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Validating non-motivated methods and equipment for studying mouse olfactory behavior

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Validating Non-Motivated Methods and Equipment for Studying Mouse Olfactory Behavior

by

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Thesis

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in

Biology

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For Mom, Dad, and Cee

For all the help and support, thank you.

Abstract

Mouse olfactory behavior has traditionally been difficult to assess due, in part, to the expensive nature of behavioral equipment and the lengthy process of training animals. The present study aims to validate a new behavioral paradigm requiring no prior animal training using existing liquid dilution behavioral olfactometers. We also aim to validate Triton-100x, a detergent, as a new anosmia inducing agent, as well as self-built, Do-it-yourself (DIY) behavioral olfactometers. Equipment and methods were tested using a variety of common-discrimination and detection-threshold assays. Difficulties maintaining stimulus control arose during testing as mice routinely detected volume-to-volume concentrations of amyl acetate diluted in mineral oil below reported thresholds (1×10^{-8} : $n = 8$, $p < 0.05$). Stimulus control was corrected by using individual vials for each odor presentation. These results demonstrate that non-motivated behavior using existing equipment is an effective alternative to traditional training methods when stimulus control is properly accounted for. Furthermore, intranasal irrigation with 0.1% Triton successfully induced recoverable anosmia in mice (Day 6: $p > 0.05$, $n = 6$; Day 7: $p < 0.05$, $n = 6$; PBS: $p < 0.05$, $n = 6$). Finally, a behavioral olfactometer was successfully constructed from Arduino microcontrollers for ~\$750. At a fraction of the cost, our DIY behavioral olfactometer produced behavioral data comparable to commercial equipment in common olfactory assays. We hope this cost-effective, easy-to-use equipment will be used for both research and teaching purposes.

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Chapter 1: Introduction and Background

Olfaction and the Methods of Study

Olfaction, or the sense of smell, is the detection of chemical stimuli in the environment (Huart et al. 2009; Escanilla et al. 2009). Olfaction guides many behaviors such as foraging, predator avoidance, and mating (Ache and Young 2005). As environments and their chemical signatures change, the olfactory bulb also consistently alters neuronal physiology in response (Coppola 2012). This feature underlies the role of plasticity in the sense of smell.

Olfaction begins with environmental chemicals entering the nasal cavity, and then binding to and activating olfactory-sensory neurons (OSN). OSN then project axons into the main olfactory bulb (MOB) (Huart et al. 2009). The axons synapse with output neurons in glomeruli within the MOB (Huart et al. 2009). The MOB output neurons then project to other cortical structures for integration and conscious perception (Aungst et al. 2003; Huart et al. 2009). However, the olfactory bulb, even a glomerulus itself, processes a lot of sensory information (Wilson and Mainen 2006). The details underlying this initial sensory processing circuit, as well as how this processing circuit is altered in response to a changing environment, are not well understood.

Olfactory processing is often investigated via psychophysical testing in rodents (Restrepo and Slotnick 2005), and colloquially referred to as olfactory-mediated behavior. Psychophysical methods in olfaction have traditionally used rodents trained in olfactory discriminations tasks reminiscent of operant conditioning. This is known as behavioral olfactometry. This learning technique is commonly used to strengthen or weaken desired behaviors through consequence, such as reward or punishment (Schacter et al. 2011). The careful analysis of the behavioral

responses in these tasks are then used to elucidate the underlying circuits that drive the initial olfactory behavior.

Behavioral methods are not without their limitations. First, manual methods can be slow, and the experimenter may inadvertently introduce error. Second, most automated (that is nearly anything considered behavioral olfactometry) methods use trained animals, which requires a significant time investment (Qiu et al. 2014). Third, behavioral olfactometry equipment is cost prohibitive. Even “inexpensive” equipment can cost several thousand dollars, and may not include all necessary software. Lastly, current behavioral methods rely on external motivation to promote animal behavior. Mice undergoing operant-conditioning tasks are continuously evaluating their likelihood of obtaining reward. With the reward pathways potentially influencing or overriding olfactory perception, fully isolated study of the MOB is difficult (Kepecs et al. 2008). The predominant goals of this work have focused on validating new and inexpensive methods to study the initial sensory processing circuits in the MOB.

