Rodriguez, 2010).

Encoding and Processing of Olfactory Cues

In order to appropriately respond to the environment, the AOS has to encode stimuli in a manner that allows for both discrimination and integration. Enhancing discrimination allows for distinct behavioral responses, such as choosing to mate with a female versus fleeing from a predator, but at the expense of increased processing. On the other hand,

enhancing integration promotes the combination of ethologically similar stimuli with potentially lower levels of processing. Discrimination and integration are seen at both the level of the VNO and AOB, although AOB circuitry is especially dedicated to response integration.

Individual VSNs have high and variable spontaneous activity, leading to a low signal-to-noise ratio during stimulus detection (Meeks et al., 2010; Arnson & Holy, 2011). Possibly due to this the variability, the encoding of different environmental stimuli is likely accomplished through a burst code as opposed to the rate code seen in other brain regions. In response to odor stimulation, the frequency of bursts by an individual VSN increases but the individual pattern of spikes within each burst remains unchanged (Arnson & Holy, 2011). Individual VSNs also have a small dynamic range over which they will respond to an odorant or a blend of odorants like urine (He et al., 2010; Arnson & Holy, 2013). The combination of highly variable spontaneous activity, burst encoding, and small dynamic ranges mean that the accurate transmission of information about a blend of odorants requires sampling across populations of VSNs. Practically this makes it difficult to study how the AOS encodes stimuli through recording the responses of individual VSNs.

Delivery of urine and individual chemical activators to the VNO has revealed intriguing aspects of VSN encoding on the population level. As expected from the small dynamic range of an individual VSN, the concentration of an odorant can be estimated from a population of VSNs but not from individual neurons (Arnson & Holy, 2013). Sex appears to also be encoded at the population level as well. Differences in sex-responses to urine have been observed in individual VSNs, but only a few of these cells are able to respond

consistently to a given sex across individuals and mouse strains (He, Ma, Kim, Nakai, & Yu, 2008; Tolokh, Fu, & Holy, 2013). The importance of VSN population encoding has also been seen with individual odors. When a battery of 12 sulfated sterols was delivered to the VNO, a cell's individual responses were varied but on the population level VSN firing could be clustered into just 8 processing streams (Meeks et al., 2010). These results further support that even though an individual VSN can have variability in responses, at the population level robust stimulus encoding can be observed.

MCs send their dendrites to multiple glomeruli, potentially allowing them to sample and integrate information from VSNs responsive to different stimuli (Belluscio et al., 1999; Dulac & Wagner, 2006). This integration of information potentially allows for the discernment of donor identity to be the result of comparing multiple cues instead of relying on a single ligand. Reliance on a single ligand is a poor conveyor of information as an individual ligand can be present across sex or species, and it's concentration is unlikely to remain consistent across the lifespan of an animal (Kahan & Ben-Shaul, 2016). A recent behavior study has shown support for this integration hypothesis. The authors found that individual cues were not sufficient to induce male mounting behaviors, but a combination of odors was (Haga-Yamanaka et al., 2014).

Despite the importance of integrating across multiple cues, the AOB is also capable of discriminating between different odors or blends of odorants. Sex- and species-specific responses to urine have been observed in MCs (Luo, Fee, & Katz, 2003; Ben-Shaul, Katz, Mooney, & Dulac, 2010; Meeks et al., 2010; Tolokh et al., 2013). This discrimination is not specific to blends of odorants; MCs also show unique patterns of responses to individual

ligands. With a battery of 12 sulfated sterols, the majority of MCs faithfully transmitted the discriminable sulfated sterol processing streams observed in the VNO. Integration across sulfated steroid-sensitive processing streams was only detected in 10% of MCs (Meeks et al., 2010). On a large scale, this discrimination can be seen anatomically due to the differential projection of V1Rs and V2Rs to the AOB. The anterior bulb, receiving projections from V1R-expressing molecules that respond to small chemicals, may be specifically tuned to stimuli related to physiological status. Meanwhile, the posterior bulb may be more tuned to information about identity due to the targeting of VSNs that respond to proteins that vary in expression and sequence across individuals and species (Isogai et al., 2011; Chamero et al., 2012).

In order to survive, stimulus-driven activity cannot be static but instead must be responsive to changes in the environment or a mouse's own internal status. A potent way to change stimulus processing is through the use of neuromodulators like noradrenaline (NA) and acetylcholine. Unlike classical synaptic transmission, neuromodulators act over relatively large volumes of tissue to affect a neuron's activity in response to stimulation (Nai, Dong, Hayar, Linster, Ennis, 2009; Sara, 2009). Neuromodulators are typically released in response to internal state or environmental changes, such as arousal or the presentation of novel stimuli (Aston-Jones & Bloom, 1981; Sara & Bouret, 2012).

NA is one of the best-studied modulators of AOS activity. NA is produced in the hindbrain by tyrosine hydroxylase-expressing (TH) neurons in the LC, and these cells send projections throughout the brain (Fig. 1.3) (Sara, 2009). At least 30% of NAergic fibers project to the MOB and AOB, primarily to the ECL and ICL (Fig. 1.3) (Shipley, Halloran,

De La Torre, 1985). In the AOB NA is detected by α1 and α2 receptors, members of the G-protein coupled receptor family (Zhong & Minneman, 1999). Consistent with the localization of LC projections, these NAergic receptors are enriched in the ECL and ICL (Pieribone, Nicholas, Dagerlind, & Hökfelt, 1994; Rosin et al., 1996; Talley, Rosin, Lee, Guyenet, & Lynch, 1996; Domyancic & Morilak, 1997; Winzer-Serhan, Raymon, Broide, Chen, & Leslie, 1997). The exact cells that expresses these receptors has not been determined anatomically, but functionally NA appears to act primarily on GCs (Araneda & Firestein, 2006; Smith et al., 2009).

The exact role that NA plays in AOS stimulus processing is currently not well understood. Experiments in male and female AOB slices have shown that NA increases the spontaneous activity of granule cells, leading to non-specific suppression of all MC activity (Araneda & Firestein, 2006; Smith et al., 2009). This is different from MOB slices in which NA has been shown to have heterogeneous effects including both enhancement and suppression of MC activity (Trombley & Shepherd, 1992; Jiang, Griff, Ennis, Zimmer, & Shipley, 1996; Ciombor, Ennis, & Shipley, 1999; Hayar et al., 2001; Nai, Dong, Hayar, Linster, & Ennis, 2009; Nai, Dong, Linster, & Ennis, 2010; Linster, Nai, & Ennis, 2011; Devore & Linster, 2012). In the intact female mouse, NA release leads to the suppression of responses to any stimulus delivered coincident with LC activation (Brennan, Kendrick, & Keverne, 1995; Brennan & Keverne, 1997; Brennan & Binns, 2005). This phenomenon underlies the pregnancy block (Bruce) effect, in which a female mouse AOB cells will show responses to a novel male's odors but not her mate's. Activation in response to a novel male leads to a reduction in prolactin and progesterone, preventing embryo implantation (Bruce,

1959; Brennan & Binns, 2005; Brennan, 2009). Due to the dramatic nature of the pregnancy block effect, most studies on NA's actions in the AOB have focused on female mice and little is known about the role of NA in modulating male AOS activity.

Olfactory-Driven Behaviors

Traditionally, the AOS is associated with modifying innate behaviors and internal states while the MOS is linked to associative learning (Stowers, 2015). This distinction is not entirely pure, the AOS can exhibit learning behaviors as seen with the pregnancy block effect and activity in the MOS can trigger innate behaviors like predator aversion (Brennan, 2009; Kobayakawa et al., 2007). All known AOS-driven behaviors are related to other animals and they include changes in the internal status or crucial survival related behaviors like mating and fighting.

The first identified role of the AOS was in inducing hormonal or physiological changes in the detecting mouse, so-called primer effects (Bruce, 1959; Koyama & Kamimura, 2000; Koyama, 2004). The best-studied hormonal effect is the pregnancy block effect discussed above, in which a novel male's odors trigger a pregnancy failure (Bruce, 1959; Brennan & Keverne, 1997; Brennan, 2009). The identification of novel male versus mate appears to be through peptide ligands, as the addition of an unfamiliar mouse's MHC peptides to a mate's urine can trigger the pregnancy block effect (Leinders-Zufall et al., 2004). Male urine can also trigger the acceleration of female puberty (Vandenbergh effect) and synchronize estrous cycles (Whitten effect), possibly through the detection of small

chemicals bound to MUPs (Whitten, 1958; Vandenbergh, 1969; Novotny, Ma, Wiesler, & Žídek, 1999; Koyama, 2004).

Mating is one of the best-studied behaviors regulated by the AOS, leading to the discovery of multiple mating-related ligands. The detection of male-specific ESPs by female mice promotes lordosis, the bending of the back performed by females during mating (Haga, 2010). ESPs can also affect mating behaviors, such as mate selection, in males. Juvenile mice secrete a protein named ESP22 before puberty, and this protein produces a profound inhibition of mounting attempts in males (Ferrero et al., 2013). Other known regulators of male mating include a blend of unknown odorants in urine that is required for virgin males to begin mating with a female mouse. Both sulfated and carboxylic acid sterols have been implicated in this behavior, but other components of the blend are unknown (Haga-Yamanaka et al., 2014; Fu et al., 2015). Despite these discoveries, the relative roles of the AOS and MOS in regulating mating are not completely understood (Serguera, Triaca, Kelly-Barrett, Banchaabouchi, Minichiello, 2008; Baum & Kelliher, 2009). Mice born without a functional VNO through the knockout of TRPC2, a cation channel necessary for VSN signal transduction, are able to mate and produce offspring. In fact, male mice lacking TRPC2 will attempt to mate with both female and male mice (Stowers et al., 2002). In other rodents the VNO is necessary for the induction of mating behaviors, but on only in virgin males (Meredith, 1986).

In addition to regulating mating, AOS odorant detection can trigger animal-related fight-or-flight responses. Although small chemicals have been reported to increase male-male aggression, recent research has focused on peptides as the major drivers of aggression

and avoidance behaviors (Novotny et al., 1985). Androgen-regulated MUPs are potent regulators of aggressive behaviors in males; detection promotes both urine counter-marking and territorial aggression (Chamero et al., 2007; Kaur et al., 2014). Male-male aggression can also be triggered by the detection of the same ESPs that enhance sexual receptivity in females (Hattori et al., 2016). Intact vomeronasal signaling is also necessary for maternal aggression, although the exact olfactory cues are currently unknown (Kimchi, Xu, & Dulac, 2007; Chamero et al., 2011). In addition to aggressive responses, the AOS can also regulate avoidance behaviors through the detection of largely unknown cues. The only known cue is a protein found in cat saliva, which produces potent fear and avoidance behaviors in mice (Papes et al., 2010). Through currently unknown odorants, the urine of sick conspecific mice can also trigger avoidance behaviors (Boillat et al., 2015).

Although known AOS-mediated behaviors are potent and largely innate, they can also be heavily modulated by a mouse's internal state (Stowers & Liberles, 2016). Female mice change their responses to AOS cues based on both their estrous cycle and previous mating history (Chamero et al., 2011; Wu, Autry, Bergan, Watabe-Uchida, & Dulac, 2014; Dey et al., 2015). These alterations in activity are not unique to females; male mice also have context-dependent changes in responses. Urine countermarking is affected by a mouse's position in the cage's social hierarchy and mating switches a male from infanticidal to parental behaviors (Tachikawa, Yoshihara, & Kuroda, 2013; Kaur et al., 2014; Wu et al., 2014). These studies demonstrate that the AOS is not only a potent driver of behaviors crucial to survival, but the exact behavioral outputs can be affected by a mouse's individual history.

Summary

The rodent AOS is a crucially important olfactory subsystem involved in the detection of and response to odors released by other animals. Odorants released into the excretions of animals are detected by sensory neurons in the VNO that project to the AOB. These odorants are encoded by a combinatorial code within the AOB that is subject to alterations by neuromodulators like NA, and downstream stimulus-driven activity leads to social behaviors like mating and aggression. Despite decades of research there are only a few identified ligands for this system, severely limiting our ability to understand how this system encodes cues both at rest and during neuromodulation.

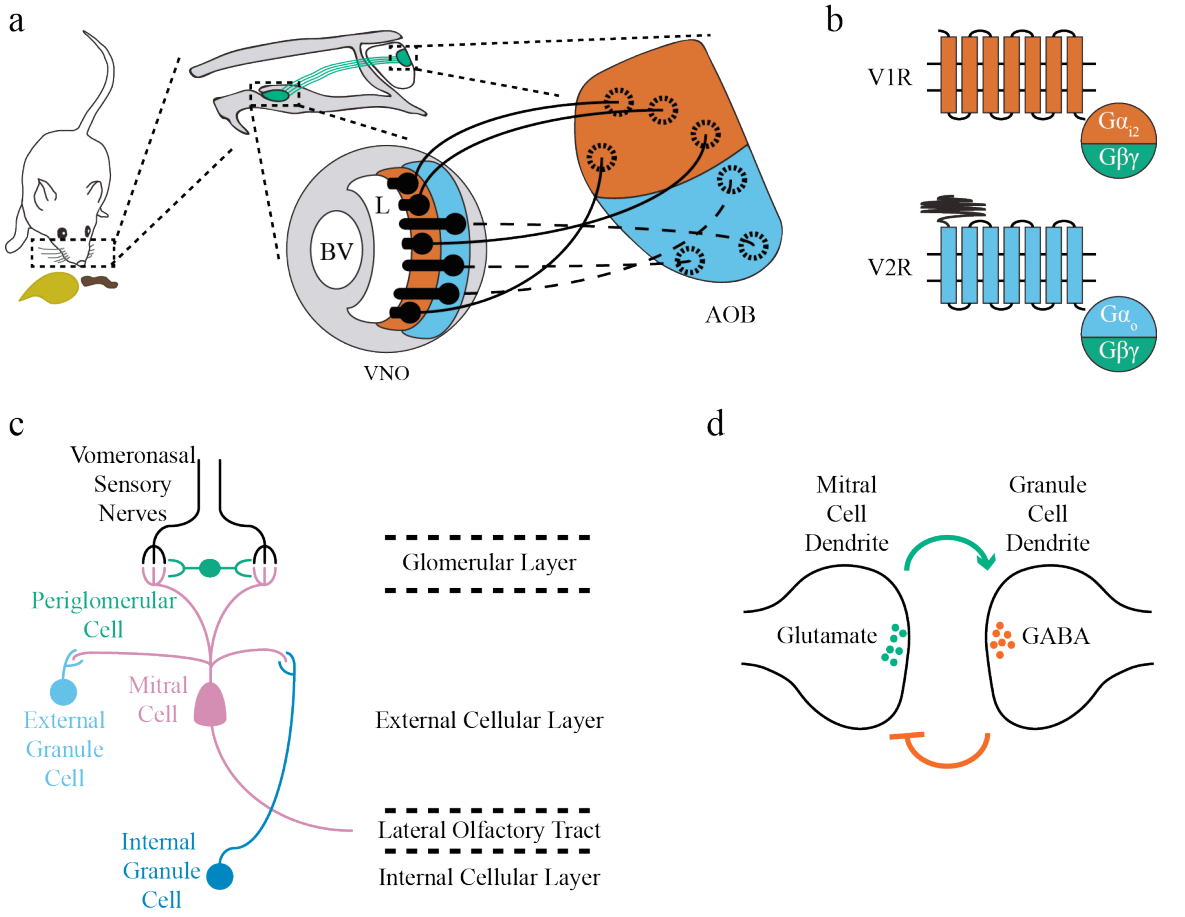

Figure 1.1 The Mouse AOS Detects Animal-Related Odors

a: AOS anatomy. Odorants are pumped into the lumen of the VNO and are detected by VSNs. VSNs send their axons to the AOB. BV: blood vessel, L: lumen.
b: Major vomeronasal receptors. V1Rs have a small extracellular domain, couple to $G\alpha_{i2}$, and respond to low-molecular weight ligands. V2Rs have a large extracellular domain, couple to $G\alpha_{o}$, and respond to high-molecular weight ligands.
c: AOB microcircuit. VSN axons synapse with MCs, which send their axons to higher brain regions. Local inhibitory interneurons can modify MC activity.
d: MC and GC dendrites form unique reciprocal dendrodendritic synapses. MC's release glutamate and activate GCs, leading to the release of GABA and inhibition back onto the MC.

	Ligand	Source	Action	References
Low Molecular Weight	isobutylamine	urine	puberty acceleration	Del Punta et al. 2002
	2-sec-butyl-4,5-dihydrothiazole	urine	estrous synchronization, pubery acceleration, male aggression	Novotny et al. 1985; Leinders-Zufall et al. 2000; Del Punta et al. 2002
	2,3-dehydro-exo-brevicomin	urine	estrous synchronization, pubery acceleration, male aggression	Novotny et al. 1985; Leinders-Zufall et al. 2000; Del Punta et al. 2002
	α- and β-farnesenes	urine	estrous synchronization, pubery acceleration	Leinders-Zufall et al. 2000; Del Punta et al. 2002
	2,5-dimethylpyrazine	urine	puberty delay	Novotny et al. 1986; Leinders-Zufall et al. 2000; Del Punta et al. 2002
	2-heptanone	urine	puberty delay	Novotny et al. 1986 Leinders-Zufall et al. 2000; Boschat et al. 2002; Del Punta et al. 2002
	trans-4-hepten-2-one	urine	puberty delay	Novotny et al. 1986
	trans-5-hepten-2-one	urine	puberty delay	Novotny et al. 1986
	n-pentyl acetate	urine	puberty delay	Novotny et al. 1986
	cis-2-penten-1-yl acetate	urine	puberty delay	Novotny et al. 1986
	6-hydroxy-6-methyl-3-heptanone	urine	puberty acceleration	Leinders-Zufall et al. 2000; Del Punta et al. 2002
	sulfated sterols	urine	mating (estrogens), unknown (others)	Nodari et al. 2008; Haga-Yamanaka et al. 2014
	carboxylic acid sterols	urine	mating	Fu et al. 2015
High Molecular Weight	MHC peptides	urine	mating, pregnancy block	Leinders-Zufall et al. 2004
	MUPs	urine	mating, aggression, countermarking	Chamero et al. 2007; Roberts et al. 2010; Kaur et al. 2014
	Feld4	saliva	avoidance	Papes, Logan, Stowers 2010
	ESPs	exocrine glands	aggression, mating	Kimoto et al. 2005; Kimto et al. 2007; Haga et al. 2010; Ferrero et al. 2013; Hattori et al. 2016
	bacterial peptides	unknown	unknown	Rivière et al. 2009; Liberles et al. 2009
Other	aciditiy	urine	unknown	Cichy et al. 2015

Table 1 Known AOS Ligands

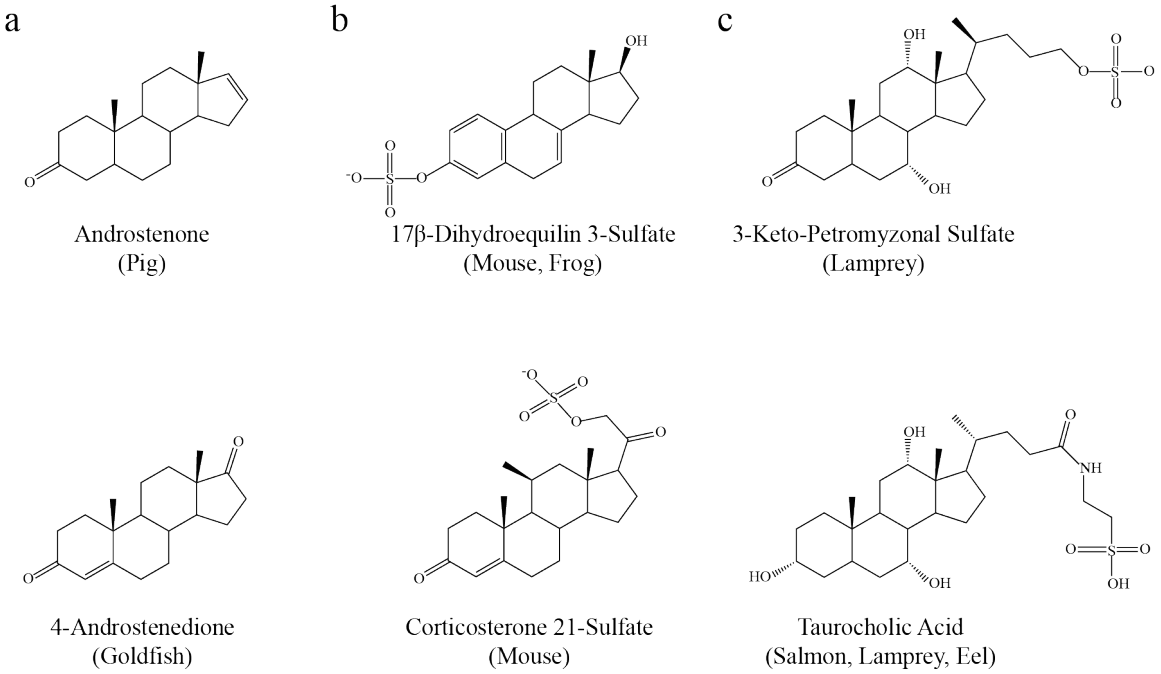

Figure 1.2 Sterols Are Potent Olfactory Cues Across the Animal Kingdom

a: Unconjugated sterol ligands. Androstenone is a boar pheromone that activates the MOE. 4-Androstenedione is a pheromone that triggers aggression in goldfish.
b: Sulfated sterol ligands. 17β-dihydroequilin 3-sulfate is a sulfated estrogen detected by both frogs and mice. Corticosterone 21-sulfate is a mouse cue first discovered in urine.
c: Bile acid ligands. 3-keto-petromyzonal sulfate is a mating pheromone in sea lamprey. Taurocholic acid is a taurine-conjugated bile acid detected by the olfactory epithelium of many fish species.