Chapter 2: Review of Relevant Literature

2.1 The Olfactory Pathway Review

Environmental chemicals enter the nasal cavity during inspiration, where they can interact with the olfactory epithelium. Olfactory sensory neurons (OSN) line the epithelium with cilia. The cilia express G-protein coupled receptors, called odorant receptors, and initiate adenylyl cyclase catalyst pathways when bound to an odorant (Buck and Axel 1991). There are between 300–1300 receptor genes depending on species (Ben-Arie et al. 1994; Buck and Axel 1991; Zhang and Firestein 2002). Each OSN expresses only one receptor type (Chess et al. 1994), establishing a one cell–one receptor rule. Encoding odors (sensation), however, is combinatorial. A single odor can activate multiple OSN, and a single OSN is activated by multiple odors (Malnick et al. 1999). The OSN are minimally organized by receptor type into large, distinct, and mirrored zones along the bilateral nasal epithelium, but the specific arrangement of OSN in these zones is random (Ressler et al. 1993). OSN axons bundle together to form the olfactory nerve that projects towards the main olfactory bulb (MOB) (Wilson and Mainen 2006).

As the olfactory nerve arrives at the MOB, it defasciculates into individual fibers that target and synapse in discrete neuropil structures called glomeruli (Figure 1). These structures surround the entire periphery of the MOB, and comprise the glomerular layer (Wilson and Mainen 2006). Each OSN receptor type has a specified pair of mirrored and interconnected glomeruli in each hemisphere of the MOB (Jan et al. 2008). This organization has allowed for the careful mapping of olfactory receptor topography on the MOB (Ressler et al. 1994; Vassar et al. 1994). There are between 1100–2200 glomeruli, and they range between 55 and 120 μm in

diameter (Pinching and Powell 1971). OSN axons form excitatory synapses on the dendrites of the mitral/tufted (M/T) cells within the glomeruli (Pinching and Powell 1971; Pinching and Powell 1972; Treloar et al. 2002). A glomerulus also contains interneuron fibers, some of which project laterally to other surrounding, and sometimes distant, glomeruli. This interconnected network of interneurons allows for lateral communication and inhibition among glomeruli (Nagayama et al. 2014; Kiyokage et al. 2010), and is largely thought to comprise an initial sensory processing network within the bulb (Banerjee et al. 2015; Ennis et al. 2001; McGann et al. 2005; Vaaga et al. 2017; Wachowiak and Shipley 2006), though this is discussed in greater detail later in this review.

From the glomeruli, M/T cells project sensory information towards several structures including the anterior olfactory nucleus (Brunjes et al. 2005; Brunjes and Illig 2008; Schoenfeld and Macrides 1984), piriform cortex (Scott et al. 1980), and amygdala for perception (Kevetter and Winans 1981; Licht and Meredith 1987). There are further interneuron processing components in the granule cell layer of the MOB. GABAergic granule cells are shown to further modulate outgoing M/T cell signaling to the cortex (Wilson and Mainen 2006); however, this deeper circuit is outside the focus of this review.

In total, only two neurons and one synapse are required to propagate olfactory sensory information to the brain for perception. As a result, much of the initial sensory processing falls on the interneurons that connect to all layers of the olfactory bulb (Banerjee et al. 2014; Aungst et al. 2003). How the interneurons collectively process sensory information, and how interneuron processing influences olfactory behavior, is not well understood.

2.2 The Glomerular Interneurons, Signal Modulation, and Dopamine

2.2.1 Glomerular Interneurons and Sensory Processing

The glomerular interneuron network is responsible for MOB sensory processing (Aungst et al. 2003; Banerjee et al. 2015; Ennis et al. 2001; McGann et al. 2005; Vaaga et al. 2017; Wachowiak and Shipley 2006). In addition to M/T cells, OSN co-excite a diverse network of glomerular interneurons that both surround the glomeruli, and span horizontally across the glomerular layer (Figure 1) (Kiyokage et al. 2010; Nagayama et al. 2010; Spors et al. 2012). Certain subsets of these interneurons pre-synaptically inhibit their own local M/T cell signal output (Figure 2A, Aungst et al. 2003; Banerjee et al. 2015; Ennis et al. 2001). Other interneuron subsets inhibit adjacent and sometimes distant glomeruli, and their corresponding M/T signal output (Figure 2B, Banerjee et al. 2014; Kiyokage et al. 2010; Vaaga et al. 2017). These findings, in part, have helped establish the gain control and center surround inhibition models of early sensory processing in the glomerular layer.

Gain control is a sensory processing model of interneuron dictated feedback inhibition of presynaptic OSN terminals (Figure 2A; Banerjee et al. 2014; Olsen and Wilson 2008). This inhibition is compensatory to OSN activation, and scales with OSN excitation strength (Olsen and Wilson 2008). Strong OSN activation will overcome inhibition, and successfully drive M/T activation. However, weak OSN activation may be incapable of overcoming gain control, and M/T cells receive no excitation. These interneurons, producing a detection threshold, are thought to prevent OSN signal saturation from concentrated or strong olfactory stimuli while simultaneously removing background noise.

Center-surround inhibition is a mechanism of feed-forward inhibition (Aungst et al. 2003), which targets the interneurons, presynaptic OSN terminals, and postsynaptic M/T cell

terminals of adjacent and distant glomeruli (Figure 2B; Kiyokage et al. 2010; Vaaga et al. 2017). Center-surround inhibition is similarly compensatory to OSN activation, and scalable to OSN excitation strength (Aungst et al. 2003; Vaaga et al. 2017). All glomeruli initiate feed-forward inhibition, though some OSN have greater excitation than others. A strongly-activated OSN will inhibit the feed-forward inhibition from other surrounding glomeruli with weaker OSN activation, thereby increasing signal to noise ratio.

Both gain control and center-surround inhibition may permit initial detection and discrimination between environmental odorants (Su et al. 2009). These mechanisms are traditionally GABAergic in function (Ennis et al. 1996; McGann et al. 2005), but an additional dopaminergic role is under study. Furthermore, how the sensory processing behind these mechanisms and circuits coalesce into complex olfactory behaviors is not fully understood.

2.2.2 The Dopamine and TH Connection

The MOB undergoes extensive plasticity in response to reduced environmental stimuli (Coppola 2012). Interestingly, histological analysis of MOB layers in anosmic mice demonstrates significant depression of both the dopamine (DA) synthesis enzyme tyrosine hydroxylase (TH) (Baker et al. 1983), and the gamma-Aminobutyric acid (GABA) synthesis enzyme glutamate decarboxylase 67 (GAD 67) (Parrish-Aungst et al. 2011). DA and GABA are principle neurotransmitters with neuromodulatory and inhibitory functions, respectively (Escanilla et al. 2009; Watanabe et al. 2002). Physically, depression in TH and GAD 67 manifest in a shrinking glomerular layer (Figure 3). It is not well understood why TH and GAD 67 are targeted through sensory deprivation, though this loss is associated with a specific glomerular interneuron subtype.

The glomerular interneurons were first categorized and visualized in the 1970s through Golgi staining analysis (Price and Powel, 1970A, B). In the glomerular layer, these cells are collectively referred to as the juxtglomerular (JG) neurons, and consist of periglomerular (PG) cells, external tufted (ET) cells, and short axon (SA) cells (Figure 4: Nagayama et al. 2014). Interestingly, only SA cells express TH to a large degree.

Short axon (SA) cells are categorized into two groups dependent on axon length and neurotransmitter type. Classic SA cells extend to only 1 or 2 adjacent glomeruli, and are singularly GAD 65 expressing (An alternative GAD enzyme, Nagayama et al. 2014). In contrast, some SA cells branch to adjacent, distant, and occasionally very distant glomeruli (~1mm axon, ~50 glomerular contacts) (Kiyokage et al. 2010), and are notably GAD 67 and TH co-expressing neurons. TH and GAD67 are the primary rate-limiting enzymes in the synthesis of the neurotransmitters dopamine (DA) and GABA, respectively. It is the TH+/GAD67+ SA cells that connect the other interneurons of the glomerular network, but the nature of these cells, especially with regards to the presence of TH, is not fully understood (Aungst et al. 2003; Kosaka and Kosaka 2011; Nagayama et al. 2014).

2.2.3 Dopamine and Olfactory Function

DA can alter olfactory behavior. Psychophysical experiments utilizing diffuse, in-vivo delivery of DA agonists and antagonists have shown significant depression of rat odor discrimination performance (Yue et al. 2004; Escanilla et al. 2009). Furthermore, DA function is substantiated via the presence of dopamine receptors on the presynaptic OSN (Ennis et al. 2001), M/T cells (Huart et al. 2013; Kiyokage et al. 2014), and on the many JG cell subtypes (Kiyokage et al. 2014). Activating presynaptic DA receptors inhibits OSN activity, and thus serves as a

mechanism for gain control (Ennis et al. 2001; Vaaga et al. 2017). However, these results are controversial (for review, see McGann 2013).