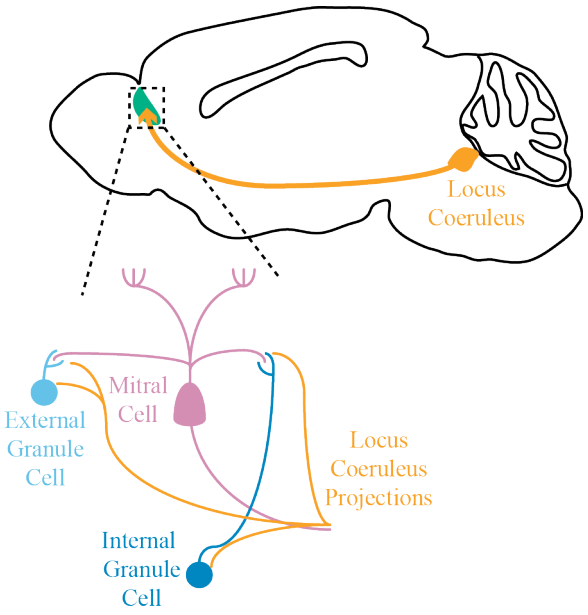

Figure 1.3 NA Is a Potent Modulator of AOS Activity

NAergic projections target the AOB. NA is produced by TH-positive cells in the LC. These cells send targeted projections to the ECL and ICL of the AOB.

CHAPTER TWO

FECAL BILE ACIDS ACTIVATE THE ACCESSORY OLFACTORY SYSTEM

Summary

Despite decades of research there are only a handful of known ligands for the AOS, severely limiting our ability to understand how this system functions in the mouse. I have discovered that mouse feces are a novel source of AOS odorants, and feces produce distinct responses in the AOB compared to urine. Fecal responses are also sex-selective, supporting a potential role in guiding mouse behaviors. I found that mouse feces are enriched for a class of sterols called bile acids. Bile acids, which vary in identity and concentration by both sex and species, produce unique and overlapping patterns of activity within the AOB. These data indicate that fecal bile acids are a previously unknown class of pheromones and kairomones. This chapter is reproduced, with modifications, from Doyle et al., 2016.

Introduction

Social communication between most mammals relies heavily on olfaction. Terrestrial mammals employ multiple olfactory pathways for social communication, including the AOS (Munger et al., 2009). The AOS processes social chemosensory information from conspecifics (via pheromones) and heterospecifics (via kairomones), and is important for many behaviors, including mating, territorial aggression, pregnancy maintenance, and predator avoidance (Brennan & Keverne, 1997; Stowers et al., 2002; Dulac & Torello, 2003;

Papes et al., 2010; Haga-Yamanaka et al., 2014). A major barrier to understanding AOS function has been lack of knowledge about the system's full complement of natural ligands.

Most of the known AOS ligands were identified through screening excretions from mice and mouse predators (Kimota et al., 2005; Leinders-Zufall et al., 2004; Spehr et al., 2006; Chamero et al., 2007; Nodari et al., 2008; Rivière et al., 2009; Papes et al., 2010; Roberts et al., 2010; Ferrero et al., 2013). Discovering new natural AOS ligands has proven to be difficult, in large part due to technical barriers to recording the activity of AOS neurons that have only recently been overcome (Holy et al., 2000; Spehr, Hatt, & Wetzel, 2002; Luo et al., 2003; Chamero et al., 2007; Rivière et al., 2009; Ben-Shaul et al., 2010; Isogai et al., 2011; Turaga & Holy, 2012). AOS sensory signaling begins in the VNO where VSNs detect ligands through the expression of just one or two G protein-coupled vomeronasal receptors (Dulac & Axel 1995; Herrada & Dulac, 1997; Matsunami & Buck, 1997; Ryba & Tirindelli, 1997; Pantages & Dulac, 2000) or formyl peptide receptors (Liberles et al., 2009; Rivière et al., 2009). VSNs project their axons to the AOB the first and only major dedicated neuronal circuit for information processing in the AOS (Luo et. al, 2003; Dulac & Wagner, 2006; Munger et. al, 2009; Meeks et al., 2010). VSNs are rather noisy signal detectors (Holy, et al., 2000; Arnson and Holy, 2011) but their downstream synaptic targets, the AOB MCs, demonstrate high signal/noise ratios by virtue of a high degree of synaptic convergence from many VSNs expressing the same receptor (Luo, et al., 2000; Meeks, et al., 2010; Rodriguez, Feinstein, and Mombaerts, 1999; Belluscio, Koentges, Axel, and Dulac, 1999). I used an *ex vivo* preparation of the AOS (Meeks & Holy, 2009; Doyle, Hammen, & Meeks, 2014) that

makes use of this network feature in a screen aimed at determining whether feces contained novel AOS ligands.

There are two main reasons why I investigated feces as a potential source of AOS activation. First, it has long been known that soiled cage bedding is one of the most potent activators of AOS activity (Kimoto & Touhara, 2005; Isogai, et al., 2011). Bedding contains a mixture of mouse excretions, the most abundant of which are urine and feces. Urine, currently the best-studied source of AOS ligands, contains a number of unique ligands including sulfated steroids and major urinary proteins (Leinders-Zufall et al., 2004; Chamero et al., 2007; Nodari, et al., 2008), but mammalian fecal chemosignals have not yet been systematically investigated. Secondly, molecular components of feces vary across many biological states, including sex and species (Turley, Schwarz, Spady, & Dietschy, 1998; Moschetta et al., 2005; Hagey, Vidal, Hofmann, & Krasowski, 2010; Hoffman, Hagey, & Krasowski, 2010), and information gleaned from fecal constituents might regulate animal behavior.

I found that feces are a robust source of AOS ligands that produce largely distinct patterns of activity from urinary ligands. The most prominent active components of mouse feces are bile acids and AOS neurons discriminate between bile acids that vary with sex and species. The discovery of bile acids as natural AOS ligands derived from feces reveals a previously unrecognized external link between the gut and brain that may inform mouse social and reproductive behaviors.

Materials and Methods

Animals

Electrophysiology experiments were performed with male C57Bl/6J and B6DF2F1/J mice between 6 and 15 weeks of age. VNO imaging experiments were performed with Omp$^{tm4(cre)Mom}$/J knock-in mice (*OMP-Cre* mice; Jackson Laboratory Stock #006668; Li, Ishii, Feinstein, & Mombaerts, 2004) mated to Gt(ROSA)26Sor$^{tm38(CAG-GCaMP3)Hze}$/J mice (Ai38 mice; Jackson Laboratory Stock # 014538; Zariwala et al., 2012). Feces and urine were collected from male and female BALB/cJ mice aged 6-13 weeks. Mice were provided food and water ad libitum and kept on a 12:12 light cycle. Mice were fed 16-18% protein by weight chow (Harlan Teklad Global Rodent Diet). All procedures were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee and follow guidelines from the National Institutes of Health.

Stimuli and Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Stimuli were dissolved to their final concentrations in Ringer's solution containing (in mM): 115 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 $NaHCO_3$, 10 HEPES, and 10 glucose. Mouse urine and feces were collected from 20 female and 10 male BALB/cJ mice for 2-6 weeks. Mice were suspended in a wire-bottomed cage over liquid nitrogen for 4-8 hours per day. At the end of collection, frozen urine and feces were stored at -80°C until extraction. Urine was extracted as previously described (Nodari et al., 2008; Meeks et al. 2010). Urine was thawed, pooled and centrifuged at 80 x G for 2 minutes. The supernatant

was removed and filtered through a 0.22 μM filter, aliquoted, and stored at -80°C until use. For all physiology experiments, urine was diluted 1:100 in Ringer's solution.

Fecal particles were diluted 1:10 in dH_2O, homogenized, and left overnight at 4°C on an orbital shaker. The suspension was homogenized again and centrifuged at 2400 x G for 10 minutes and 2800 x G for 30 minutes. The supernatant was filtered through a 0.22 μM filter, aliquoted, and stored at -80°C. Polar fecal extracts were obtained from the aqueous phase of Bligh-Dyer (methanol:chloroform:water) extractions of whole fecal extracts. Whole fecal extract was extracted with chloroform:methanol:water at a ratio of 2:2:1.8. Whole extracts of mouse feces were used as stimuli at 1:300 and polar extracts were used at 1:100.

Cat urine/feces was purchased from BioreclamationIVT (Westbury, NY, USA) and used at a dilution of 1:100. As indicated by the vendor, cat urine was collected after it had been in direct contact with cat feces, thus allowing for direct mixing between the two substances.

All sulfated steroids were purchased from Steraloids, Inc. (Newport, RI, USA). Steroids used included the epitestosterone-17-sulfate (A6940), testosterone sulfate (A7010), 17α-estradiol-3-sulfate (E0893), corticosterone-21-sulfate (Q1570), and hydrocortisone-21-sulfate (Q3910). 20 mM stock solutions of A6940, A7010, E0893, and Q3910 were prepared in methanol. 20 mM Q1570 stock was prepared in dH_2O. All sulfated steroids were diluted to 10 μM for experiments.

Cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), lithocholic acid (LCA), α-muricholic acid (α-MCA), β-MCA, and ω-MCA were purchased

from Sigma-Aldrich. 20 mM bile acid stocks were prepared in methanol and were diluted to 10 μM for experiments.

Ex Vivo Preparation

Mice were anesthetized with isofluorane and decapitated into ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 3 *myo*-inositol, 2 sodium pyruvate, and 0.5 sodium ascorbate. An additional 9 mM of $MgCl_2$ was added to the dissection aCSF to limit excitotoxicity. Half of the mouse skull, from the snout to the olfactory bulbs, was dissected out and adhered to a small plastic plank with tissue adhesive. The plastic plank was inserted into a custom-built dissection chamber where it was superfused with room temperature aCSF. A secondary microdissection was performed to expose the vomeronasal nerves and accessory olfactory bulbs. A thin 0.0045-inch cannula (A-M Systems, Carlsborg, WA, USA) was placed into the VNO with a steady stream of fresh VNO Ringer's. The cannula was subsequently used for the delivery of stimulus batteries through the use of a pressurized system (AutoMate Scientific, Berkeley, CA, USA). Stimulus batteries were delivered to the VNO in a randomized, interleaved manner. Stimuli were delivered for 3 to 5 seconds, and each cell was exposed to no fewer than 3 stimulus repeats.

Electrophysiology

Extracellular recordings were made from the accessory olfactory bulb through glass electrodes with resistances between 1.5 and 8 MΩs. Electrodes were filled with 0.22 μm

filtered aCSF and advanced into the AOB by a micromanipulator (Siskiyou Corporation, Grants Pass, OR, USA). All recordings were made from neurons generating large positive spikes that were located between 94 and 351 μm from the AOB surface, characteristic of MCs in the AOB external cellular layer (Meeks et al., 2010). Signals were amplified through a Cornerstone BVC-700A amplifier (Dagan Corporation, Minneapolis, MN, USA), digitized by an analog-digital device (National Instruments Corporation, Dallas, TX, USA), and controlled by custom software written in LabVIEW. Recordings were sorted for single-unit activity using custom MATLAB (MathWorks, Inc., Boston, MA, USA) programs (Meeks et al., 2010; Hendrickson et al., 2008). Cells included in analysis had clearly separable waveforms (evaluated by principal component analysis, spike autocorrelograms, and spike crosscorrelograms). All cells included in analysis responded to at least one stimulus and completed at least 3 complete stimulus trials.

Mass Spectrometry

I analyzed mass spectrometry data generated by Jordan Dinser and Ian Riddington at University of Texas at Austin. For female mouse fecal analysis and the initial detection of bile acids, liquid chromatography was performed as previously described (John et al., 2014), with minor variations. All experiments were run with an injection volume of 5 μL. Standards were prepared by diluting 5 μL of 5 μg/mL of the standard with 20 μL of 2:1.8 MeOH:H_2O. Biological extracts were injected with no dilution. Chromatographic separation was performed on a C18 HPLC column with embedded polar amide groups (Accucore Polar Premium, 2.6 μm particle size, 150 x 2.1 mm). The injected volume was eluted at a flow rate

of 0.3 mL/min using a gradient method where mobile phase A was water and mobile phase B was methanol (Fisher). Both mobile phases contained 0.1% formic acid (Thermo Scientific) and 5 mM ammonium acetate (Fisher). Elution was performed with 60% mobile phase B for two minutes, followed by a gradient increase in mobile phase B to 100% over 18 minutes. The column was held at 100% mobile phase B for 30 minutes and then returned to 60% mobile phase B over 3 minutes followed by a 13-minute equilibration period.

For the comparison of DCA and CDCA content in male versus female mouse fecal extracts, an isocratic liquid chromatography method was used in order to resolve the closely eluting, isobaric DCA and CDCA peaks. For comparison of male fecal samples to DCA and CDCA standards, the standard samples were prepared by adding 0.8 µL of 5 µg/mL DCA or CDCA (in 2:1.8 MeOH:H_2O) to 45 µL of H_2O. The male fecal extracts were prepared by adding 0.8 µL of 2:1.8 MeOH:H_2O to 45 µL of extract. All samples were analyzed with 30 µL injection volumes. For comparison of female fecal samples to DCA and CDCA standards, the standard samples were prepared by adding 4 µL of 5 µg/mL DCA or CDCA (in 2:1.8 MeOH:H_2O) to 20 µL of H_2O. The female fecal extracts were prepared by adding 4 µL of 2:1.8 MeOH:H_2O to 20 µL of extract. All samples were analyzed with 12 µL injection volumes. Separation was performed on a C18 HPLC column (Zorbax Eclipse Plus, 5 µm particle size, 50 x 2.1 mm). Elution was performed with the same mobile phases as initial female fecal analyses. Phases were delivered at a ratio of 35:65 (mobile phase A to mobile phase B) at a flow rate of 0.7 mL/min.

Eluting species were detected by an Agilent Technologies 6530 Accurate Mass Q-TOF equipped with an electrospray ion source in negative mode. Ion source settings were:

capillary voltage 5000 V, gas temperature 350 °C, gas flow 12 L/min, nebulizer pressure 40 PSI. For male mouse versus female mouse analysis the nebulizer pressure was 50 PSI. Analyte identification was performed through comparison of elution time and mass spectra to standard samples. Targets in standards and in extracts were observed as $[M-H]^-$ (deprotonated species) and $[M+CHOO]^-$ (formate adducts). Data was analyzed with MassHunter Qualitative Analysis (Agilent) and custom MATLAB programs.

In Vivo Exposure to Fecal Extracts and Bile Acids and Fos Immunostaining

Male B6D2F1/J mice were exposed to fecal extracts and pure bile acids in two separate assays. In the first, 20 mice were placed into a standard mouse cage containing a 10 cm petri dish with a test or control stimulus, and were allowed to freely interact with the stimuli for 10 minutes. The negative control stimulus was 8-10 g of clean corncob-style bedding (same as the standard bedding on which they were raised) that had been moistened by 1 mL of distilled, filtered water prior to the test (to match the wetness of test bedding). The positive control stimulus was 8-10 g of corncob bedding that had been soiled by 3-5 BALB/cJ female mice for 48-72 hours and frozen at -80 °C until use. All stimuli were dampened with a total volume of 1 mL of water or diluted stimuli 15 minutes prior to each test. The two test stimuli were clean bedding to which either 100 µL of pure BALB/cJ female mouse urine or fecal extracts plus 900 µL of water had been added prior to the test. Each interaction was video monitored to ensure that each animal interacted with the bedding using its nose during the test period. Following the 10-minute interaction period, animals were placed in a cage with clean bedding for 90 minutes prior to transcardial perfusion (see

below). Natasha Browder and Hillary Cansler, in the laboratory of Dr. Julian Meeks, performed these experiments.

In the second test, 10 μL of a control or test stimulus was directly pipetted onto the nares of 15 B6D2F1/J male mice following light isofluorane anesthesia (5 μL per nostril). The negative control stimulus was 10% methanol (v/v), and test stimuli were 10-fold diluted fecal extract or a mixture of 4 pure bile acids, CA, DCA, CDCA, and LCA, all at 1 mM. Following stimulus exposure, mice were placed onto an empty cage containing only clean bedding for 90 minutes prior to transcardial perfusion.

Ninety minutes following free-moving or direct stimulation, animals were anesthetized by ketamine/xylazine (120 mg/kg ketamine, 16 mg/kg xylazine) and perfused transcardially with 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were extracted and post-fixed in 4% paraformaldehyde in PBS overnight. Following a 3x rinse in PBS, brains were cryoprotected in PBS containing 25% sucrose, then mounted in OCT compound and cut sagittally into 20 μm sections using a cryostat (Leica). Sections were permeabilized with 0.1% Triton-X 100, blocked with 10% goat serum (Sigma), and immunostained with primary antibodies against Fos (Abcam rabbit polyclonal antibody #ab7963, 1:200 dilution), and secondary antibodies conjugated to Alexa Fluor 633 (Thermo Fisher Scientific #A-21070, 1:2000 dilution). Following immunostaining, 3-4 sections from each animal were mounted on slides and counterstained with mounting medium containing DAPI to help identify cell nuclei and the boundaries between AOB sublaminae. Slides were coverslipped and imaged using an AxioScan slide scanner equipped with a 20x, 0.8 NA objective (Zeiss). Daniel Dinh and Hillary Cansler performed these experiments.

Quantification of Fos-staining in AOB sections was performed manually by Dr. Julian Meeks and myself. Boundaries were drawn around the AOB internal and external cellular layers and a curved line was traced along the lateral olfactory tract using basic tools in FIJI/ImageJ. We each counted the number of stained neurons in the AOB, referring to the DAPI nuclear image to avoid double-counting cells. Our scores were strongly positively correlated and the mean of our scores were used for further analysis. The position of each identified Fos+ cell along the anterior/posterior axis was determined by calculating the shortest distance between the marked cell and the line drawn along the LOT. Differences between experimental conditions were assessed by one-way ANOVA (total cell density) and Kruskal-Wallis test (anterior-posterior positions).

Electrophysiology and Imaging Data Analysis

All data analysis was performed in software written in MATLAB. Electrophysiological spike responses to stimulus delivery were analyzed within a defined 4 s window beginning 1 s after stimulus onset (to allow for complete evacuation of the fluid dead volume within the cannula). The change in spike firing rate (ΔR) was determined by subtracting the firing rate within the analysis window by the baseline firing rate before stimulus delivery. Spiking responses were compared to the response to Ringer's control, and significance was evaluated within this window by a two-sample Student's *t*-Test ($p < 0.05$ threshold). To be considered for further analysis, the average spiking rate increase in this 4 s window must also have exceeded 1 Hz. Cells were excluded from analysis that did not demonstrate clearly separable waveforms, either from noise or from other cells. Cells

demonstrating no discernable stimulus response to any stimulus or which responded to Ringer's control stimuli were discarded. Peristimulus time histograms (PSTHs) were generated from the spiking responses within a 20 second window around the stimulus onset (5 seconds before onset, 15 seconds after onset). Spiking was evaluated in 1 s bins. Spike rate was averaged across all stimulus repeats to determine the average firing response, which was plotted in a colorized heat map.

Cluster analysis using a modified version of the mean shift method was performed as previously described (Meeks, et al., 2010). Briefly, ΔR responses on a per-cell basis were normalized to the maximum response across stimuli. Normalized ΔR values across cells were passed into the custom clustering algorithm, which compiled a similarity matrix across multiple (typically hundreds to thousands) non-deterministic clustering calculations. The similarity matrix was then analyzed by mean shift clustering. This similarity matrix was also displayed in some cases via nonclassical multidimensional scaling (*mdscale* function in MATLAB), which produces 3-dimensional visualizations of the differences between clusters.

To determine the ability of two stimuli to be discriminated from one another I utilized the discriminability index (d'). I calculated the d' statistic using the following formula:

$$d' = \frac{\Delta R_1 - \Delta R_2}{\sqrt{\frac{\sigma_1^2 + \sigma_2^2}{2}}}$$

Where ΔR_1 and ΔR_2 represent the mean changes in firing rate to the two stimuli being compared and σ_1^2 and σ_2^2 are the variances of ΔR across repeated trials. To quantify the significance of the observed d' values, I performed a shuffle test in which 100,000 model populations containing the same number of cells as the observed population were randomly

assigned one of the normalized ΔR values from each of the compared stimuli. A d' value was calculated for each simulated neuron, and a Kruskal-Wallis test performed between each of the 100,000 randomly-shuffled model populations and the observed population. I report percentages of these model populations that achieved statistical significance at the 5% criterion. A higher percentage of statistically significant differences from the model indicate that the observed discriminability was not attributable to random integration of combinations of independent variables, but instead reflected a systematic discrimination between them.

A pairwise comparison matrix was calculated to demonstrate the overlap in responses between bile acids. For this calculation, the number of cells that significantly responded to both bile acids was determined, then divided by the number of cells responding to just one of them (the row variable).

Results

VNO Stimulation with Female Fecal Extracts Activates Anterior AOB Neurons

I investigated the potential for fecal chemosignals to activate the AOS using single cell electrophysiological recordings from *ex vivo* preparations that maintain functional connectivity between the VNO and AOB (Fig. 2.1) (Meeks & Holy, 2009; Meeks et al., 2010; Doyle et al., 2014). I developed an aqueous extraction protocol to isolate water-soluble chemosignals from female BALB/cJ feces. I chose an aqueous extraction because AOS ligands must dissolve in the aqueous nasal mucus to be carried from the nares into the VNO *in vivo*.