Outside the localization of DA receptors within the glomeruli, there is still a significant lack of understanding regarding a direct link between dopamine inhibition and TH+/GAD67+ cells. It is also not fully understood how changes to these cells affect olfactory behavior. The pharmacological methods discussed previously can be “messy.” Diffuse delivery of dopamine compounds, while complex, is imprecise in target and action. No specific cell type was identified from these studies (Escanilla et al. 2009; Kepecs et al. 2008), and the resulting behavior may not represent true dopamine influence. This is an important distinction. Glomerular inhibition is traditionally viewed as a function of GABAergic SA cells, with no DA role (Banerjee et al. 2014; McGann et al. 2005). Furthermore, the behavioral methods used to study dopamine and this interneuron network are limited, expensive, and significantly time intensive. To better understand the role of TH and SA cells in sensory modulation, and the environmental change consequences on this network, better behavioral methods and dopamine manipulation techniques must be developed.

2.3 How to Study the Interneuron Circuit and a Dopaminergic Role

2.3.1 Behavioral Olfactometry and Current Method Limitations

Multiple behavioral olfactometry techniques have been developed and improved upon since the 1970s to study olfactory processing (Restrepo and Slotnick 2005; Slotnick and Nigrosh 1974). Behavioral olfactometers are machines that first present odorized air to subjects, carefully controlling the physical and temporal delivery of odorants, and subsequently record a subject’s corresponding behavioral response (Figure 5; Restrepo and Slotnick 2005). Care is taken to

activate OSN while also preventing confounding signal input. Since glomerular activity and subsequent behavioral response is dependent on the specific odor, stimulus control is crucial for adequate data collection.

Behavioral olfactometry experiments typically use operant conditioning techniques to train animals in detecting and discriminating between odors (Frederick et al. 2012). These operant conditioning tasks include the Go/No Go (GNG) and Two-alternative Forced Choice (2AFC) tasks (Clevenger and Restrepo 2006; Mihalick et al. 2000; Nigrosh and Slotnick 1975; Slotnick and Nigrosh 1974). Using these techniques, rodents routinely discriminated between chemically similar odorants, odorants at low concentrations, and even the same odorants of varying brands (Abraham et al. 2004; Gamble and Smith 2009). However, current behavioral olfactometry techniques have limitations.

GNG and 2AFC require, in principle, motivated animals. Problems arise when animals lose motivation during a behavioral task. This can occur either through distracting external stimuli, or an inability to predict reward outcome from the given stimulus (Kepecs et al. 2008). To motivate animals, operant condition requires training to associate successful task completion with reward (Frederick et al. 2011). One critique of these methods is the significant training time required for rodents to learn the behavioral tasks (Qiu et al. 2014). Perhaps of greater importance is the reliance on reward, which intertwines the reward pathways with the olfactory system (Kepecs et al. 2008), and may further confound behavioral data. Water or food deprivation has been the standard of motivational incentive in GNG and 2AFC task for many years (Slotnick and Nigrosh 1974). However, this has been shown to alter behavior in trained tasks, and thus prevents isolated study of the olfactory bulb circuits (Kepecs et al. 2008). Therefore, a behavioral model that does not require training or the use of reward is highly desirable.

Fortunately, a new task that relies only on innate animal curiosity, such as environment exploration, to drive behavior was recently developed. This task, referred to here as PROBES, eliminates operant conditioning and reward association (Qiu et al. 2014). PROBES used non-motivated, non-trained animals, but required the subjects be naïve to both the odorants, and the behavioral task. Using simple habituation/dishabituation tasks, the study found that unmotivated rodent models can produce behavioral data with outcomes comparable to both GNG and 2AFC (Qiu et al. 2014). Rodents are no longer bound to respond to a task based on their confidence of obtaining a reward (Qiu et al. 2014). Unfortunately, the PROBES model of non-motivated animal behavior has received little study outside of this original publication. In addition, the PROBES apparatus costs nearly \$30,000 to build. We seek to improve upon this methodology in a cost-effective manner by first determining the efficacy of non-motivated behavior using modified commercial behavioral olfactometers. We will then determine the feasibility of producing in-house behavioral olfactometers at a significantly decreased cost relative to commercial equipment.

2.3.2 Novel Anosmia Induction Agents: Triton-100x

Behavioral olfactometry needs to be biologically relevant. To validate the relevance of our procedure, we altered both the patency and chemical composition of the olfactory system, and measured changes in olfactory-guided behavior. We propose to produce a temporarily anosmic mouse through a novel method: sensory deprivation using the detergent Triton-100x (Triton). Intranasal irrigation of 0.1% Triton is proposed to cleave OSN cilia as it washes through the nasal epithelium, resulting in temporary anosmia (Adamek et al. 1984). This concentration of Triton spares the OSN, and allows cilia to regrow within 48 hours (Adamek et

al. 1984). This recoverable nature of Triton improves the ability to perform proper control experiments before, during, and after treatment. This new technique, if sufficient, will allow for the improved study of a dopaminergic role within the MOB, where anosmia is known to reduce dopamine (Baker et al. 1983; Coppola 2012). Recoverable anosmia coupled with behavior may facilitate the study of dopamine and glomerular processing in the bulb

2.4 Specific Aims

1. Are non-motivated animal models effective?
 - a. We hypothesize that non-motivated animal models are a reasonable means to study animal behavior, and that non-motivated behavioral experiments with rodents will produce comparable behavioral outcomes in odor threshold detection and odor discrimination tasks.
2. Is modification of existing equipment for non-motivated behavior models possible?
 - a. We hypothesize that modification of existing behavioral olfactometers is an effective, affordable alternative to purchasing new equipment for use in non-motivated behavioral experiments.
3. Can Triton-100x treatment alter mouse investigative behavior?
 - a. We hypothesize that intranasal delivery of 0.1% Triton will significantly change mouse detection threshold and discrimination performance.
4. Is the production of in-house inexpensive behavioral olfactometers possible?
 - a. We hypothesize that it is possible to manufacture ultra-low cost and easy to use behavioral olfactometers in house from Arduino microcontrollers, DIY hardware supplies, and python programming language.

- b. We predict this equipment will produce comparable behavioral outcomes in detection threshold and discrimination experiments when compared to our modified commercial equipment.