I delivered dilute BALB/cJ female fecal extract to the VNO of *ex vivo* preparations from male B6D2F1/J mice while making single-unit electrophysiological recordings from downstream AOB MCs (Fig. 2.1a). As a positive control, I stimulated the VNO with 100-fold diluted BALB/cJ female urine, a robust source of AOS chemosignals (Fig. 2.1b) (Nodari et al., 2008; Ben-Shaul et. al, 2010; Isogai et al., 2011; F, et al., 2015). 300-fold diluted fecal extracts produced robust, but selective activity in AOB MCs (Fig. 2.1c-i). I observed MCs that responded exclusively to urine or feces (Fig. 2.1c), MCs that selectively responded to urine or feces (Fig. 2.1d-e) and MCs that responded to both (Fig. 2.1f). Across the population, 21.4% of AOB MCs responded selectively to 300-fold diluted BALB/cJ female feces, nearly equivalent to the selective activation by 100-fold diluted BALB/cJ female urine (24.7%; 89 cells from 56 animals). 15.7% of MCs responded nonselectively to fecal extracts and urine, while 38.2% of MCs did not respond to either of these stimuli, but did respond to other chemosignals in the stimulus battery (Fig. 2.1g-h). Among MCs that responded to urine and/or feces, 34.5% of the cells responded selectively to feces, 40% responded selectively to urine, and 25.5% responded to both (55 cells from 40 animals, Fig. 2.1i). In separate *in vivo* experiments, male mice were exposed to BALB/cJ female soiled bedding and to clean bedding mixed with BALB/cJ female urine or fecal extracts. Expression of the immediate-early gene Fos was increased in the AOBs of these mice, confirming that fecal chemosignals activate the AOS *in vivo* (Fig. 2.2).

Fecal extracts and urine at these dilutions were equally potent. The apparent selectivity of many MCs indicated that urine and feces produce unique information in the AOS. I quantified the capacity for this information to be used to discriminate urine from

feces by calculating the discriminability index (d') for all MC responses to urine and feces based on the change in firing rate (ΔR) elicited by each. The d' values I observed across the MC population showed a strong bias towards high discriminability between urine and feces (Fig. 2.1j). 98.9% of 100,000 simulated MC populations (each with 55 cells from 46 animals showed d' distributions that were statistically lower than the observed population. In sum, the strong but differential activation of MCs by fecal and urinary chemosignals indicated that urine and feces provide unique information to the AOS.

Polar Molecules Are a Primary Source of Fecal-Driven Activity

Sulfated glucocorticoids are known urinary AOS chemosignals that robustly activate the anterior AOB (Nodari et al., 2008; Meeks et al., 2010; Hammen, Turaga, Holy, & Meeks, 2014). The partial overlap in urine- and feces-driven anterior AOB activity suggested that they may share common ligands, so I tested whether urinary sulfated steroids were present in both urine and feces. Consistent with this hypothesis, I encountered some MCs that responded to corticosterone-21-sulfate (Q1570, 10 μM) and 300-fold diluted fecal extracts (Fig. 2.3). Cells that responded to both fecal extracts and glucocorticoids were more rare (5/87 MCs) than MCs responding to glucocorticoids but not feces (10/87 MCs; Fig. 2.3c) I used clustering algorithms to classify the tuning curves of 87 MCs recorded during VNO stimulation with mouse urine, mouse fecal extracts, and two sulfated glucocorticoids (Fig. 2.3c-d, 87 cells from 55 animals). Multidimensional scaling, a method that assists in the visualization of multi-factor tuning differences, highlights the approximate magnitude of tuning differences between cells in each of the 8 identified clusters (Fig. 2.3d). Though MCs

tuned to both feces and glucocorticoids were rare, these results suggested that glucocorticoids may be shared ligands between urine and feces, or alternatively that glucocorticoids at 10 μM activate receptors that are also activated by different fecal ligands.

Sulfated steroids are highly polar molecules, and were originally discovered in the polar fraction of chloroform:methanol extractions of mouse urine (Hsu et al., 2008; Nodari et al., 2008). To determine whether fecal AOS ligands were also polar, I compared MC tuning to polar fractions of fecal chloroform:methanol extractions to the whole fecal extracts. Polar fecal extracts strongly co-activated feces-tuned MCs in the anterior AOB (Fig. 2.3e-g). Of the 12 cells tested with the whole extracts and polar fraction, 66.6% of cell's responsive to whole fecal extract also responded to the polar fraction (12 cells from 6 animals Fig. 2.3g). Furthermore, discriminability analysis showed that tuning to whole fecal extract and polar fraction was similar to tuning curves chosen randomly (statistically significant in only 17.4% of 100,000 12-MC simulations; Fig. 2.3h). Thus, polar molecules caused the majority of fecal extract activity in the anterior AOB. To determine whether these polar molecules contained sulfated steroids, I collaborated with Ian Riddington and Jordan Dinser of the University of Texas at Austin to perform high resolution, accurate-mass liquid chromatography-mass spectrometry (LC-MS) on the fecal polar fractions. We did not detect appreciable levels of sulfated glucocorticoids, androgens, or estrogens (Fig. 2.3i), indicating that sulfated steroids are not a major source of feces-driven activity in the anterior AOB.

The Active AOS Ligands in Female Fecal Extracts Are Bile Acids

LC-MS revealed the presence of distinct, abundant peaks for molecules with mass-to-charge (m/z) ratios consistent with unconjugated bile acids (Fig. 2.4a). Bile acids are polar sterols vital for lipid and vitamin absorption in vertebrates, and are known to be excreted in feces (Turley et al., 1997; Chiang, 2009; Griffiths & Sjovall, 2010). I compared the spectra and elution times of several pure bile acids, finding that the most abundant molecules within the female feces polar fraction were ω-muricholic acid (ω-MCA, Fig. 2.4b), β-muricholic acid (β-MCA, Fig. 2.4c), cholic acid (CA, Fig. 2.4d), and deoxycholic acid (DCA, Fig. 2.4e). These results suggested that bile acids might be the active polar ligands in fecal extracts.

I initially sought to determine whether the most abundant bile acids indicated by LC-MS were natural AOS ligands. CA is a primary bile acid produced by the liver, whereas DCA is a secondary bile acid produced by CA dehydroxylation by gut microbes (Fig. 2.5a) (Hagey & Krasowksi, 2013; Hoffman & Hagey, 2014; Dawson & Karpen, 2015). CA and DCA were delivered to the VNO at 10 μM, a concentration that for pure sulfated steroids produced strong but selective activation of AOS neurons (Meeks et al., 2010; Turaga & Holy, 2012). Both CA and DCA produced robust responses within the anterior AOB, and MCs exhibited specific tuning to these ligands (29 cells from 23 animals, Fig. 2.5b-d). Many MCs that responded to 10 μM CA or DCA were co-activated by 300-fold diluted female fecal extracts. Not all MCs that responded to 10 μM CA or DCA also responded to the diluted fecal extracts, suggesting that, at this dilution, the effective concentration of these ligands was less than 10 μM. Among the 20 MCs responsive to feces, CA, or DCA, 10% responded exclusively to CA, 35% responded exclusively to DCA, and 35% responded

exclusively to female feces. 15% of the cells examined responded to CA, DCA, and female feces (Fig. 2.5e). These results indicate that though CA and DCA are structurally similar, the AOS is capable of differentiating between these two compounds at 10 μM. Responses to ω-MCA, a rodent-enriched bile acid, were also observed, but were much more rare than for CA and DCA (Fig. 2.5f). A mixture of four pure bile acids, all at 1 mM, delivered directly to the nares *in vivo* effectively induced Fos expression in anterior AOB neurons (Fig. 2.6), confirming that these natural ligands activate the AOB following dilution and transport via the nasal mucus.

To directly investigate the concentrations at which CA and DCA were active, I evaluated the concentration-dependence of MC responses to DCA and CA in a subset of *ex vivo* experiments (4 cells from 3 animals, Fig. 2.7). Neurons that responded to both CA and DCA at 10 μM showed strong selectivity for CA at 3 μM and ceased to respond below 1 μM (3 cells from 3 animals Fig. 2.7a). A cell that was exclusively tuned to DCA failed to respond to either bile acid at concentrations below 10 μM (Fig. 2.7b). Thus, bile acids indeed produce selective, concentration-dependent MC activation, consistent with selective activation of different vomeronasal receptors by bile acids.

AOB Neurons Discriminate Between Male and Female Feces

Animal secretions (e.g., tears, urine) contain sex-specific AOS cues (Kimoto, et al. 2005; Haga et al., 2010; Haga-Yamanaka et al., 2014), and previous studies indicated that the bile acid pool also varies with sex (Turley et al., 2008). Therefore, I investigated whether male and female feces differentially activate the AOS. I delivered 300-fold diluted fecal

extracts from BALB/cJ males and females to male *ex vivo* preparations, and recorded sex-specific activation of AOB MCs (Fig. 2.8). Many MCs were selective for fecal extracts from a specific sex (Fig. 2.8a) and others responded to fecal extracts of both sexes (Fig. 2.8b). I classified MC tuning curves to male and female urine and feces using clustering algorithms, revealing variable tuning patterns (70 cells from 44 animals Fig. 2.8c). For 29 cells that responded to male or female feces, 34.5% of cells responded selectively to female feces, 20.7% responded selectively to male feces, and 44.8% responded nonselectively to both (Fig. 2.8d). I quantified the discriminability of male and female feces by AOB MCs, finding that, as a population, sex discrimination based on feces was lower than between female urine and feces, but that the presence of many MCs with high d' values was significant (67.9% of 100,000 simulated MC populations with randomly chosen tuning curves showed statistically lower discriminability than observed; Fig. 2.8e). These results indicated that there are sex-specific differences in the concentrations and/or identities of fecal chemosignals.

To determine whether male and female feces contained different bile acids, I analyzed male and female fecal extract polar fractions by LC-MS (Fig. 2.9). I found that one major sex-specific difference was the presence of chenodeoxycholic acid (CDCA) in male but not female fecal extracts (Fig. 2.9a-b). CDCA, like CA, is a primary bile acid produced in the liver, but CDCA and its secondary derivative lithocholic acid (LCA) are most commonly associated with non-rodent species (Moschetta, et al., 2005; Hagey et al., 2010; Hoffman et al., 2010). CDCA and LCA levels are low in most mice, which is thought to be due to rapid conversion of CDCA to muricholic acids in mice and other rodents (Botham & Boyd, 1983). The presence of CDCA in BALB/cJ male feces indicated that the detection of CDCA may be

one mechanism by which AOS neurons could discriminate between BALB/cJ males and females. Moreover, since CDCA is present in the feces of many heterospecifics, this bile acid may also contribute in a combinatorial fashion to other chemosensory discrimination tasks.

AOB Neurons Discriminate Between Conspecific and Heterospecific Bile Acids

I investigated AOB MC tuning to CDCA, mouse feces, and cat urine that had been collected after being in direct contact with feces. I found individual MCs that responded to 10 μM CDCA, male feces, and 100-fold dilutions of the cat urine+feces sample (Fig. 2.9c). I also investigated AOB neuronal tuning to LCA, which was not detectable in mouse feces, but is present in other species (Moschetta et al., 2005; Hagey et al., 2010; Hoffman et al., 2010). I encountered MCs that were responsive to both 10 μM CDCA and 10 μM LCA (Fig. 2.9d), as well as cells that were exclusively responsive to 10 μM LCA (Fig. 2.9e). I evaluated MC tuning to CA, DCA, CDCA, and LCA for 25 MCs exposed to all of these ligands using cluster analysis (Fig. 2.9f). MCs responding to CDCA and/or LCA rarely responded to female mouse feces (Fig. 2.9g). Pairwise comparisons of bile acid responsiveness showed that LCA-responsiveness was mutually exclusive with CA responsiveness (Fig. 2.9h). These results indicate that the AOS discriminates between bile acids present in conspecific and heterospecific feces.

Finally, I investigated MC tuning across all the polar sterols in the stimulus battery, including primary and secondary bile acids and sulfated glucocorticoids (Fig. 2.10a). Cluster analysis revealed rich combinatorial tuning across sterols, but clearly showed that MCs tuned

to bile acids were almost completely separable from MCs tuned to sulfated glucocorticoids (Fig. 2.10b). Overall, these results indicated that AOS bile acid tuning is not limited to molecules excreted by conspecifics, and that bile acids produce a complex combinatorial code in AOB MCs, similar to codes produced by other AOS ligand classes including sulfated steroids, major urinary proteins, formyl peptides, major histocompatibility proteins, and exocrine-secreting gland peptides (Kimoto et al., 2005; Leinders-Zufall et al., 2004; Spehr et al., 2006; Chamero et al., 2007; Liberles et al., 2009; Rivière et al., 2009; Nodari et al., 2008; Haga et al., 2010; Meeks et al., 2010; Turaga & Holy, 2012; Ferrero et al., 2013; Kaur et al., 2014).

Discussion

Despite two decades of research since the discovery of vomeronasal receptors, our understanding of the repertoire of ligands for this behaviorally-relevant neural pathway remains incomplete (Dulac & Axel, 1995; Herrada & Dulac, 1997; Matsunami & Buck, 1997; Ryba & Tirindelli, 1997; Kimoto et al., 2005; Chamero et al., 2007; Leinders-Zufall et al., 2004; Nodari et al., 2008; Liberles et al., 2009; Rivière et al., 2009; Haga et al., 2010; Ferrero et al., 2013). Technical improvements have made it possible to conduct AOS ligand screens using VSNs as bioassays (Leinders-Zufall et al., 2004; Chamero et al., 2007; Nodari et al., 2008; Papes et al., 2010; Isogai et al., 2011; Kaur et al., 2014; Fu et al., 2015). However, VSNs are notoriously noisy (Holy et al., 2000; Meeks et al., 2010; Arnson & Holy, 2011; Arnson & Holy, 2013) and require extensive controls to avoid false positive results. AOB MCs, in contrast, have dramatically improved signal/noise ratios by virtue of synaptic

pooling/averaging (Meeks et al., 2010). In this study, I utilized MC recordings from the *ex vivo* preparation (which maintains functional connectivity between the VNO and AOB) as the platform of a screen for AOS ligands.

Simple aqueous extraction procedures isolate AOS ligands from BALB/cJ female mouse feces that, at 300-fold dilutions, produce equivalent AOB neuronal activity to 100-fold diluted BALB/cJ female mouse urine. This indicates that mouse feces are rich in AOS ligands, and that the activity stimulated by these ligands is roughly equivalent to mouse urine, which is currently the best-known source of AOS ligands (Holy, et al., 2000; Leinders-Zufall et al., 2004; Chamero et al., 2007; Nodari, et al., 2008; Rivière, et al., 2009). The concentration-related differences in stimulus potency are likely related to the relative dryness of raw feces compared to mouse urine and the specific ratio of feces:water used in extractions. That said, feces are dry in the natural environment, so fecal ligands are likely to be highly concentrated before dissolution in nasal mucus. *In vivo* studies confirmed that dilute fecal extracts produced AOB activation similar to soiled bedding, further indicating that the fecal molecules in these aqueous extracts are biologically active. AOB MCs readily discriminate urine from feces, indicating that fecal chemosignals produce unique information in the AOS.

The discovery of feces as a source of distinct AOS ligands is noteworthy for several reasons. First, feces are plentiful in natural environments and can persist for long periods of time. Secondly, feces contain a biochemical readout of internal digestion through molecules that are distinct from other known classes of AOS ligands, such as urine and tears. Thirdly, there is increasing evidence that internal gut-brain feedback influences many neurobiological

processes (Mayer, Knight, Mazmanian, Cryan, & Tillisch, 2014). Our data reveal that fecal chemosignals also activate sensory processing neurons in the AOS, establishing a link between the gut physiology of an animal and its own brain (i.e., external gut-brain feedback) or another animal's brain (i.e., as a source of pheromones or kairomones).

MC recordings revealed that fecal chemosignals strongly activate the anterior AOB, which receives selective innervation from VSNs that express members of the V1R subfamily of VRs and certain formyl peptide receptors (Dietschi et al., 2013; Wagner et al., 2006; Rodriguez et al., 1999; Belluscio et al., 1999). Previous studies have strongly implicated V1Rs and the anterior AOB in the detection of low-molecular weight ligands, including urinary sulfated steroids (Meeks et al., 2010; Hammen et al., 2014). Urine- and feces-responsive AOB were physically collocated, and many AOB MCs were activated by both feces and urine. Taken together, these indicate that AOB neurons process information about urinary and fecal chemosignals in the same V1R-receiving subcircuit, and that these two distinct natural ligand sources produce partially overlapping information.

The majority of feces-driven AOS activity was maintained in the polar fraction of fecal extracts. A small population of MCs was co-activated by sulfated glucocorticoids and fecal extracts, suggesting that sulfated steroids may be fecal AOS ligands. LC-MS revealed no detectable sulfated steroids in the polar fraction of these extracts, but instead revealed the presence of bile acids, of which CA and DCA were among the most abundant. Bile acids are cholesterol derivatives that are produced in the liver and excreted into the gut, where they aid in the absorption of lipophilic substances and act as signaling molecules for a diverse number of metabolic processes (Hylemon, 2009; Russell, 2009; Kuipers, Bloks, & Groen, 2014; Li

and Chiang, 2014; Copple and Li, 2016). The complement of excreted bile acids in feces varies across sex and species, and secondary bile acids like DCA depend on dehydroxylation and other transformations by gut microbes (Turley et al., 1998; Hofmann et al., 2010; Philipp, 2011; Hagey and Krasowski, 2013; Hoffman & Hagey, 2014). As such, bile acids, as a class, possess many features that would make them potentially instructive chemosignals. That these molecules could be important chemosignals is not totally unprecedented, as bile acids are known to act as pheromones in fishes (Doving et al., 1980; Huertas et al., 2010; Buchinger et al. 2014). I found robust, selective, concentration-dependent activation of MCs by DCA and CA, and confirmed that a mixture of bile acids generates robust AOB following *in vivo* exposure. These data confirm that fecal bile acids are natural AOS ligands, and reveal that bile acid chemosignaling is conserved between fishes and mammals.

I tested the capacity of bile acids to serve as readouts of biologically relevant features (e.g., sex, species, etc.). First, I observed MCs that can discriminate between CA, a primary bile acid produced in the liver, and DCA, which is produced by gut microbes (Ridlon, Kang, and Hylemon, 2006; Hagey & Krasowski, 2013). This indicates that gut microbiota influence AOS activation by feces. Next, I investigated whether fecal ligands varied with sex. I compared MC tuning for BALB/cJ male and female fecal extracts and found that many MCs can discriminate between male and female feces at equal dilutions. Although there may be many ligands that underlie this effect, I confirmed by LC-MS that the primary bile acid CDCA, which was undetectable in female fecal extracts, was present in male fecal extracts. Many MCs that were activated by 10 μM CDCA were also activated by male, but not female,

feces. This indicates that CDCA is one ligand that varies with sex, and suggests that CDCA signaling may underlie sex discrimination from feces.

Whereas CA and DCA are present in a number of species and in both sexes, CDCA is rapidly converted to muricholic acids in rodents (Botham & Boyd, 1983). In other mammals, including mouse predators, CDCA is more prominent (Moschetta et al., 2005; Hagey et al., Hofmann et al., 2010). Consistent with these reports, I found that several MCs that responded to CDCA and male feces also responded to cat feces. Interestingly, I found that AOB responses to rodent-specific muricholic acids were quite sparse. In contrast, LCA, a secondary bile acid and CDCA metabolite that is absent in mice but present in other mammalian species, including humans, caused robust, highly selective MC activation at 10 μM. The biological significance of these differentially tuned MC populations remains to be discovered, but these data indicate that conspecific and heterospecific bile acids generate distinct signals in AOB neurons. Although additional studies will be necessary to test this hypothesis, these observations suggest that bile acids may also serve as kairomones.

MC tuning across bile acids and sulfated glucocorticoids shows that bile acids, similar to other classes of AOS ligands, activate the AOB with a specific, but complex, combinatorial code (Meeks et al., 2010; Hammen et al., 2014; Kaur et al., 2014; Fu et al., 2015). The capacity of the AOS to distinguish between individual bile acids that differ across biological states (e.g., sex, species, etc.) indicates that these ligands may drive state-specific behaviors. Future studies that investigate the behavioral impact of the combinations of bile acids found in natural samples will be necessary to identify these state-specific behaviors. It is worth noting that common themes of sex, species, and other biological state-related

differences exist across AOS ligand classes, and in nearly all cases, ligands that vary with biological state have been shown to influence mouse behaviors (Chamero et al., 2007; Haga et al., 2010; Papes et al., 2010; Ferrero et al., 2013; Haga-Yamanaka et al., 2014; Kaur et al., 2014; Boillat et al., 2015; Fu et al., 2015;). The apparent redundancy of sex, species, and other biologically relevant information across AOS ligand classes raises the question: what is the biological benefit of processing all this redundant information? The answer is likely to require detailed investigation of the specific social contexts in which the information is encountered.

In summary, I have demonstrated that feces are a natural source of AOS chemosignals, and that bile acids are a prominent class of AOS ligands. AOB MCs readily discriminate between components of feces and urine, and between male and female mouse feces. MCs discriminate between individual bile acids that vary with sex, species, and gut microbiota. This discovery reveals a signaling pathway linking gut physiology to the brain, and opens up new avenues for studying the impacts of gut metabolism on mammalian behavior.