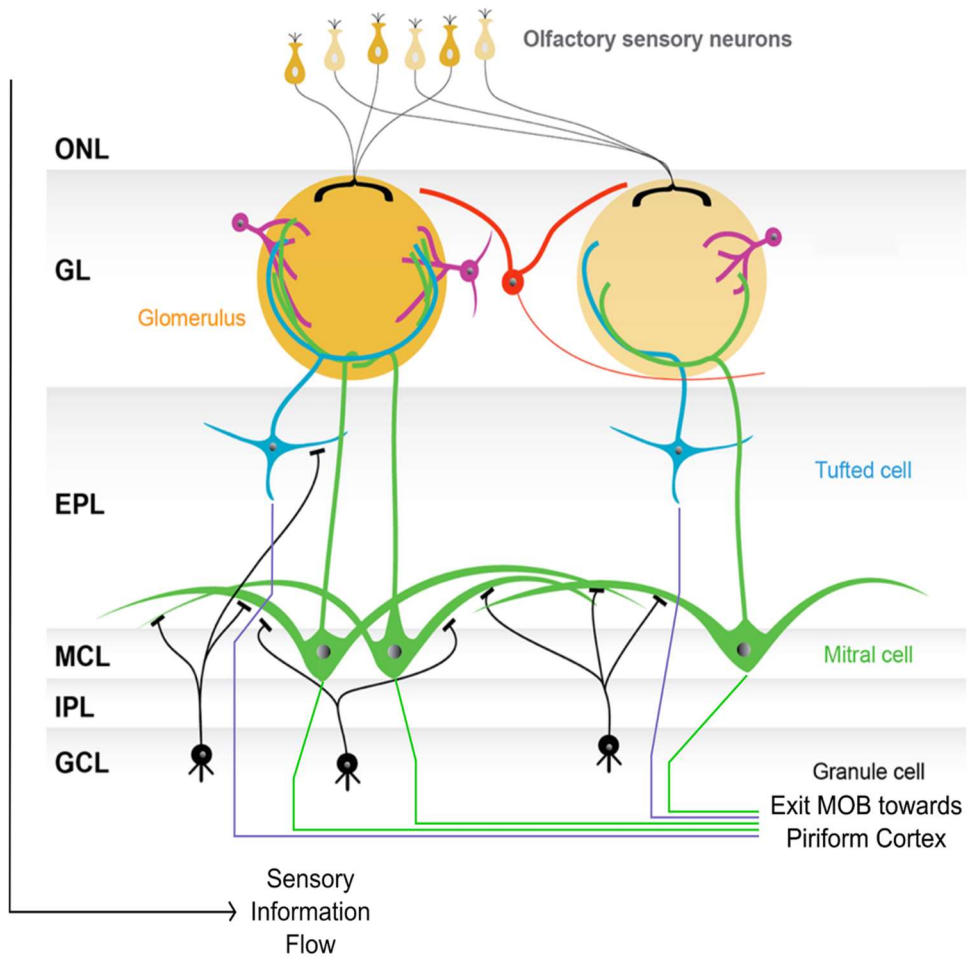


Figure 1: The primary olfactory pathway. This diagram depicts the layers of the main olfactory bulb (MOB), as well as the direction of sensory information flow. The MOB is comprised of three primary cell layers: the glomerular (GL), mitral (MCL), and granule cell layer (GCL). Three fibrous layers also exist consisting of the olfactory nerve, external plexiform (EPL), and internal plexiform layers (IPL). Mitral and tufted cell axons project deeper into the olfactory bulb where they cluster and project towards the piriform cortex.

Source: Nagayama et al. 2014.

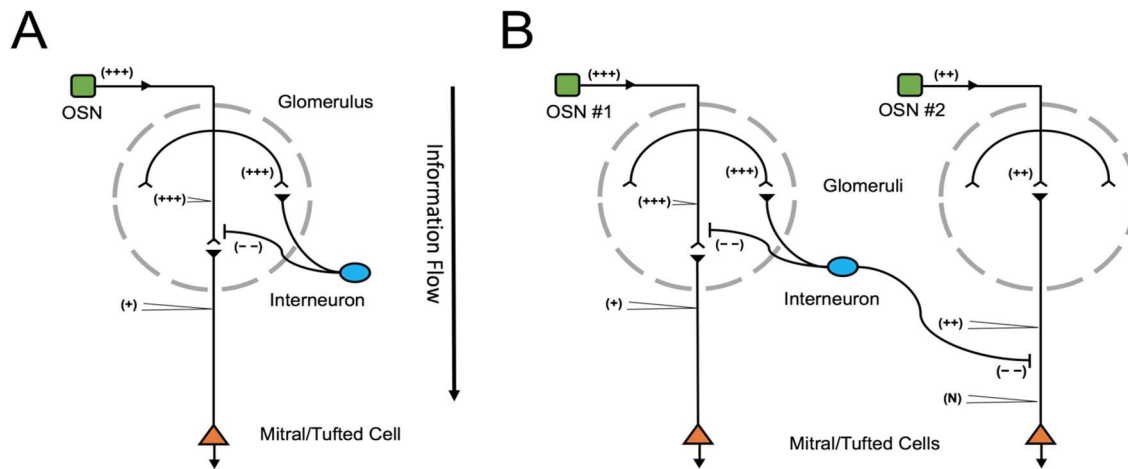


Figure 2: Models of glomerular olfactory sensory processing. **A.** Gain control: Strong OSN activation co-stimulates interneurons resulting in feedback inhibition of the OSN pre-synaptic terminals. **B.** Center-Surround Inhibition: Strong OSN activation co-stimulates interneurons resulting in feedforward inhibition of surrounding glomeruli. This feed-forward inhibition will completely inhibit adjacent M/T cell output (OSN #2) if the initial glomerular (OSN #1) activation is stronger.