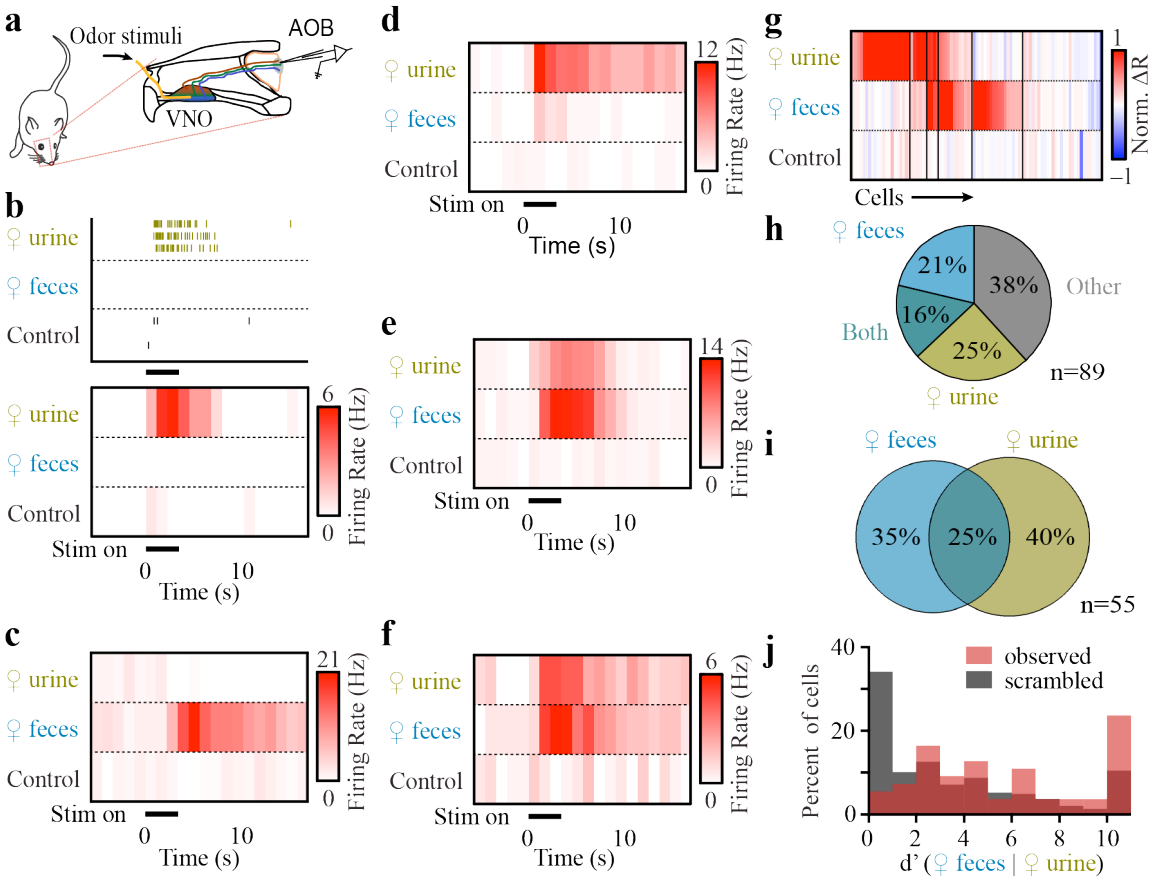

Figure 2.1 Female Fecal Extracts Activate the AOS

a: Overview of the AOS and the *ex vivo* preparation.
b: Example single-unit recording of a male mouse AOB neuron that responded to female mouse urine. Top is a raster plot, bottom is an average peristimulus time histogram (PSTH) from the same cell.
c: An AOB neuron exclusively responsive to female fecal extracts.
d: A neuron that selectively responded to urine.
e: Neuron that selectively responded to feces. (f) A cell that responded equally to both urine and feces.
g: Heat map of normalized change in firing rate (Norm. ΔR) following VNO stimulation with female urine or feces. Thin black lines indicate divisions between clusters (89 cells from 56 animals).
h: Percentage of cells that responded exclusively to urine, exclusively to feces extracts, to both urine and feces extracts, or to other compounds in the stimulus battery (89 cells from 56 animals).
i: Venn diagram of response overlap for the pool of neurons that responded to urine and/or feces (55 cells from 40 animals).

j: Histogram showing the discriminability index (d^{l}) for the observed AOB neuron population (red) compared to the mean of 100,000 scrambled populations (gray; 55 cells from 40 animals).

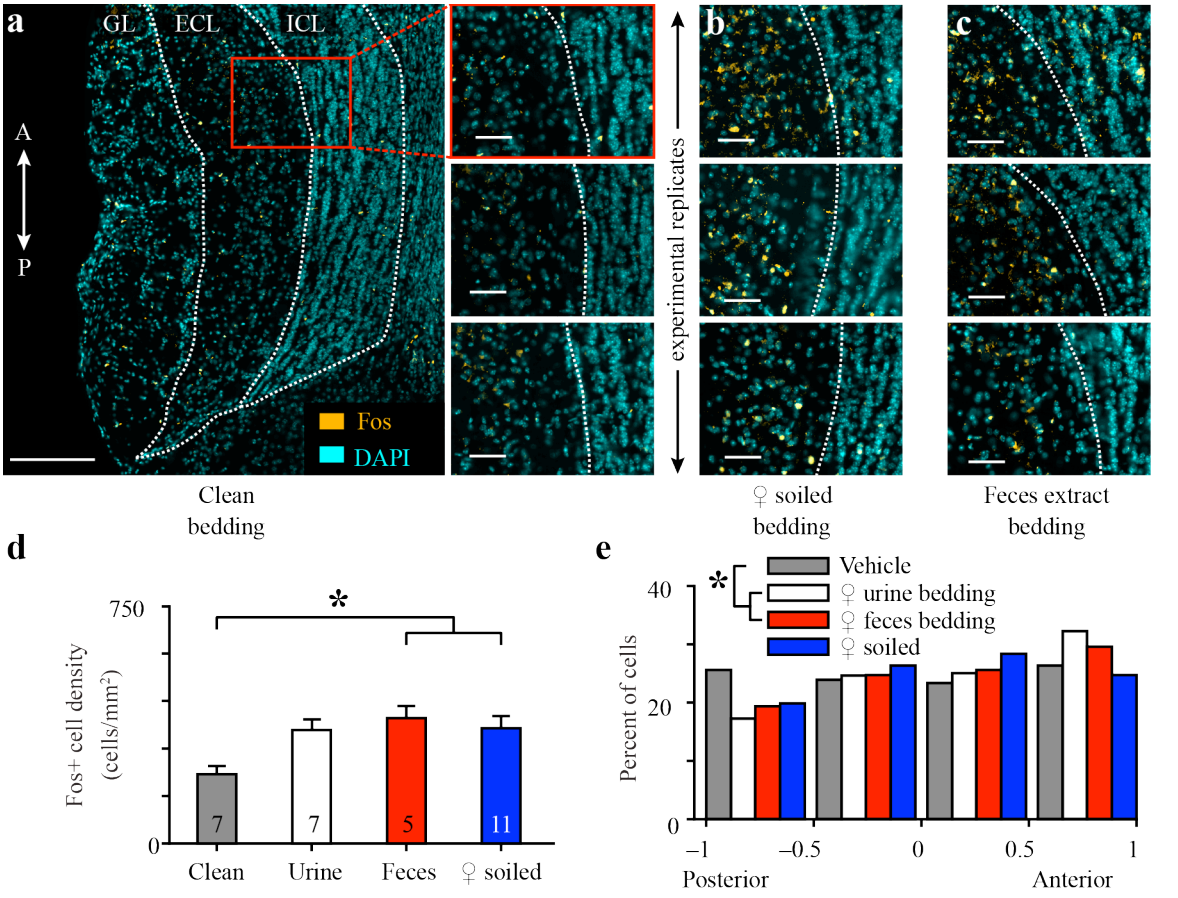

Figure 2.2 Fecal Extract Activates the AOS *In Vivo*

a: Left: Fluorescence micrograph showing Fos immunostaining (orange) in the AOB following 10 minute *in vivo* exposure to clean bedding. DAPI counterstaining is shown in cyan. Scale bar 200 µm. A: anterior. P: posterior. Right: zoomed views of the ECL/ICL border for the image shown in (a) (top) and two other experimental replicates (middle, bottom). Scale bars: 50 µm.
b: Views of the ECL/ICL border for animals exposed to BALB/cJ female soiled bedding (3 replicates). Scale bars: 50 µm.
c: Views of the ECL/ICL border for animals exposed to feces-extract doped bedding (3 replicates). Scale bars: 50 µm.
d: Total Fos+ neuron density following *in vivo* exposure to clean bedding (gray), urine-doped bedding (UB, white), feces extract-doped bedding (FB, red), and BALB/cJ female soiled bedding (blue) conditions. Asterisk indicates $p < 0.05$ (one-way ANOVA corrected for multiple comparisons). Overlaid numbers indicate replicates.
e: Histogram of Fos+ neuron position along the AOB anterior-posterior axis. Asterisk reflects $p < 0.05$ (Wilcoxon rank sum test, cumulative data from 20 animals).

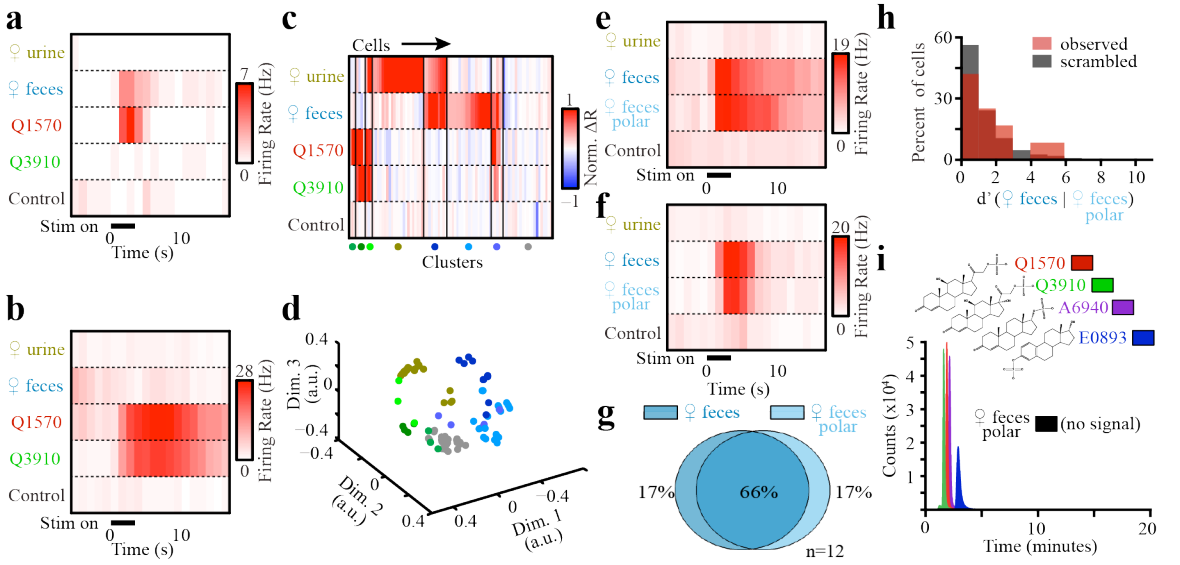

Figure 2.3 Female Fecal Extract Activity Is Not Due to Sulfated Steroids

a: PSTH of AOB neuron that responded to 10 µM corticosterone-21-sulfate (Q1570) and 300-fold dilute BALB/cJ female mouse fecal extract.
b: Cell that responded to 10 µM Q1570 and 10 µM hydrocorticosterone-21-sulfate (Q3910) but not to female mouse fecal extract.
c: Heat map of responses to female mouse urine, fecal extract, Q1570, and Q3910. Thin black lines indicate divisions between clusters (87 cells from 55 animals).
d: Multidimensional scaling of the AOB responses to feces, urine, and glucocorticoids. Colors correspond to the clusters in (c).
e-f: Two AOB neurons that responded to female mouse feces (1:300 dilution) and female mouse feces polar fraction (1:100 dilution).
g: Venn diagram showing overlapping responses between female fecal extract and its polar fraction (12 cells from 6 animals).
h: Histogram of the d' values for female feces extract and its polar fraction. Red bars indicate the observed d'. Gray bars indicate the average d' of 100,000 shuffled populations.
i: Overlaid extracted ion chromatograms (EICs) showing [M-H]- signals for standards: Q3910 ($[C_{21}H_{30}O_8S\text{-}H]^-$, green), Q1570 ($[C_{21}H_{30}O_7S\text{-}H]^-$, red), epitestosterone-17-sulfate (A6940, $[C_{19}H_{28}O_5S\text{-}H]^-$, purple), 17α-estradiol-3-sulfate (E0893, $[C_{18}H_{24}O_5S\text{-}H]^-$, blue), and female feces (black, not visible). Female feces polar extract at these $[M\text{-}H]^-$ values produced no detectable signal.

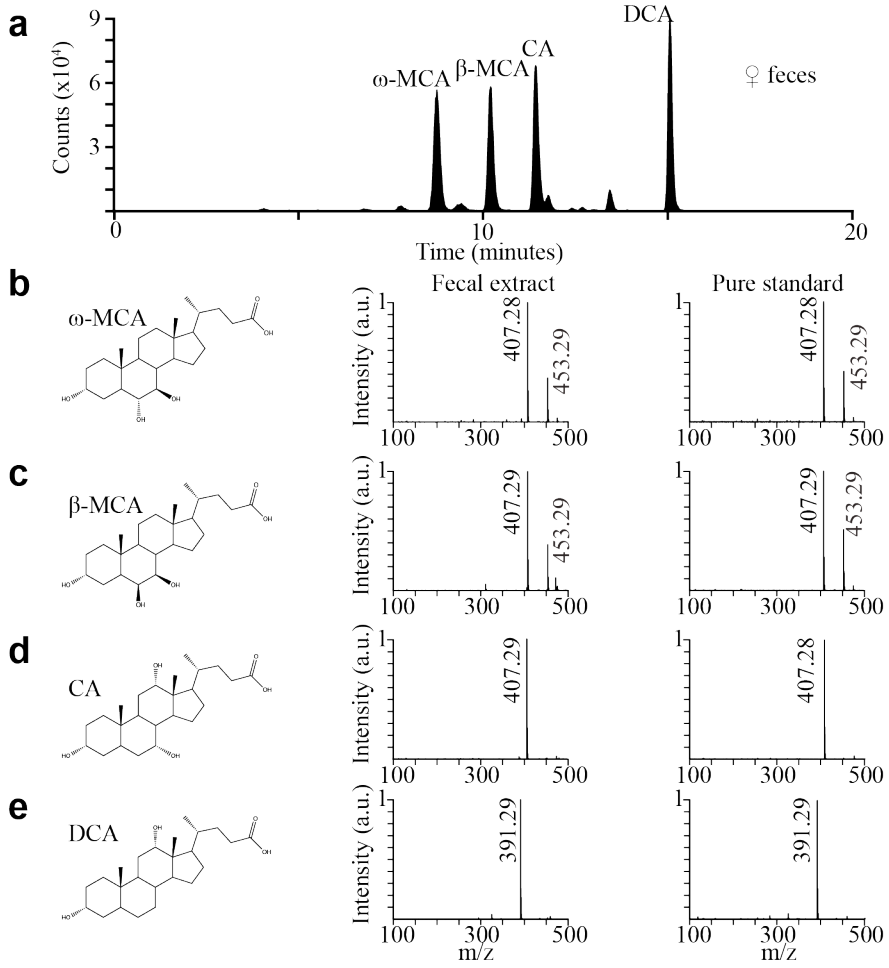

Figure 2.4 Female Fecal Extracts Contain Unconjugated Bile Acids

a: Extracted ion chromatograms (EICs) showing $[M-H]^-$ signals for $C_{24}H_{40}O_4$ (deoxycholic acid) and $C_{24}H_{40}O_5$ (cholic and muricholic acids) in female mouse fecal extract. Identified compounds are indicated above each peak.
b-e: Mass spectra of peaks for bile acid standards and fecal extracts. Peak times refer to times from (a). Peaks at m/z 453.29 indicate the presence of formate adducts.
b: ω-muricholic acid (ω-MCA at 8.8 minutes
c: β-muricholic acid (β-MCA) at 10.2 minutes.
d: Cholic acid (CA) at 11.4 minutes.
e: Deoxycholic acid (DCA) at 15.0 minutes.

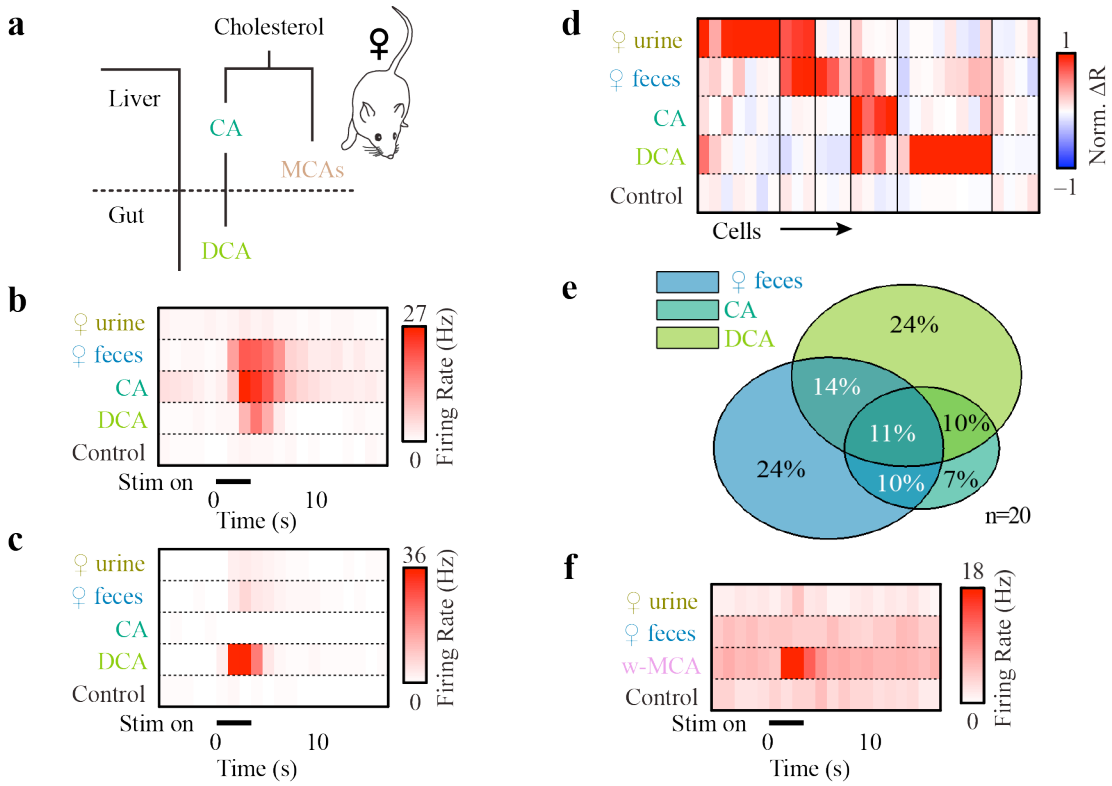

Figure 2.5 The AOS Is Activated by Bile Acids Present in Female Mouse Feces

a: Simplified synthesis pathway for CA, DCA, and MCAs in the female mouse.
b: PSTH of a cell that responded to feces, CA, and DCA.
c: A cell that responded to DCA but not to CA.
d: Heat map of AOB responses to urine, feces, CA, and DCA. Thin black lines indicate divisions between clusters (29 cells from 14 animals).
e: Venn diagram of responses to feces, CA, and DCA (20 cells from 11 animals).
f: An AOB neuron that responded to the rodent-specific bile acid ω-MCA.

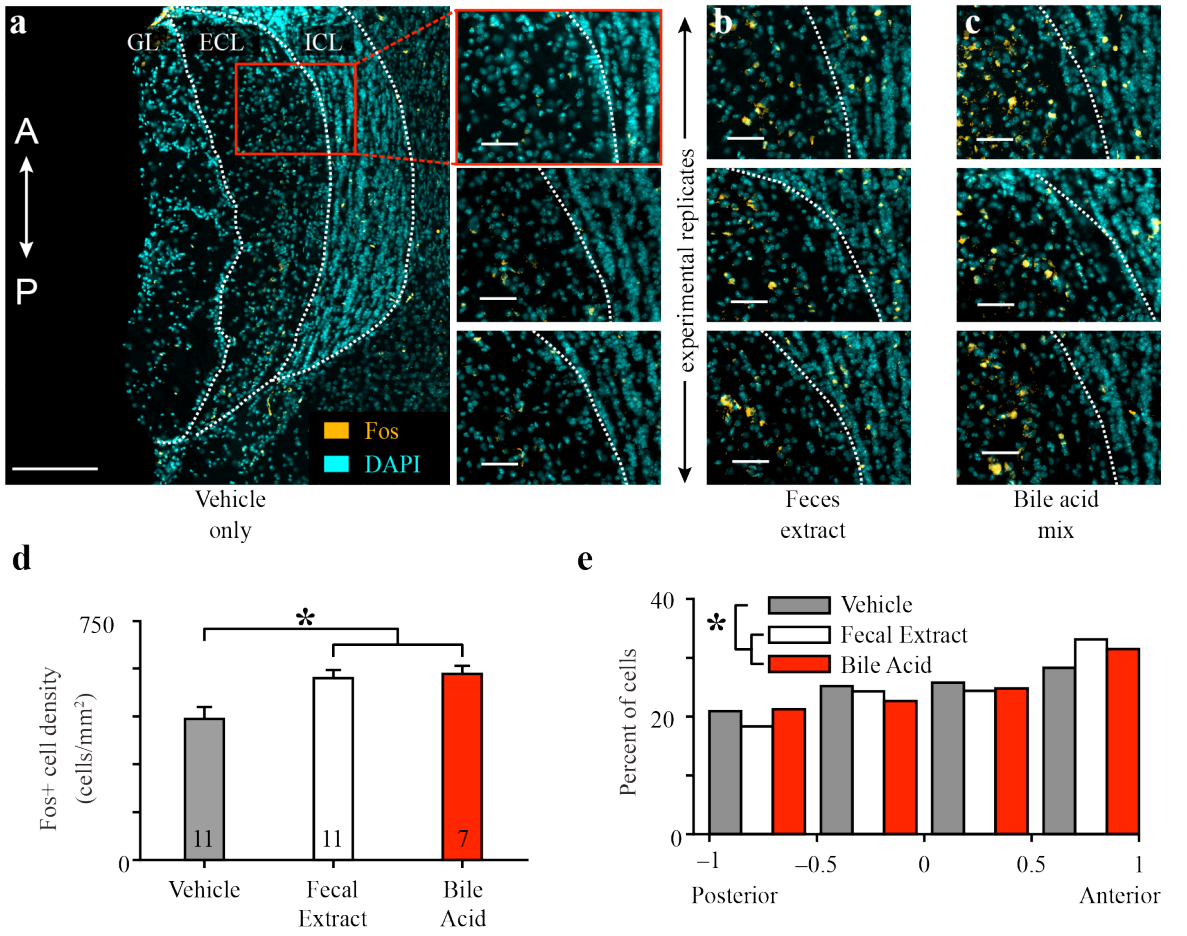

Figure 2.6 Bile Acids Activate the AOS *In Vivo*

a: Left: Fluorescence micrograph showing Fos immunostaining (orange) in the AOB 90 minutes after direct stimulation of the nares with methanol/water (vehicle). DAPI counterstaining shown in cyan. Scale bar 200 µm. Right: zoomed views of the ECL/ICL border for the image shown in Panel a (top) and two other experimental replicates (middle, bottom). Scale bars: 50 µm.
b: Views of the ECL/ICL border for animals exposed to BALB/cJ female feces extract dissolved in vehicle (3 replicates). Scale bars: 50 µm.
c: Views of the ECL/ICL border for animals exposed to a mixture of 4 bile acids (CA, DCA, CDCA, and LCA), each at 1 mM (3 replicates). Scale bars: 50 µm.
d: Fos+ cell density for direct stimulation conditions. Asterisk indicates $p < 0.05$ (one-way ANOVA corrected for multiple comparisons). Overlaid numbers indicate replicates.
e: Histogram of Fos+ neuron position along the AOB anterior-posterior axis. Asterisk reflects $p < 0.05$ (Wilcoxon rank sum test, cumulative data from 15 animals).

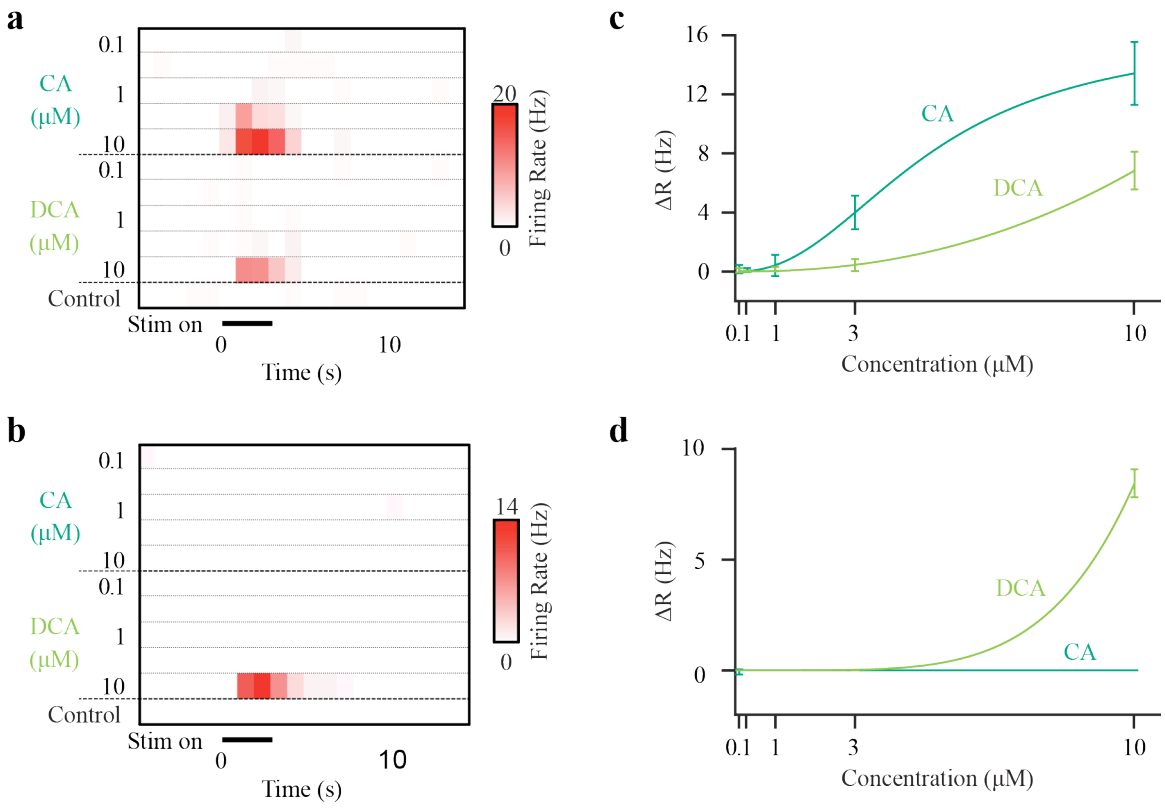

Figure 2.7 Dose-Responses to CA and DCA

a: PSTH of an AOB neuron that responded to both CA and DCA across a range of concentrations.
b: PSTH of a cell that responded to DCA only at 10 μM.
c-d: Dose response curves for the cells in (a) and (c), respectively (4 cells from 3 animals).

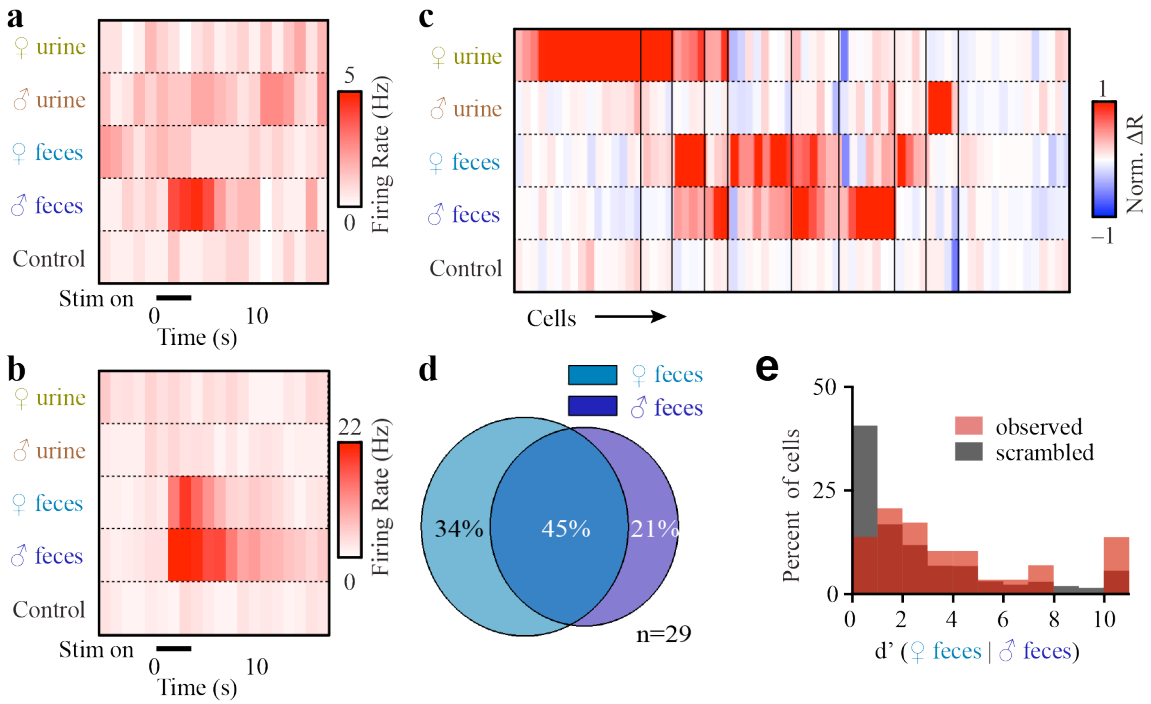

Figure 2.8 AOB Neurons Discriminate Between Male and Female Fecal Extracts

a: An AOB neuron that responded exclusively to male mouse fecal extract.
b: An AOB neuron that responded to both female and male feces.
c: Heat map of neuronal responses to female and male urine and feces. Thin black lines indicate cluster divisions (70 cells from 44 animals).
d: Venn diagram of AOB responses to female and male feces (29 cells from 23 animals). **e**: Histogram of d' values for male and female mouse feces. Red bars indicate the observed d' values. Gray bars indicate the average d' for 100,000 shuffled populations (29 cells from 23 animals).

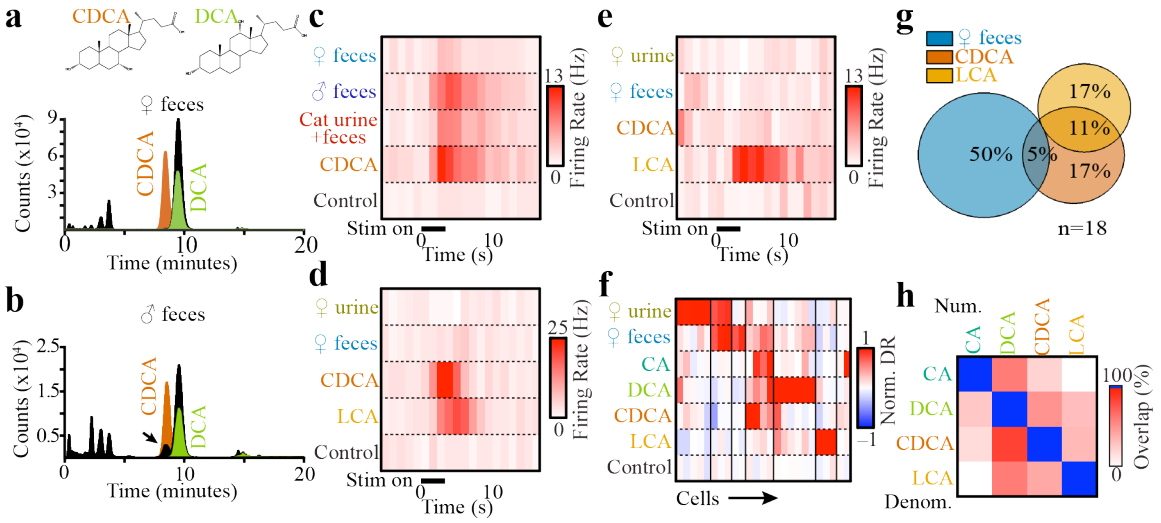

Figure 2.9 CDCA and LCA Are Detected and Discriminated by the AOS

a: EICs showing $[M\text{-}H]^-$ signals for $C_{24}H_{40}O_3$ (LCA) and $C_{24}H_{40}O_4$ (DCA) for female mouse fecal extract (black). Overlaid are EICs for CDCA (orange) and DCA standards (green). Female fecal extract contains DCA and not CDCA.
b: EICs showing $[M\text{-}H]^-$ signals for $C_{24}H_{40}O_3$ (LCA) and $C_{24}H_{40}O_4$ (DCA) for male fecal extract (black). Overlaid are EICs for CDCA (orange) and DCA standards (green). Male feces contain both CDCA and DCA.
c: An AOB neuron that responded to male mouse feces, a cat urine+feces mixture, and CDCA.
d: PSTH of a cell that responded to CDCA and its derivative LCA.
e: A cell that responded to LCA and not to CDCA.
f: Heat map of AOB responses to female mouse urine, female feces, CA, DCA, CDCA, and LCA. Thin black lines indicate divisions between clusters (25 cells from 13 animals). **g**: Venn diagram of shared responses among female feces, CDCA, and LCA (18 cells from 10 animals.
h: Pairwise comparisons of neuronal responsiveness to CA, DCA, CDCA, and LCA. Percentages indicate the number of neurons that were co-activated by the row/column pair divided by the total pool of neurons that responded to the row stimulus (25 cells from 13 animals).

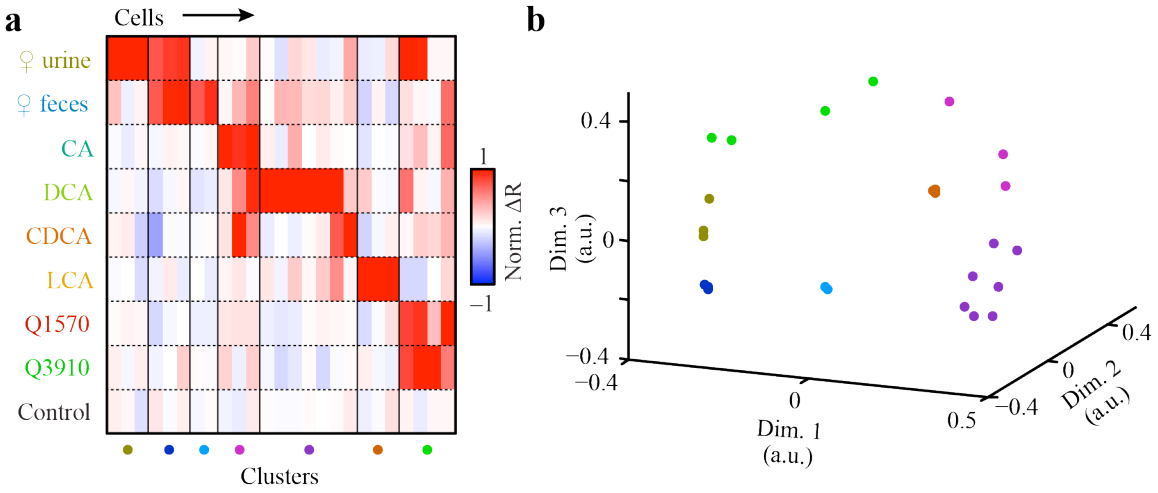

Figure 2.10 Tuning of AOB Neurons to Female Mouse Urine and Feces, Sulfated Glucocorticoids, and Bile Acids

a: Heat map of AOB responses to female mouse urine, female feces, CA, DCA, LCA, Q1570, and Q3910. Thin black lines indicate cluster divisions (25 cells from 13 animals). **b**: Multidimensional scaling of the AOB responses to feces, urine, glucocorticoids, and bile acids. Colors correspond to the clusters in (a).

CHAPTER THREE

NORADRENALINE MODULATES ACCESSORY OLFACTORY SYSTEM PROCESSING

Summary

NA is a known and potent modulator of neural activity in multiple brain regions, including the olfactory bulbs. In the AOB, NA is hypothesized to increase levels of the inhibitory neurotransmitter GABA leading to the suppression of spontaneous and evoked activity. I delivered NA to *ex vivo* preparations of the early AOS to test this hypothesis and discovered that NA had heterogeneous and nuanced effects on AOB activity. I observed an overall suppression of stimulus-evoked activity, with limited effects on spontaneous activity. NA specifically promotes a form of evoked activity suppression that monotonically develops over repeated deliveries of a stimulus. This monotonic suppression is heterogeneous and is not seen with every stimulus response, even within a single cell. In a subset of AOB principal cells there is a significant increase in spontaneous activity that leads to a decrease in chemosignal discriminability. This chapter is reproduced, with modifications, from Doyle and Meeks, 2017.

Introduction

NA and other neuromodulators strongly influence neuronal function throughout the brain. NA is released in response to states of arousal and novelty, and contributes to region-

specific forms of neural plasticity (Bouret & Sara 2005). Such impacts are seen even at early stages of sensory processing, indicating that centrifugal NAergic signaling influences sensory perception and behavioral responses to sensory stimuli (Bennet, Huguenard, & Prince, 1998; Bouret & Sara 2002; Devilbiss & Waterhouse 2004; Hirata et al., 2006; Linster & Fontanini, 2014). In the mammalian MOB and AOB, NA is released by centrifugal fibers originating in the LC (Fallon & Moore, 1978; Rosser & Keverne, 1985; McLean et al., 1989). In the AOB, the first and only dedicated neural circuit in the AOS, NA release occurs during social encounters, including mating, and is critical for the expression of certain forms of pheromone-mediated social learning (Bruce, 1959; Rosser and Keverne, 1985; Brennan & Keverne, 1997; ; Otsuka et al., 2001; Matsuoka et al., 2004; Brennan & Binns, 2005; Brennan, 2009; Linster, et al. 2011). Because it directly links the sensory periphery to the limbic system, the AOB is an attractive system in which to investigate the influence of NAergic signaling on information processing and behavior.

The mouse AOS detects and interprets information about social chemosignals, including pheromones (intra-species social cues) and kairomones (cross-species cues that benefit the detector; reviewed in Liberles, 2014). In the natural environment, mice encounter AOS ligands in blends of socially informative cues, which typically originate in animal excretions like urine (Leinders-Zufall et al., 2004; Chamero et al., 2007; Nodari et al., 2008; Fu et al., 2015), tears (Kimoto et al., 2005), or feces (Doyle et al., 2016). Certain environmental nonvolatiles elicit innate behaviors, including courtship and mating (Roberts et al., 2010; Haga-Yamanaka et al., 2014; Fu et al., 2015), territorial aggression (Chamero et al., 2007; Kaur et al., 2014; Hattori et al., 2016), and predator avoidance (Papes et al., 2010).

The strong behavioral influence of the AOS is well appreciated, but the circuit mechanisms linking sensory detection to behavior remain weakly understood.

The behavioral impacts of NA release in the AOB can be profound. For example, in female mice, chemosensory cues from a recently mated male drive AOB activity coincident with centrifugal NAergic drive from the LC. The coincidence of peripheral sensory input and centrifugal neuromodulation can generate a stable memory of the mated male's chemosignals, which prevents subsequent exposure to the mated male or its excretions from triggering pregnancy termination (Rosser & Keverne, 1985; Brennan et al., 1995; Brennan & Keverne, 1997; Brennan & Binns, 2005). Despite its importance, the capacity of NAergic signaling to shape information processing has not been thoroughly investigated.

Several studies have provided insights into cellular and synaptic changes that accompany neuromodulatory signaling via NA, acetylcholine, and oxytocin receptors, (Kaba & Keverne, 1988; Araneda & Firestein, 2006; Fang, Quan, and Kaba, 2008; Smith et al., 2009; Smith & Araneda 2010; Smith et al., 2015). A common conclusion of each of these inquiries is that neuromodulation changes the balance of excitation and inhibition between the principal neurons of the AOB, MCs, and local GABAergic interneurons (Kaba & Keverne, 1988; Brennan & Keverne, 1997; Brennan, 2009).

AOB MCs and interneurons communicate via a dense network of reciprocal, dendrodendritic synapses (Jia, Chen, & Shepherd, 1999; Taniguchi & Kaba, 2001; Larriva-Sahd, 2008). At these synapses, glutamate release by MC dendrites excites terminals of local GABAergic interneurons that can, in turn, release GABA back onto the same MC dendrite (Rall, Shepherd, Reese, Brightman, 1966; Jia et al., 1999; Taniguchi & Kaba, 2001).

Targeted investigation into the impacts of NA on AOB MCs and interneurons revealed opposing effects on MC and interneuron excitability (Otsuka et al., 2001; Araneda & Firestein, 2006; Brennan, 2009; Leszkowicz et al., 2012). In the MOB NA's effects are also variable, with some studies showing a NA-driven increase in MC activity (Jahr & Nicoll, 1982; Trombley & Shepherd, 1992; Jiang et al., 1996; Hayar et al., 2001), other studies showing a decrease (Shea, Katz, Mooney, 2008; Zimnik, Treadway, Smith, Araneda, 2013), and yet other studies showing mixed effects (Nai et al., 2009). One hypothesis emerging from the AOB studies is that NAergic activation depolarizes IGCs, comprising the largest AOB interneuron population, resulting in increased GABAergic inhibition of MCs (Araneda & Firestein, 2006; Smith et al., 2009). However, it remains unclear how NA impacts MC activity in the context of chemosensory processing. For example, it is not known whether NA causes widespread, nonselective suppression of MC activity, or whether MC suppression is selective for the cues present during NA release.

Here, I report the results of a targeted investigation into the impacts of NA on MC chemosensory tuning. To do so, I utilized *ex vivo* preparations of the functionally connected VNO and AOB (Meeks & Holy, 2009; Meeks et al., 2010; Doyle et al., 2014). Single unit electrophysiological recordings of MCs during naturalistic chemosensory stimulation of the VNO revealed heterogeneous effects of NA on MC spontaneous and stimulus-driven activity. I found that the net impact of NAergic signaling in the AOB was a reduction in the stimulus-evoked MC activity and chemosensory discriminability. These results support the hypothesis that NA sculpts MC activity via inhibition, but the non-uniformity of MC responses to NA may help to reconcile seemingly contradictory models of pheromone learning.

Materials and Methods

Animals

All procedures were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. Mouse urine and feces were collected from male and female BALB/c mice aged 6 to 12 weeks. All electrophysiological experiments were conducted on C57Bl/6J and B6D2F1 male mice aged 6 to 10 weeks.

Urine and Feces Collection and Chemosignal Extraction

Male and female urine and feces were collected from 20 female and 10 male BALB/c mice over approximately 2 months. Mice were suspended in a wire-bottomed cage over liquid nitrogen for 3-8 hours per day. At the end of collection, frozen urine and feces were collected and stored at -80°C until extraction. Domestic cat urine was purchased from BioreclamationIVT (Westbury, NY). According to the vendor, cat urine was collected in a manner such that it came in contact with cat feces prior to collection.

Urine was processed as previously described (Nodari et al. 2008; Meeks et al., 2010). In brief, the urine was thawed, pooled, and centrifuged at 80 x g for 2 minutes. The supernatant was removed and filtered through a 0.22 μM filter, aliquoted, and stored at -80°C. Freshly unfrozen mouse and cat urine were diluted 1:100 in Ringer's saline before experiments.

Mouse fecal extracts were prepared as described previously (Doyle et al., 2016). Feces were diluted 1:10 in dH_2O (w/v), homogenized, and left overnight at 4°C on an orbital shaker. The fecal slurry was then homogenized and centrifuged twice at 2400 x g for 10

minutes, and 2800 x g for 30 minutes. The supernatant was filtered through a 0.22 μM filter, aliquoted, and stored at -80°C. Freshly unfrozen feces extracts were diluted 1:300 in Ringer's saline before experiments.

Reagents and Solutions

Ex vivo preparations were superfused with artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 3 *myo*-inositol, 2 sodium pyruvate, and 0.4 sodium ascorbate. For the duration of *ex vivo* experiments the peripheral sensory epithelium was continuously perfused with a solution of Ringer's saline containing (in mM): 115 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 $NaHCO_3$, 10 HEPES, and 10 glucose. All stimuli were diluted into this Ringer's saline.