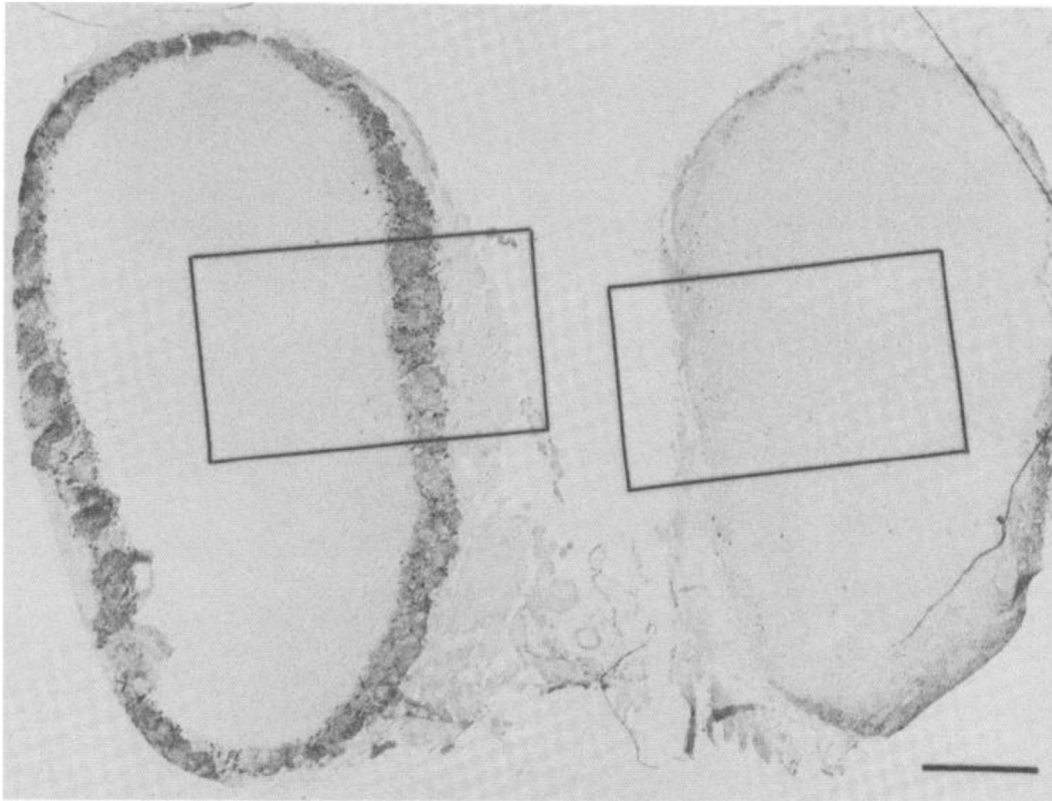


Figure 3: MOB Tyrosine hydroxylase following unilateral naris occlusion. **Left:** Histological analysis of the unoccluded MOB reveals a large, dark staining band in the glomerular layer. This indicates a large concentration of TH, and likely the dense presence of TH+/GAD67+SA neurons. **Right:** Analysis of the occluded MOB reveals significantly altered MOB morphology. This is immediately evident by the lack of a dark staining glomerular layer TH ring. The width of the glomerular layer has also shrunk in size.

Source: Baker et al. 1983.

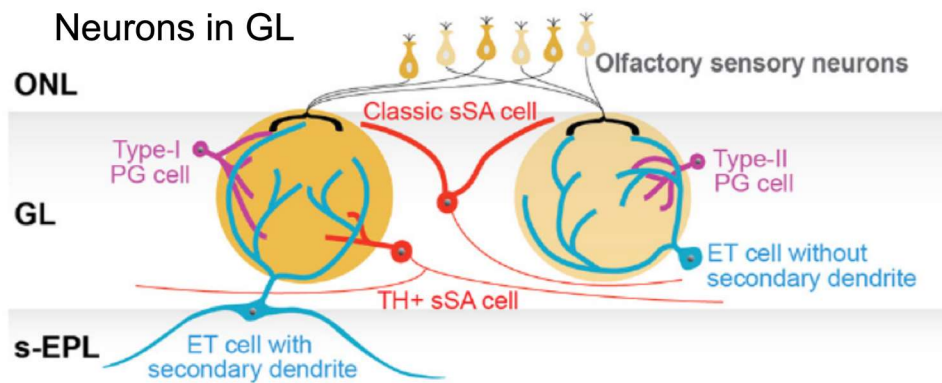


Figure 4: The interneurons of the glomerular layer. Juxtglomerular (JG) interneurons are divided into three groups consisting of periglomerular (PG), external tufted (ET), and short axon (SA) cells. PG cells are defined by their dendrodendritic synapses. Type-I PG cells receive input from both OSN and other interneurons. Type-II only receive input from other interneurons. SA cells are grouped by neurotransmitter type. Classic SA cells are GABAergic, though new evidence shows they can also express tyrosine hydroxylase (TH). TH is the rate limiting enzyme in dopamine synthesis. There are further subdivisions of each JG cell subtype, although the distinguishing factors are beyond the scope of this project.

Source: Nagayama et al. 2014.