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Corticosterone 21-sulphate (Q1570) and hydrocortisone 21-sulphate (Q3910), epitestosterone sulphate (A6940), testosterone sulphate (A7010), and 17α-estradiol sulphate (E0893) were purchased from Steraloids (Newport, RI). Q1570 was prepared in water, while all other sulfated sterols were dissolved in methanol to a concentration of 20 mM. Steroids were diluted to 10 μM in Ringer's immediately before each experiment. Sulfated androgens and estrogens were omitted from some batteries.

Pharmacology

NA (Abcam, Cambridge, MA) was prepared in water to a concentration of 10 mM, and diluted to a concentration of 10 μM in aCSF for experiments. The rate of superfusion

was 6-10 mL/min, and the aCSF stream was aimed directly at the AOB surface. This physical setup encourages convective exchange at the AOB surface, and was previously shown to allow for the pharmacological disruption of GABAergic signaling at drug concentrations comparable to those used in brain slice experiments (Meeks and Holy, 2009). These *ex vivo* conditions are similar – if not slightly improved compared – compared to *in vivo* pharmacological experiments that were effective at disrupting NA signaling in the olfactory bulb (Eckmeier & Shea, 2014). After completing 3 trials of the stimulus battery to establish baseline responses, NA was washed in for 10 minutes without stimulus delivery. The 10-minute wash-in is similar to the time courses utilized *in vitro* (Araneda & Firestein, 2006; Meeks & Holy, 2009; Smith et al., 2009). After the wash-in period, a second stimulus battery was delivered to the VNO that contained the same stimuli in a different order.

Because these experiments lasted up to 90 minutes, it was important to control for potential run-down (or run-up) of spontaneous activity and/or stimulus responses over time. Control recordings were performed using the exact same wash-in procedures used to deliver NA to the tissue, but NA was omitted from the superfusing aCSF. Statistical tests of NA effects were compared to these controls (see Data Analysis and Statistics).

Bath application of NA could theoretically lead to unintended activation of adrenergic receptors in the VNO. This is made unlikely, however, because my setup allows continuous, independent perfusion of the VNO with a drug-free Ringer's saline solution such that 1-2 VNO volumes are exchanged per second, exiting the VNO into the bath (i.e., generating a constant stream of drug-free saline that starts inside the VNO and exits into the bath). Moreover, in my *ex vivo* preparation the VNO is left inside its outer bony capsule, which

serves as an additional barrier for NA diffusion into the VNO from the bath. Combined, these aspects of my *ex vivo* prevent bath-applied pharmacological reagents from passively diffusing into the VNO.

Ex Vivo Preparations

Ex vivo preparations were performed as previously described (Meeks & Holy 2009; Doyle et al., 2014). Mice were anesthetized with isofluorane and decapitated into ice-cold aCSF containing an additional 9 mM of $MgCl_2$ to limit excitotoxic damage. Half of the mouse skull from the snout to the olfactory bulbs was dissected out, and adhered to a small plastic plank with Vetbond surgical glue (3M, St. Paul, MN). The plastic plank was inserted into custom-built dissection chamber and superfused with room temperature aCSF. A secondary microdissection was performed to expose the vomeronasal nerves and accessory olfactory bulb. A 0.0045-inch internal diameter polyimide cannula was placed into the VNO with a steady stream of fresh Ringer's saline at a rate of approximately 0.2 mL/min, driven by a pressurized stimulus delivery system (Automate Scientific, Berkeley, CA). The cannula was subsequently used for the delivery of stimulus batteries to the VNO. Batteries consisted of stimuli delivered in a random, interleaved order produced by custom software written in MATLAB (MathWorks, Natick, MA). Stimuli were delivered for 3 times per battery for 3 seconds with a 12 second wait period between stimuli.

Electrophysiology

Extracellular recordings were made with glass electrodes with a resistance of 1.5 to 6 MΩ as previously described (Meeks & Holy 2009; Meeks et al., 2010). Electrodes were filled with aCSF and advanced into the AOB by a micromanipulator, with distances measured by a micrometer (Siskiyou Corporation, Grants Pass, OR). All recordings were made from neurons in the AOB ECL (between 125 and 375 μM from the AOB surface) that produced large, positive-going spikes, consistent with MCs (Meeks & Holy 2009; Meeks et al., 2010). Signals were amplified through a Cornerstone BVC-700A amplifier (Dagan Corporation, Minneapolis, MN), converted to digital signals by a data acquisition card (National Instruments, Austin, TX), and saved with custom LabVIEW software. Recordings were sorted for single-unit activity in custom MATLAB programs per previously described reports (Meeks & Holy 2009; Meeks et al. 2010). Cells that responded to at least one stimulus and that had separable waveforms lasting throughout the entire baseline and NA application period and were considered for analysis.

Data Analysis and Statistics

All data analysis was performed with custom MATLAB software. Electrophysiological responses to VNO stimulation were analyzed during a 3 second window. Because subtle differences in cannula placement and pneumatic pressure across experiments resulted in small shifts in the time delay between stimulus valve switching and the onset of neuronal activity in the AOB, I chose the onset of the 3-second averaging window based on the spike timing statistics. Specifically, the window was chosen at the time

point at which the instantaneous spike frequency (as measured by inter-spike intervals, or ISIs), increased beyond the 95 percent confidence interval expected from measurements of the cell's spontaneous activity. Spontaneous activity for each cell was calculated by determining the spontaneous rate in a defined 3-second window before each stimulus onset. Statistical significance for spontaneous rate changes between baseline and test conditions was determined by Mann-Whitney-Wilcoxon tests.

The change in spike firing rate (ΔR) was calculated by subtracting the baseline-firing rate from the average firing rate during the 3-second window. I restricted my analysis to strong and reproducible responses by setting my threshold for significance to include only those ΔR values that exceeded 2 Hz and were statistically significant ($p < 0.05$) compared to responses to control Ringer's saline (unpaired, two-tailed Student's *t*-test). Cells that did not demonstrate responses exceeding these criteria were excluded from analysis (32/55 total cells). ΔR ratios were calculated by calculating the mean change in ΔR between the control and test conditions, then dividing by the mean ΔR in the baseline condition. To avoid divide-by-zero errors in the ratio calculations, all ΔR values less than a value of 1 Hz were replaced with a value of 1 Hz. Within-cell statistical significance was evaluated by paired Student's *t*-tests. Across-population differences were evaluated by Mann-Whitney-Wilcoxon tests.

Stimulus response suppression and enhancement were categorized as either monotonic or non-monotonic. Stimulus suppression was categorized as monotonic if the response to each stimulus repeat during a single battery was decreased compared to the previous repeat. Monotonic enhancement exhibited the reverse trend (activity to each stimulus repeat was increased compared to the previous stimulus). Stimulus responses were

categorized as undergoing non-monotonic suppression or enhancement if the responses did not undergo monotonic suppression or enhancement, but did have significantly different average responses between the two batteries by an unpaired, two-tailed Student's *t*-test. Per-cell modulation was categorized as being enhanced or suppressed if it experienced monotonic or non-monotonic changes to any of the stimuli to which it responded. Any cell that showed enhancement to one stimulus and suppression to another was considered to have a mixed response.

The discriminability of a response to baseline firing was determined using a discriminability index (d', Davison & Katz, 2007; Gale & Perkel 2010). The sensitivity index was calculated by the formula:

$$d' = \frac{\Delta R_s - \Delta R_c}{\sqrt{\frac{\sigma_s^2 + \sigma_c^2}{2}}}$$

where ΔR_s represents the mean firing response to a given stimulus and ΔR_c represents the mean firing response to the paired Ringer's control. σ_s^2 and σ_c^2 are the across-trial variances of ΔR_s and ΔR_c, respectively.

A shuffle test was used to determine whether response enhancement or suppression was statistically different than expected from controls (in which cells experienced the same procedures as in NA conditions, but no NA was applied; see Pharmacology). For this test, the percentage of cells or stimulus responses showing enhancement, suppression, etc. was calculated from the control recordings. These control percentages were used as the basis for the generation of 100,000 simulated data sets (containing the same number of observations as my experimental data) in which each simulated cell was assigned the identity of enhanced,

suppressed, or unchanged based on the control percentages. Random assignment into each category was achieved using MATLAB's uniform distribution random number generator ("rand"). These simulated data sets established a distribution of values for the amount of enhancement and suppression expected in the absence of NA. I calculated the statistical probability of observing increased levels of enhancement or suppression in NA conditions compared to controls using the formula:

$$p_{shuffle} = \frac{(N_{sim \geq obs}) + 1}{N_{sim} + 1}$$

where $N_{sim \geq obs}$ is the number of simulated sets in which the number of cells or stimuli showing suppression or enhancement exceeded observations, and N_{sim} is the total number of simulated sets (North, Curtis, and Sham, 2002). If the observations indicated a decrease in enhancement or suppression, I used the following formula:

$$p_{shuffle} = \frac{(N_{sim \leq obs}) + 1}{N_{sim} + 1}$$

where $N_{sim \leq obs}$ is the number of simulated sets in which the number of cells or stimuli showing suppression or enhancement was less than observations. These results were effectively equivalent to those expected from a binomial distribution (MATLAB's "binocdf" function). Shuffle test and binomial p-values are reported for each comparison.

Results

Studying Neuromodulation and Sensory Tuning in the AOS Ex Vivo Preparation

A major limitation of slice-based electrophysiological studies of neuromodulation is a lack of capacity to investigate neuronal responses to naturalistic stimuli. The *ex vivo* preparation of the AOS retains the functional connections between the peripheral sensory neurons in the VNO and their downstream targets in the AOB. This preparation also avoids severing intrinsic fibers of the AOB, leaving it physically intact. In these experiments, chemosensory stimuli were delivered to the VNO through a thin cannula while extracellular single-unit electrophysiological recordings of AOB MCs were made (Fig. 3.1a). This reduced AOS preparation is uniquely suited for examining the effects of neuromodulators on stimulus tuning because it allows pharmacological agents to be applied via the rapidly circulating bath while the periphery is stimulated with defined concentrations of chemosignals (e.g., pure sulfated steroids) and/or chemosignal blends (e.g., dilute mouse urine).

I delivered a panel of known AOS chemosignals to the VNO in 3 blocks of randomized, interleaved trials to the *ex vivo* preparation before ("baseline") and during ("test") 10 μM NA delivery to the AOB (Fig. 3.1b). These experiments required sustained isolation of spikes from a single neuron over relatively long time periods (approximately one hour per cell). I therefore performed parallel control experiments in which I repeated the exact same procedures but excluded NA from the "test" superfusate. This approach allowed us to compare the spontaneous and stimulus-evoked activity in AOB neurons that were exposed to NA to neurons that underwent the same recording procedures for the same length of time (n = 11 cells control; n = 12 cells NA).

As in previous studies, I encountered MCs demonstrating specific response profiles across stimuli ("tuning curves"), and the tuning of a given MC remained constant over the time course of these experiments (Fig. 3.1c-e) (Meeks & Holy, 2009; Meeks et al., 2010; Doyle et al., 2016). MCs responded to chemosignal blends (e.g., dilute mouse urine and/or feces), and/or pure chemosignals (sulfated sterols), with a high degree of selectivity (Fig. 3.1f). The majority of cells responded to a single stimulus, with only a few cells in control and experimental groups responding to 3 or more stimuli. The across-trial reliability of the neuronal responses added confidence that this *ex vivo* approach would provide an appropriate platform in which to monitor the impacts of NAergic signaling on AOB function.

NA Increases Spontaneous Activity and Decreases Response Discrimination in Some AOB Neurons

NA has been shown to increase the spontaneous activity of inhibitory interneurons in the AOB *in vitro*, leading to a widespread decrease in MC spontaneous activity (Araneda & Firestein, 2006; Smith et al., 2009). In my experiments, I found that the population of NA-exposed cells exhibited more varied changes in spontaneous activity between the baseline and test period (Fig. 3.2). Across the population, the control group showed a small but significant decrease in spontaneous activity during the test period compared to the baseline (baseline = 1.71 ± 0.13 S.E.M, test = 1.28 ± 0.13 S.E.M., $p = 0.008$, Mann-Whitney-Wilcoxon test). In contrast, NA-exposed cells showed a small but significant increase in spontaneous activity in the test period (baseline = 1.36 ± 0.16, test = 2.24 ± 0.20, $p = 0.032$; Mann-Whitney-Wilcoxon test). To evaluate the likelihood of observing a specific amount of

spontaneous rate suppression or enhancement, I compared observed results to (1) the binomial distribution and (2) a shuffled population based on control recordings (see Materials and Methods).

I observed a significantly higher incidence of increased spontaneous activity in NA conditions (Fig. 3.2b, $p = 0.0185$ shuffle test; $p = 0.003$ binomial test, $n = 12$). Importantly, many cells exposed to NA did not demonstrate any change in spontaneous activity (4/12 cells). A potential explanation for these results is that, in this experimental preparation, pharmacological agents in the superfusate may not uniformly penetrate the full depth of the AOB. This could result in recording depth-dependent differences in the effect of NA on spontaneous firing. To test whether this was the case, I tested for correlations between recording depth and spontaneous firing rate changes. I found that spontaneous activity was not correlated with the depth of the recording electrode ($r^2 = 0.008$, $p = 0.787$, $n = 12$ cells), indicating that variable effects of NA are not explained by variable NA penetration into the *ex vivo* preparation. These results indicate that NA does not produce uniform, unidirectional changes in spontaneous activity in all AOB MCs, and that, as a whole, MC spontaneous activity is increased by exposure to NA.

NA has been hypothesized to promote suppression of stimulus-driven activity in addition to any effects on spontaneous activity (Brennan, 2009). Across the population, ΔR responses in baseline and test periods were consistent in both control and NA conditions (Fig. 3.2c). However, when grouped based on the cell's change in spontaneous firing rate, I found that cells with decreased spontaneous activity in NA conditions showed decreased stimulus evoked ΔR compared to baseline (Fig. 3.2d; 6/6 responses; $p = 0.01$, paired

Student's *t*-test). The same was not seen for cells with increased spontaneous activity in NA conditions (1 of 9 responses).

The increased baseline activity of some MCs in NA conditions may result in a paradoxical decrease in these cells' ability to discriminate cues from background. I therefore calculated the discriminability index (d') for stimulus responses in control and NA conditions (comparing across-trial stimulus responses to control Ringer's stimulation). In the absence of NA, the d' statistic remained unchanged between the baseline and test period, even in cells that underwent spontaneous rate decreases (Fig. 3.2e). In contrast, NA-exposed cells that showed enhanced spontaneous activity showed a significant decrease in d' (Fig. 3.2f, $p = 0.0133$, paired, two-tailed Student's *t*-test, $n = 9$ responses, $n = 4$ cells). These data indicate that NA-associated increases in spontaneous activity result in decreased chemosignal discriminability.

Most AOB Neurons Do Not Respond to NA With Immediate Response Suppression

In AOB slices, NA has been reported to promote global suppression of MC activity upon NA wash-in (Araneda & Firestein 2006). Given the heterogeneity of spontaneous and stimulus-driven activity during NA application in the *ex vivo* preparations, I further investigated AOB neuronal responses to stimuli during NA application (Figs. 3.3 - 3.5). As noted above, my experimental design allowed us to evaluate the responsiveness of AOB neurons to the same stimuli across repeated trials. Across the population, I found that the changes in the average ΔR between baseline and test conditions were not significantly different ($p = 0.5848$; Mann-Whitney-Wilcoxon test) between control and NA-exposed

responses (0.0233 ± 0.5749 S.D. for control; -0.0806± 0.5104 S.D. for NA). I investigated the impact of measured recording depth on evoked changes, finding a trend towards decreased ΔR ratio (i.e., increased inhibition by NA) correlation between cell depth and the degree of suppression (r^2 = 0.204, p = 0.0524, n = 19 responses). This effect is small, but indicates that NA penetration into the ECL of the AOB in the *ex vivo* preparation is effective, and may indicate that deeply situated MCs are more likely to undergo response suppression in the presence of NA. The lack of a strong enhancing or suppressing effect of NA was initially surprising, given the previous evidence of MC and IGC modulation by NA (Araneda and Firestein 2006; Smith et al. 2009). When I inspected the NA-associated responses more closely, I observed two forms of apparent NA-associated changes: one that was immediate and non-monotonic, and the other that was gradual and monotonic.

I defined a "non-monotonic" change as a stimulus-driven response that showed a statistically significant difference in the ΔR value in the test period compared to the baseline period without a monotonic change across the 3 repeated stimulus trials (see Materials and Methods; Fig. 3.3). Non-monotonic changes in ΔR occurred in both control and NA-exposed groups (Fig. 3.3d). On a per-stimulus basis, non-monotonic ΔR suppression was not statistically significant compared to shuffle test expectations (p = 0.2078; Fig. 3.3e). The same lack of effect was also seen on a per-cell basis (p = 0.1611; Fig. 3.3f). I did observe a slightly lower number of stimulus responses that underwent non-monotonic ΔR enhancement in NA conditions (p = 0.0317 shuffle test; p = 0.03 binomial test; Fig. 3.3e), but this effect was modest. Overall, the data indicate that NA application does not uniformly induce

immediate/non-monotonic stimulus suppression as might have been expected based on previous slice studies (Araneda & Firestein, 2006; Smith et al., 2009).

Many AOB Neurons Respond to NA With Gradual, Monotonic Stimulus Suppression

Monotonic response suppression has been observed in brain regions other than the AOB (Devilbiss & Waterhouse, 2004). In this form of suppression, stimulus-driven activity decreases with each delivery of a stimulus (Fig. 3.4). Unlike non-monotonic suppression, monotonic suppression is cumulative and experience-dependent, and may be especially relevant to neuronal plasticity that develops over longer and/or more sustained exposure to environmental chemosignals. A caveat to this form of suppression is that the gradual reduction in response magnitudes may not always produce a statistically significant across-trial change in ΔR over the time course of these experiments (Fig. 3.3c).

Monotonic suppression occurred rarely in control cells (3/16 responses), but was more prevalent in NA-treated cells (9/19 responses; $p = 0.0041$ shuffle test; $p = 0.009$ binomial test; Fig. 3.3f). This increase in monotonic suppression was also significant when evaluated on a per-cell basis (8/12 cells; $p = 0.005$ shuffle test; $p = 0.008$ binomial test; Fig. 3.3g). In contrast to the relatively mild effects of NA exposure on immediate, non-monotonic suppression, these analyses revealed that many AOB neurons undergo gradual, experience-dependent response suppression in the presence of NA.

To assess the cumulative influence of NA on non-monotonic and monotonic suppression, I applied a binary classification to each stimulus response in control and NA-exposed conditions (Fig. 3.5). Using this binary classification strategy allowed us to include

monotonic suppression in my analysis that, as mentioned previously, was not always mirrored by a strong change in the average ΔR (Fig. 3.3c). On both a per-stimulus and a per-cell basis, I found a significant increase in the amount of combined suppression during NA exposure ($p = 0.00075$ shuffle test, $p = 0.0002$ binomial test per stimulus; $p = 0.0083$ shuffle test, $p = 0.001$ binomial test per cell; Fig. 3.5b). The increased overall suppression was paralleled by a decrease in the number of unchanged responses ($p = 0.0355$ shuffle test, $p = 0.04$ binomial test). It was noteworthy that the responses to many stimuli remained unchanged in the presence of NA (Fig. 3.5c), further evidence that NA does not uniformly suppress AOB neuronal responses to chemosensory stimuli.

Discussion

Studying the effects of NA on the AOB in *ex vivo* preparations has several advantages over both acute slice and *in vivo* approaches. Principally, the *ex vivo* preparation allows one to study the responses of AOB neurons to naturalistic chemosensory stimulation of the VNO, which is impossible in acute slices. Moreover, with *ex vivo* approach one can precisely control the concentration of delivered chemosignals and the timing of stimulus delivery. This is also possible using anesthetized *in vivo* preparations, but the impacts of systemic anesthetics on GABAergic signaling (Ishizawa, 2007) may interfere with the normal function of the reciprocal synapses between AOB MCs and interneurons. A final advantage of this preparation is that it directly exposes the AOB, a structure that is only 800 μm deep, to rapid bath superfusion and pharmacological administration. A noteworthy limitation of this approach, however, is that the experimental dissection severs centrifugal fibers, including

NAergic fibers, and in doing so may alter basal neuromodulatory tone. Another caveat is that my extracellular single unit electrophysiological recordings, which isolate MCs based on electrode depth and extracellular spike waveform (Hendrickson et al., 2008; Meeks & Holy, 2009, Meeks et al., 2010), do not exclude the possibility that some of the recorded neurons were from cells other than MCs (e.g., EGCs, Larriva-Sahd, 2008). On balance, I have found that for many experiments the benefits outweigh the limitations, and open new routes to studying unanswered questions about AOB circuit function.

In the MOB, NA-associated increases in GABA lead to a decrease in spontaneous MC activity and enhanced signal-to-noise ratios for detected odorants (Linster et al., 2011; Linster & Fontanini, 2014). In the AOB, some studies have indicated that NA causes disinhibition of MCs, which may contribute to potentiation of MC activity (Dong et al., 2009). Others, however, have found that NA application increases GABAergic tone, leading to a general suppression of MC activity (Araneda & Firestein, 2006; Smith et al., 2009). In *ex vivo* preparations, I found that spontaneous activity is slightly increased in the AOB. A similar increase in spontaneous MC activity was observed after artificial mating, but it was not clear whether this effect was dependent on NAergic signaling (Otsuka et al., 2001). Importantly, I found that the subpopulation of MCs that experience spontaneous increases in firing rate also show a diminished capacity to discriminate stimuli from background. Overall, these data indicate that the effects of NA on spontaneous AOB neuronal activity are subtle and non-uniform, and result in a net decrease in the signal-to-noise ratio for detected chemosignals.

One hypothesis of NA-associated pheromonal memory formation suggests that NA promotes selective suppression of stimuli that are encountered during the period of NA release (Araneda & Firestein, 2006; Brennan & Binns, 2005; Brennan, 2009). Consistent with this hypothesis, I found increased suppression of many stimuli that were delivered during NA application. However, this suppression is heterogeneous and does not occur with all stimuli. Many single cells that responded to several stimuli in my panel showed NA-associated suppression of some stimuli while others were unchanged. This apparent stimulus specificity was unexpected. This observation may indicate that some MC-GC synapses are primed for NA-associated suppression but others are not.

The mechanisms of such a phenomenon are unclear. MC-GC reciprocal synapses are located along the multiple primary dendrites of AOB MCs, and AOB MCs integrate across chemosignal classes by innervating multiple glomeruli (Wagner et al., 2006; Meeks et al., 2010; Tolokh et al., 2013; Kahan & Ben-Shaul, 2016). Heterogeneous NA-mediated suppression may result from differential inhibition of MC dendrites by internal or EGCs, or may even occur at the level of glomerular inputs, as has been reported in the MOB (Eckmeier & Shea, 2014). Since adrenergic receptors are localized to the ECL and ICL of the AOB (Domyancic & Morilak, 1997; Rosin et al., 1996; Talley et al., 1996), it seems most likely that IGCs or the more recently described EGCs (Larriva-Sahd, 2008) are the prime targets of NAergic signaling.

The lack of suppression of some stimulus responses during NA exposure also raises questions related to the potential biological implications of this resistance to suppression. The persistence of NA-independent stimulus responses may be important for sustaining the

capacity to detect chemosignals containing important information that should not be suppressed by an LC-engaging experience. For example, it might be disadvantageous to suppress MC activation by chemosensory cues that are unrelated to mating (e.g., predator-associated cues) that happen to be detected while NA is present in the AOB. A broader inquiry into the relationship between stimulus identity and the capacity for NA-mediated suppression is needed to determine whether this is the case.

In addition to stimulus-specific suppression, I also found that NA promoted distinct modes of inhibition in the AOB. The most prominent form of NA-mediated suppression was a gradual, monotonic decrease in response magnitude over repeated trials of the same stimulus. This effect has been reported in cortical regions (Devilbiss & Waterhouse, 2004), but has not been reported in the AOB. Monotonic suppression is an activity-dependent process that blunts the effects of chemosensory stimuli over time and/or repeated exposure. The slow accumulation of stimulus-dependent suppression seems to be a strong match for the slow actions of chemosignals on the AOB. The process of dissolving environmental chemosignals in nasal/vomeronasal mucus and pumping them actively into the VNO takes many seconds, and MC responses to brief chemosensory encounters can last many seconds to minutes (Luo et al., 2003). More studies will be needed to determine whether this gradual, monotonic form of suppression is seen *in vivo*, and, if so, whether it is associated with experience dependent forms of AOB plasticity and pheromonal learning.

In summary, my data indicate that NA is not a simple suppressive gate for chemosensory information flowing through the AOB. Instead, NA has nuanced, heterogeneous effects that include changes in both spontaneous and stimulus-driven activity.

Future experiments will be necessary to tease apart the distinct mechanisms, but my findings suggest that specific MCs – and perhaps specific reciprocal synapses – respond differently to NAergic modulation.

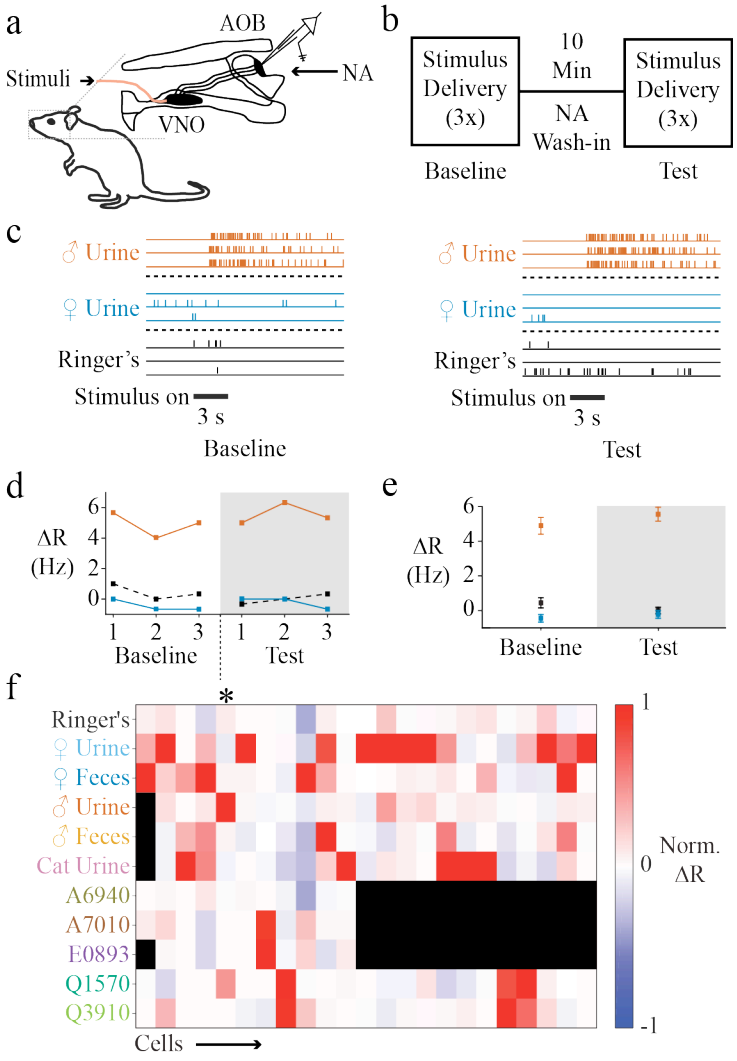

Figure 3.1 Stimulus Tuning and Modulation in the AOS *Ex Vivo* Preparation

a: Diagram of the *ex vivo* preparation of the mouse AOS. Stimuli were delivered to the VNO while single-unit electrophysiological activity was recorded in the AOB.
b: Experimental overview. Three rounds of randomized stimulus trials were conducted before (baseline) and during (test) NA application.
c: Raster plot of stimulus-driven single-unit responses in the AOB in baseline and test periods in a control experiment (not exposed to NA). Response magnitude and stimulus selectivity remained constant over time.
d: Changes in firing rate (ΔR) across stimulus responses for the same cell shown in (c). Numbers on x-axis refer to the stimulus repeat within each stimulus battery.
e: Average ΔR per stimulus battery for the cell represented in (c-d). Error bars reflect SE.

f: Heat map representation of stimulus responsiveness of 23 cells. Each row is a different stimulus; each column is a different cell. Black indicates stimuli that were not delivered to that particular cell. Asterisk indicates the cell shown in (c-e). Norm. ΔR, normalized ΔR.

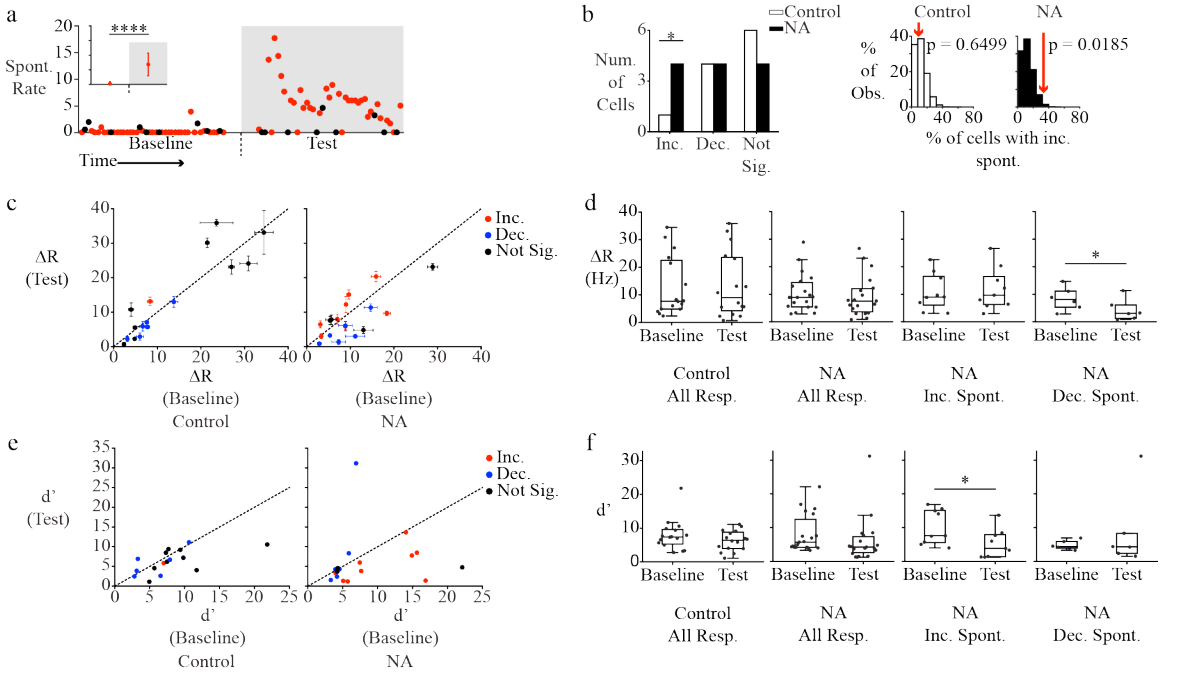

Figure 3.2 Spontaneous Activity Is Increased in a Subpopulation of AOB Neurons

a: Scatter plot of spontaneous activity in an AOB neuron that showed increased spontaneous activity (Spont. Rate) in the NA period (****$p = 1.25 \times 10^{-16}$). Red circles indicate the unstimulated firing rate (measured just before a stimulus onset). Black circles indicate firing rate measurements that happened to follow strong stimulus responses (which may not fully recover to baseline before the subsequent trial). Inset shows the mean and SD of the measured spontaneous rate for this cell.
b: The number of cells in control (n = 11) and NA-treated (n = 12) conditions that exhibited significantly changed or unchanged spontaneous activity (Num., number; Inc., increased; Dec., decreased; Not Sig., no change). There were significantly more cells that exhibited increased spontaneous activity (p = 0.0185 compared with a shuffled population).
At right are histograms relating the observed prevalence (% Obs.) of spontaneous rate increases (red arrows) to shuffle test expectations (which are based on control recordings).
c: Scatter plot of stimulus-evoked ΔR, with changes in spontaneous activity indicated by color (blue, decreased; red, increased; black, no change). Error bars represent SE.
d: Box plots showing mean stimulus responses (Resp.) for control and NA-exposed cells (left 2 graphs), and breakdowns based on spontaneous activity changes (right 2 graphs). *p = 0.01 (paired Student's t-test).
e: Scatter plots of the sensitivity index (d') for control (n = 16) and NA-treated (n = 19) conditions. Colors reflect the same parameters as (c).
f: Box plots showing d' values for all stimuli in control and NA-treated conditions, broken down by categories as in (d). *p = 0.0133 (paired Student's t-test).

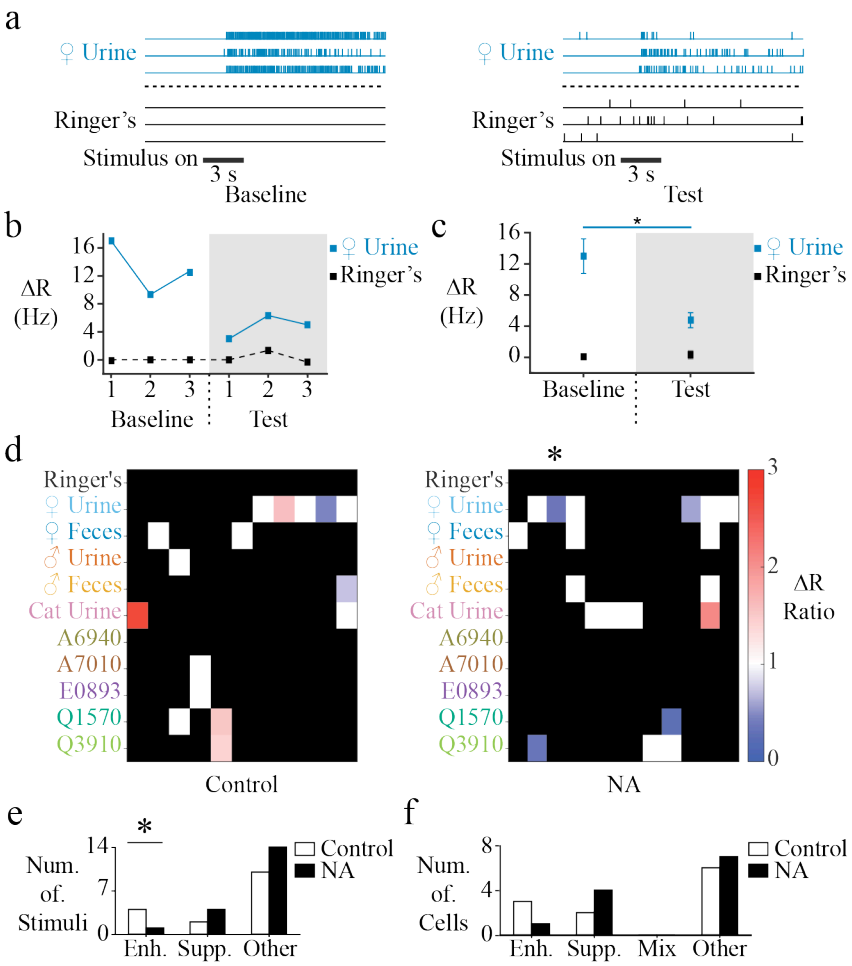

Figure 3.3 NA Elicits Immediate, Non-Monotonic Suppression in a Small Fraction of AOB Neurons

a: Raster plot showing stimulus-evoked spiking responses in a cell demonstrating immediate, non-monotonic response suppression in the presence of NA.
b: Per-trial ΔR values for the cell shown in (a).
c: Average ΔR for the cell in (a-b). *p = 0.027 (unpaired, 2-tailed Student's *t*-test).
d: Heat map of average stimulus responses in control cells (left; n = 11) and NA-exposed cells (right, n = 12). Non-monotonic enhancement is shown in red and suppression in blue. White pixels indicate no change, and black indicates that the cell did not respond to the stimulus in either the baseline or test period. Asterisk marks the column showing the response of the cell represented in (a-c).
e: Per-stimulus counts of significant non-monotonic enhancement (Enh.), suppression (Supp.), or no change (n = 16 for control, n = 19 for NA). The number of responses exhibiting non-monotonic enhancement was significantly decreased during NA exposure (*p = 0.032 shuffle test; p = 0.03 binomial test). "Other" indicates responses that did not show non-monotonic changes.

f: per-cell count of enhancement, suppression, or a mix of both (n = 11 cells for control, n = 12 for NA). “Other” indicates cells that did not demonstrate any non-monotonic changes.

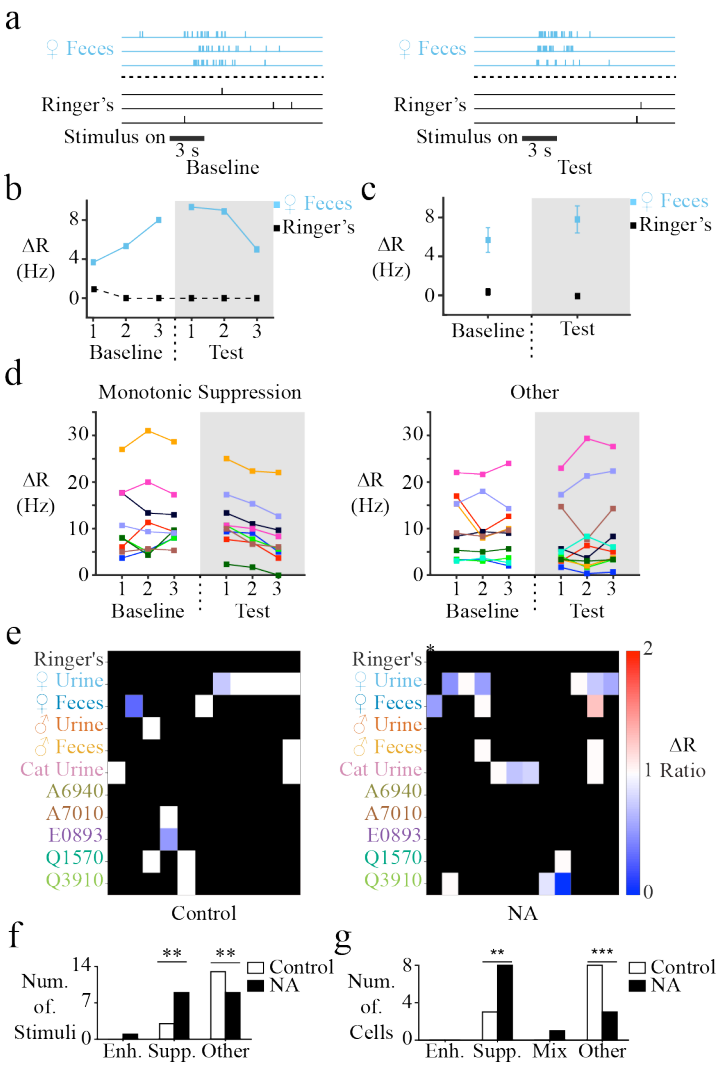

Figure 3.4 NA-Associated Monotonic Response Suppression

a: Raster plot showing stimulus-evoked spiking responses in a cell demonstrating monotonic suppression in the presence of NA.
b: Per-trial ΔR values for the cell shown in (a).
c: Average ΔR for the cell in (a-b).
d: Across-trial ΔR values for responses that either exhibited monotonic suppression during NA delivery (left; n = 9) or did not (right; n = 10).
e: Heat map of stimulus responses in control (left; n = 11) and NA-exposed cells (right; n = 12) that exhibited monotonic enhancement (red), suppression (blue), or neither (white). Hues indicate the net difference in ΔR at the third (final) stimulus delivery in test period compared with final stimulus delivery in the baseline period.
f: Per-stimulus counts of monotonic enhancement, suppression, or no monotonic change in control and NA-exposed conditions. The number of responses exhibiting monotonic

suppression was significantly increased (**p = 0.004 shuffle test; p = 0.0009 binomial test), whereas the number of unchanged responses was significantly decreased during NA exposure (**p = 0.001 shuffle test; p = 0.0009 binomial test; n = 16 for control, n = 19 for NA).
g: Per-cell counts of monotonic enhancement or suppression. There were significantly more cells with a suppressed response (**p = 0.005 shuffle test; p = 0.0008 binomial test) and significantly fewer cells with no significant changes (**p = 0.0008 shuffle test; p = 0.0008 binomial test; n = 11 for control, n = 12 for NA).

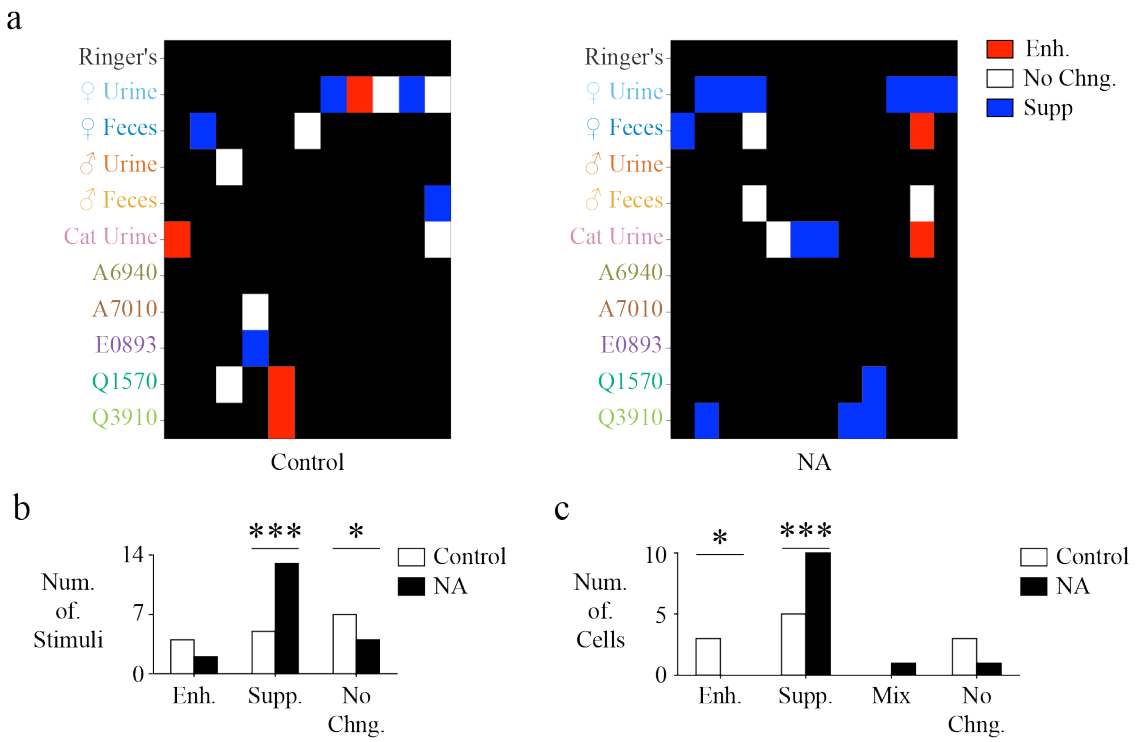

Figure 3.5 Combined Forms of Suppression During NA Exposure

a: Heat map of stimulus responses that showed monotonic or non-monotonic suppression (blue), enhancement (red), or no change (No Chng.) in control (left; n = 11) or NA-exposed cells (right; n = 12).
b: Per-stimulus counts of suppression, enhancement, or no change. The overall number of suppressed stimuli was significantly increased (***p = 0.0009 shuffle test; p = 0.0002 binomial test), and the number of unchanged responses was significantly decreased (*p = 0.035 shuffle test; p = 0.04 binomial test; n = 19 for NA, n = 16 for control).
c: Per-cell counts of enhancement, suppression, a mixture of enhancement and suppression, or no change. The number of cells with enhanced responses was significantly decreased (*p = 0.021 shuffle test; p = 0.02 binomial test), and the number with suppressed responses was significantly increased (***p = 0.008 shuffle test; p = 0.001 binomial tests).

CHAPTER FOUR

DISCUSSION AND FUTURE DIRECTIONS

In order to survive in a changing environment, animals have to be able to accurately and flexibly encode information. During my thesis work I have studied these phenomena using the mouse AOS as a model system. I have discovered that bile acids released into mouse feces are a novel class of chemosignals for this system. These bile acids, as well as whole feces, are encoded for in the AOB in a manner consistent with a combinatorial code related to the sex and species of the donor. I also found that NA affects the encoding of stimuli in the male AOS, promoting a form of suppression that requires multiple deliveries of a stimulus to develop. This suppression does not occur with every evoked response in the AOB and is also specific to stimulus-evoked activity, indicating that NA has more heterogeneous actions in the AOS than previously appreciated.

Feces Are a Novel Source of AOS Odorants

Despite the presence of ligands in other animal excretions, feces had never been explored as a source of AOS cues prior to my thesis work. Although feces had not been examined, there were some indications that it could be a potent activator of this chemosensory subsystem. Urine was previously thought to be the major driver of AOS activity with other sources of odorants providing more specific responses (Holy et al., 2000; Kimoto et al., 2005; Nodari et al., 2008). Surprisingly however, it was previously

shown that urine alone was not able to recapitulate the amount of activity in the VNO produced by soiled bedding which contains multiple animal excretions (Kimoto & Touhara 2005). Since the other known excretions in bedding, saliva and tears, contained only a few ligands I hypothesized that there must be another major source of odorants to explain the difference between urine- and bed-driven activity.

I focused on feces as a potential source of odorants for a number of reasons. Urine is potentially a powerful source of AOS odorants because it is one of the two major waste excretion pathways in the body. By sampling urine, a mouse is able to spy upon the health status of another animal helping to guide behavioral decisions. Feces are also a waste excretion pathway meaning they could provide complementary information about health status, as well as potentially unique information like recent diet history. It has also been known for decades that feces are a potent modulator of rodent behavior. Mice will actively avoid cat feces, although the exact mechanism for that avoidance is unknown (Drickamer, Mikesic, & Shaffer, 1992; Apfelbach, Blanchard, Blanchard, Hayes, & McGregor, 2005). Maternal feces are also highly appetitive to rodent pups, they will preferentially seek out and eat feces from a lactating female over other females or males (Poster & Doane, 1976; Moltz & Leidahl, 1977; Leon, 1978; Moltz, 1978; Kilpatrick, Bolt, Moltz, 1980; Moltz & Lee, 1981; Lee, Lee, & Moltz, 1982; Kilpatrick, Lee, Moltz, 1983). Importantly, this behavior is dependent on bile acids. Female rats have an increase in CA synthesis during the period in which the feces are attractive, leading to increased levels of DCA in the feces through bacterial dehydroxylation (Kilpatrick et al., 1980; Kilpatrick et al., 1983). Furthermore, when the bile from a lactating female is added to

the intestine of a male rat his feces will become appetitive to rat pups (Moltz & Leidahl, 1977). The hypothesis at the time was that this attraction was a mechanism to supplement low levels of DCA in rat pups, but the olfactory system and cue underlying this phenomenon were not determined (Kilpatrick et al., 1983).

Due to the importance of the AOS in regulating sex-related behaviors, I first examined male AOS activity to female mouse feces. The number of MCs responsive to feces was almost the same as the number responsive to urine, indicating that not only are feces a novel source of odorants but they are potentially a major source like urine. If feces contained only a few odorants and provided specialized information like other excretions, the number of MCs responsive to feces would be expected to be far lower. Instead it appears, at least in the anterior AOB, that both urine and feces are the principal drivers of AOS activity. Many of the cells that were responsive to feces were highly selective, indicating that at the level of the AOB, feces and urine are being maintained as separate sources of information.

I also tested responses to male mouse feces and found they activated the AOS, with responses only partially overlapping with female fecal activity. These results indicate that, consistent with a behaviorally relevant source of cues, fecal responses can be sex-selective. When responses were compared across male and female stimuli a combinatorial code emerged in which some cells were broadly fecal-responsive, some were broadly sex-selective, while others showed combinations between the fecal and urine responses. Overall, I have found that fecal-driven responses are consistent with feces being a major and unique driver of AOS activity.

Fecal Bile Acids Are Rodent Olfactory Cues

To identify fecal ligands I collaborated with University of Texas at Austin's mass spectrometry facility. Our analysis revealed that the fecal samples were enriched for a class of sterols called bile acids. Primary bile acids are produced in the liver from cholesterol and are released into the gut to aid in the digestion of non-polar substances like lipids and vitamins (de Aguiar Vallim, Tarling, and Edwards, 2013). Within the gut, bile acids are subject to bacterial modifications, such as dehydroxylation and epimerization, forming secondary bile acids (Alnouti, 2009; Hagey & Krasowski, 2013; Wahlström, Sayin, Marschall, Bäckhed, 2016). Most bile acids are then taken back up from the intestine and return to the liver, completing the enterohepatic bile acid cycle (Russel, 2009). The exact complement and the levels of bile acids vary by sex, species, and age. This variation indicates that bile acids as a class of chemosignals could be highly informative and produce differential activity based off ethologically important conditions. In support of this hypothesis I found that female mouse feces did not have detectable CDCA while male mouse feces did. Both CDCA and LCA, a bile acid not found in mice, were able to produce differential activity in the AOB compared to the bile acids present in the female mouse feces. My results indicate that not only are fecal bile acids a novel class of AOS ligands, but their patterns of activity are consistent with those of behaviorally relevant pheromones and kairomones.

Bile acids are only a novel olfactory ligand in terrestrial mammals, they have been known cues in fish species for decades (Doving et al., 1980; Li et al., 2002; Huertas et al., 2010; Buchinger et al., 2014). Lampreys, one of the most primitive vertebrates, use bile

acids as migratory and mating pheromones (Li et al., 2002; Buchinger et al., 2014). This detection has remained conserved throughout fish evolution and can be seen in eels, salmon, and zebrafish (Doving et al., 1980; Michel and Derbidge, 1997; Huertas et al., 2010). My discovery that bile acids have remained as olfactory cues with the transition to land has been seen with other sterol cues, such as sulfated sterols. Goldfish, frogs, and mice can all detect sulfated pregnanolones, indicating that reliance on a conserved set of odorants is common across different sterol classes (Sorensen et al., 1995; Meeks et al., 2010; Sansone et al., 2015).

Potential Information Conveyed by Bile Acids

The information conveyed by bile acids to the mouse is unknown, similar to that of other known activators of the anterior AOB. Ligands that activate the anterior AOB are hypothesized to convey information about physiological status, such as age or health (Isogai et al., 2011; Chamero et al., 2012). Many features of bile acid synthesis and excretion are consistent with this hypothesis. As seen in my own data, CDCA is present in male mice but not in females providing a potential marker of sex when combined with the rodent-enriched MCAs. Bile acids can also potentially convey information on age as the bile acid complement changes over the lifespan of the animal. Young animals have decreased levels of secondary bile acids due to low levels of intestinal bacteria, while the bile acid pool of older animals is enriched for secondary bile acids due to slower gut motility (Uchida et al., 1978; Ferdinandusse et al., 2005; Fu, Csanaky, & Klaasen, 2012). Bile acid levels can also be regulated by the diet as well as different disease states

(Chiang, 2004; Hagey & Krasowski, 2013; Ridlon 2014). Similar to other sterol activators, bile acids may be potent signals of a donor animal's physiological status.

One of the major reasons for the physiological hypothesis of anterior AOB ligands is that all of the known cues are present in multiple species, limiting their ability to convey individuality. For example, sulfated glucocorticoids are produced across the animal kingdom making them a poor marker of individuals or species (Millspaugh & Washburn, 2004; Touma & Palme, 2005; Hagey, Moller, Hofmann, & Krasowki, 2010). This is not an issue with bile acids however as many bile acids show species-specific enrichment. The MCAs I detected in mouse feces are highly enriched in rodents, hyocholic acid is found in pigs, while ursodeoxycholic is a bear-enriched bile acid (Hagey et al., 1993; Hagey et al., 2010). In fact, the structure of bile acids tends to follow a systematic pattern among taxa and at the species level there are variations in ratios of produced bile acids (Russell & Setchel 1992; Buchinger et al., 2014). I found specificity in responses to different bile acids in the AOB, with a bile acid not found in mice (LCA) producing very unique patterns of activity. This maintenance of separate processing streams for different bile acids suggests that the AOS could potentially use bile acids as indicators for the presence of specific animals in the environment, a phenomenon unique to anterior bulb activators.

As with other AOS cues, it is likely that bile acids will not convey information alone but will instead act in combination with other odorants. The AOS is particularly effective at performing integrative processing and the relative levels of multiple cues are likely compared to guide behaviors (Kahan & Ben-Shaul, 2016). This type of

combinatorial code is potentially very powerful for guiding the complex life-or-death behaviors of the AOS. For example, when deciding to mate a male mouse should not only take into account that an estrous-specific cue is present (like a sulfated estrogen) but that there is also a marker that the donor animal is actually a female mouse and not a predator (Haga-Yamanaka et al., 2014). It is likely that bile acids will fit into this combinatorial code, and provide additional support that the donor animal is of a certain sex or of a certain species.

Bile Acid Future Directions

Bile acids are a completely novel class of ligands for the AOS, meaning that there are many potential future directions for this work. As with other ligands it will be crucial to determine the full complement of bile acids that can activate the system. It is also currently unknown which VNO receptors are responsive to bile acids. Finally, there are no known behaviors regulated by bile acids in the mouse.

I have tested only a few bile acids for activity and it will be important to expand upon this initial battery. To test if bile acids are serving as species-specific signals it will be necessary to deliver bile acids that are at low levels or absent in mice. Potential bile acids of interest include hyocholic (pig), ursodeoxycholic (bear), avicholic (bird), bitocholic (snake), and pythocholic (snake) acids (Hagey et al., 2010). So far I have only tested unconjugated bile acids, but in the body they are often conjugated to different chemical moieties. In mice the majority of bile acids in the gallbladder are conjugated to taurine to increase their hydrophilicity (Dawson & Karpen, 2015; Wahlström et al.,

2016). The ratio of conjugated to unconjugated bile acids is under dietary control; there are increased levels of conjugation following feeding (Li & Chiang, 2014). Sulfate groups can also be added to bile acids, which increase their excretion into both feces and urine (Alnouti, 2009). The other known sterol activators of the AOS have different moieties attached (sulfate groups or carboxylic acid), which raises the possibility that conjugated bile acids could also be potent activators of the AOS. However, taurine-conjugated bile acids may not be as potent as the bile acids already tested. Within the gut bacteria remove conjugate groups leading to greater fecal excretion, meaning that mice are more likely to contact unconjugated bile acids in feces (Hagey & Krasowski, 2013; Wahlström et al., 2016).

Bile acids potently activated the anterior AOB, a region targeted by V1R-expressing VSNs (Rodriguez et al., 1999; Belluscio et al., 1999). V1Rs are also the likely detectors of low-molecular weight compounds, making them the likely receptors for bile acids in the VNO (Liberles, 2014). Due to the unique responses seen to different bile acids, there are likely multiple receptors that respond to these cues. Detection by multiple receptors is already known for sulfated sterols; a number of closely related V1Rs respond to multiple sulfated estrogens (Isogai et al., 2011; Haga-Yamanaka et al., 2014). Identifying the specific V1Rs responsive to bile acids is a challenge as there at least 200 functional and 100 pseudogenized V1Rs in the mouse genome (Young, Massa, Hsu, & Trask, 2010). Even among annotated V1Rs, there are concerns on the accuracy of their annotations and their expression in the VNO (Ibarra-Soria, Levitin, Saraiva, & Logan, 2014).

Even though bile acids are likely to bind to V1Rs there are two potential other candidate receptors. Amphibians are able to detect sulfated sterols in their VNO, but this structure expresses V2Rs and not V1Rs (Sansone et al., 2015). Sulfated pregnanolones also activate the mouse AOS in a manner consistent with detection by V2Rs (Hammen et al., 2014). These results indicate that, contrary to expectations, V2Rs may bind to sterols like bile acids. There is also the possibility that the mouse VNO is utilizing existing bile acid receptors in a novel manner. Known bile acid receptors include multiple nuclear receptors and a single G-protein coupled receptor named TGR5 (Kawamata et al., 2003; Bunnet, 2014; Copple & Li, 2016). The rapid onset of responses indicates that bile acids are likely detected by a G-protein coupled receptor rather than a nuclear receptor. I tested for the expression of TGR5 in the VNO in collaboration with Jie Cao in Dr. Meeks laboratory, and we did not find evidence of receptor expression (data not shown). The use of a single receptor would also not explain the specificity of responses seen at the level of the AOB, making it unlikely that TGR5 is a bile acid receptor in the VNO.

It is crucial that future experiments identify the receptors responsive to bile acids in the VNO. The best-established method for receptor-ligand pairing in the AOS is to stimulate the VNO and perform *in situ* hybridizations against different V1Rs and immediate early genes to mark recently active VSNs. Receptors can then be de-orphaned by looking for overlap between the immediate early gene and a specific V1R (Isogai, et al. 2011). This approach has inherent challenges, however. Based on sequence similarity the earliest identified receptors can roughly be grouped into 12 families, although with the discovery of more V1Rs the usefulness of this grouping has decreased (Rodriguez et

al., 2002; Young et al., 2010). Between family similarity is only between 15 and 45% and even among the identified clades given receptors can share anywhere between 40 and 99% similarity (Tirindelli, Dibattista, Pifferi, & Menini, 2009). Due to the poor sequence conservation among V1Rs, it is difficult to design a single probe against multiple receptors. This issue makes *in situ* based receptor-ligand identification extremely time-consuming. A potential alternative is to utilize recent revolutions in RNA-sequencing and perform single-cell sequencing on cells responsive to bile acids (Grün & van Oudenaarden, 2015; Kolodziejczyk, Kim, Svensson, Marioni, & Teichmann, 2015).

As AOS ligands, bile acids are likely influencing currently unknown animal-related behaviors. A major difficulty in identifying behaviors related to low-molecular weight odorants is that they appear to act in combination with other odors to guide behaviors (Haga-Yamanaka et al., 2014; Fu et al., 2015). This difficulty can best be seen with sulfated sterols, it took six years between their identification as odorants to a behavior (Nodari et al., 2008; Haga-Yamanaka et al., 2014). Despite these challenges there are several possible aspects of bile acid physiology that could implicate certain behaviors. An individual's complement of bile acids can vary by age and sex, making them potentially useful cues for guiding mating and aggression (Eyssen, Smets, Parmentier, & Janssen, 1977; Uchida et al., 1978; Turley et al., 1998; Chiang, 2004; Ferdinandusse et al., 2005; Alnouti 2009; Fu et al., 2012). Mice may use fecal particles in their environment to make decisions as to whether a potential mate is of the appropriate age or sex, similar to what has been observed for ESPs (Haga et al., 2010; Ferrero et al., 2013). The same information could also potentially guide aggressive behaviors, such as a

mouse choosing to only engage in territorial aggression when fecal signals are consistent with a potential rival. Many mouse predators have unique bile acids that could potentially alert mice to their presence, potentially inducing fear behaviors or inhibiting mating (Hagey et al., 2010).

Noradrenaline Produces Heterogeneous Effects on AOS Activity

The other major aspect of my thesis was an examination of how NA modulates spontaneous and stimulus-evoked activity in the AOB. NA has been hypothesized to broadly suppress MC activity, but I found that in the intact AOB its actions were more variable (Araneda & Firestein, 2006; Brennan, 2009). A subset of MCs showed increased spontaneous activity indicating NA was not simply acting as a gate to broad suppression. In the affected MCs, the increase in spontaneous activity leads to a functional decrease in the discrimination of stimulus-driven activity from background activity. In contrast to the effects on spontaneous activity, NA in general promoted suppression of stimulus-evoked responses. NA specifically promoted a form of monotonic suppression in which the suppression developed over repeated deliveries of a stimulus. This suppression is different from what was expected, if NA was simply increasing suppression than all responses should have been suppressed equally from the onset. Instead I observed that suppression was stimulus-specific with some stimuli immediately suppressed, others requiring multiple deliveries, while others were completely resistant. These results are more consistent to what has been observed in other brain regions, where NA has long

been known to have variable actions (Bennet et al., 1998; Devilbiss & Waterhouse, 2004).

My work on NA's actions in the AOB has also demonstrated the importance of discovering novel ligands for this system. For my stimulus batteries I included known activators of the AOS (sulfated sterols and urine) in addition to novel odorant sources (mouse feces and a mixture of cat urine and feces). Surprisingly, all of the responses to the known AOS ligands became suppressed during NA, while the new blends of odorants showed variable changes (Fig. 3.5). If I had simply used the known battery of odorants during these experiments, I would have observed NA having more consistent effects with suppression to all observed stimuli. The consistent responses to known odorants would have lead to a misinterpretation on the actions of NA in the AOB, limiting our ability to understand how the AOS processes stimuli. It is crucial in the future that the field continues to discover new cues so more subtle aspects of AOS physiology can be discovered.

Potential Roles of Noradrenaline in the Male AOS

Previous work on NA's roles in the AOS has focused on female mice and the pregnancy block effect. During this phenomenon, NA promotes suppression allowing for the formation of inhibitory memory that prevents a mate's odors from producing activity and preventing embryo implantation (Brennan & Keverne, 1997; Brennan & Binns, 2005; Brennan, 2009). The role of NA-mediated suppression outside of mating or even in male mice is unknown. My results demonstrate that stimulus suppression is not specific

to females and can also occur in males, although the exact role for this suppression is unknown. One hypothesis is that the suppression in males serves a similar purpose in females, namely to recognize and suppress activity to a mate. This could allow a male to then focus his attention on females he has not mated with by preferentially detecting their odorants. Potential support for this hypothesis can be seen in the female mouse urine responses, all of which became suppressed during NA delivery. Contrary to that hypothesis all responses to female mouse feces did not become suppressed, although that could reflect feces playing a different role than urine in guiding mating.

I also observed suppression to odors that were not associated with female mice and instead could be relevant during behaviors other than mating. NA is potently released during arousing events like the fight-or-flight response, and when making the decision to fight or flee it would be logical to either enhance or suppress responses to certain stimuli (Nelson & Trainor, 2007; Nai et al., 2009). Suppressing responses to stimuli that are not relevant to fighting could help focus attention, providing an alternative explanation to the suppression seen with female mouse urine. For example, during male-male aggression or predator avoidance information about females may be irrelevant with the greater threat of survival. This hypothesis, however, does not explain the benefit of suppressing responses to predator odors (cat urine and feces) or stress markers (sulfated glucocorticoids). It will be important to increase the battery of stimuli delivered during NA administration to gain a better understanding of what stimuli are more prone or more resistant to suppression.

Noradrenaline Future Directions

My experiments with NA have all used a consistent, 10 μM concentration. Future experiments will need to vary the concentration and observe how that affects stimulus encoding. In the MOB, NA's effects vary by concentration due to the activation of different receptors (Sara, 2009; Linster et al., 2011). At low concentrations NA can increase MC activity through the activation of α2 receptors, while at higher concentrations activation of α1 receptors leads to MC suppression (Linster et al., 2011; Devore & Linster, 2012). Both α1 and α2 receptor expression have been reported in the AOB, raising the possibility of a similar phenomenon in the AOB (Pieribone et al., 1994; Rosin et al., 1996; Talley et al., 1996; Domyancic & Morilak, 1997; Winzer-Serhan et al., 1997). Due to this expression it will be important, in addition to testing different concentrations of NA, to test the effects of different receptors through specific agonists. NA may not necessarily have different effects at different concentrations, one study in the MOB found that NA produced suppression at all concentrations tested (Zimnik et al., 2013).

LC activity fluctuates with different arousal states, a phenomenon which cannot easily be recapitulated by pharmacology alone. During REM sleep LC neurons are silent, and levels increase during slow-wave sleep and the transition to waking. During quiet wakefulness cells fire tonically with changes in activating anticipating changes in arousal. Finally during the presentation of novel or salient stimuli, LC neurons begin phasic bursting (Nai et al., 2009; Sara 2009). There are now mice available that express Cre recombinase under the promoter of one of the enzymes involved in NA production,

tyrosine hydroxylase (Savitt, Jang, Mu, Dawson, & Dawson, et al., 2005). These mice can be used to allow for the expression of channelrhodopsin in NAergic terminals in the *ex vivo* preparation (Boyden, Zhang, Bamberg, Nagel, Deisseroth, 2005; Wang, Piñol, Bryne, Mendolwitz, 2014). Different patterns of LC activity can then be mimicked by optogenetic stimulation, allowing for a more in-depth examination of how NA modulates stimulus processing in the AOS. These optogenetic experiments can be extended to the living mouse to test how activation of LC terminals in the AOB affects AOS-mediated behaviors like mating and male-male aggression

Conclusion

The AOS is a crucially important rodent olfactory system involved in the detection and processing of animal-related odors. My thesis work has focused on the encoding of odorants in this system and how that encoding can be affected by neuromodulation. I have discovered that feces are a previously unknown source of AOS cues, and fecal-driven activity is consistent with them being a major driver of activity. I discovered that fecal bile acids are one group of fecal ligands. These bile acids, which vary by both sex and species, produce unique patterns of activity in the AOS consistent with them serving roles as pheromones and kairomones. Bile acids are unlikely to be the only source of fecal chemosignals, however, and additional experiments are necessary to identify more cues. I also found that NA has heterogeneous effects on AOB activity, increasing spontaneous activity in some cells and suppressing activity more broadly.

Suppression was stimulus-specific and demonstrates the importance of continuing to identify novel ligands of the AOS.

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