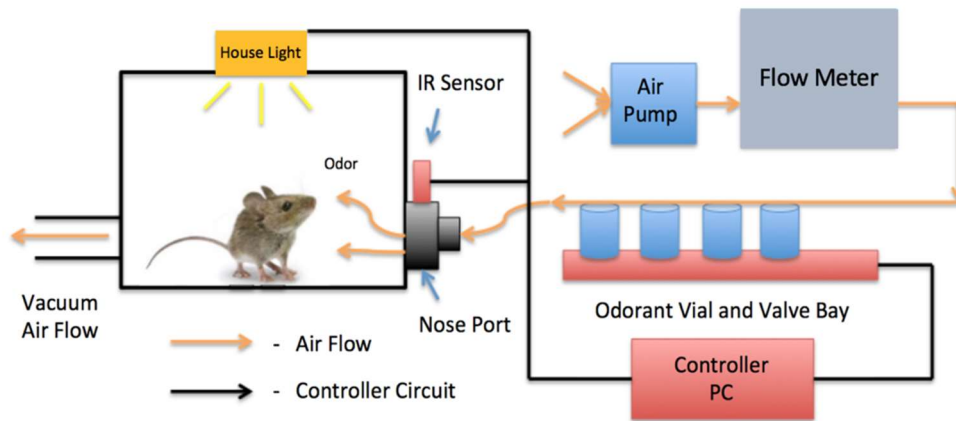


Figure 5: Typical liquid dilution behavioral olfactometer design. A behavioral chamber is used for animal experimentation. Connected to this are a house light, nose port for odorant delivery, and a vacuum port for air ventilation. Hidden within the nose-port is an IR sensor for recording animal investigation of odors. Odors are delivered using a pneumatic hose system. An air pump forces air first through a flow meter for volume control. Drierite or charcoal filters can be used for air filtration, but they are not depicted. Air is pushed into a series of odor containing vials. Tubing is fed through a series of valves to control order and timing of odor delivery. A second line of tubing connects the odor vials to the nose port for presentation. A centralized PC controls the system and collects behavioral data from the IR sensor.

Chapter 3: Research Design and Methodology

3.1 Animals

C57BL/6 male and female mice originally from Jackson Laboratories (Bar Harbor, Maine) were housed and bred in the Eastern Michigan University Vivarium. Lights were on a reverse 12-hour light-dark cycle (0700 lights off). Mice were group housed in filter top, polycarbonate cages with corn cob bedding (Bed-o'Cobs ¼", The Andersons Lab Bedding). Food and water were provided ad libitum. Only naïve animals, those never exposed to behavioral testing, were used for experimentation. Behavioral testing was performed between 0900 and 1600 hours during the dark cycle. All experimentation followed guidelines approved by the Eastern Michigan University Institutional Animal Care and Use Committee (IACUC) protocol 2014-060, as well as those outlined by the NIH.

3.2 Experimental Testing and Behavioral Tasks

Detailed behavioral assay guidelines outlined in Escanilla et al. (2009) and Qiu et al. (2014) were used throughout the testing of non-motivated animal behavior, anosmia testing with Triton, and sensory testing of animal deficits. All tasks were modified habituation/dishabituation assays. These tasks use repetitive presentation of a known stimulus (habituation trials), followed by the presentation of a novel stimulus (test trial). The animal's total response to each presentation was measured. Habituation is defined as the decrease in odor investigation (sniffing/poking) across repeated presentations of the same stimulus (such as an odorant). Dishabituation is defined as the increase in, or reinstatement of, investigatory behavior towards a novel stimulus (such as an odorant) (Bouton 2007). Depending on the animal's ability to

discriminate between habituation odor and test odor, the mouse will either detect a difference and dishabituate, or fail to detect a difference and remain habituated. Examples of simple, five-odor presentation habituation/dishabituation experiments can be seen in Figure 6, A and B. Behavioral experimentation was split between two behavioral paradigms discrimination and detection threshold tasks. Furthermore, these behavioral tasks were automated, and conducted using the behavioral olfactometer. Manual experimentation, behavior done by hand, was also performed for preliminary experiments testing non-motivated behavior and the effects of Triton treatment on behavior.

3.2.1 Discrimination Tasks

These experiments test an animal's ability to discern a difference between the final habituation odorant and the test odorant. Mice that discern this difference dishabituate to the test odor and investigate it more. The mice that do not discern a difference fail to dishabituate, and investigate the test odor the same or less than the previous trial. When given an 'easy' odor pair (mineral oil vs acetophenone) this task can detect anosmia.

3.2.2 Detection Threshold Tasks

Detection threshold tasks are designed to test mouse odor detection capabilities. These tasks utilize lengthened discrimination tasks with multiple test odorants of gradually increasing concentration (Figure 7). Mice remain habituated to test odorants at concentrations below their detection threshold for physical sensation. Likewise, test trial odorants at concentrations at or above detection threshold cause mice to dishabituate.

3.2.3 Manual Behavior

For preliminary experiments, a manual interpretation of the habituation/dishabituation assay was used, and referred to as the “Q-tip task.” This name is in reference to the experiments using cotton swabs to present odors to mice for manual behavior experiments (Escanilla et al. 2009). Mice were first habituated to a cage similar to the home cage for 10 minutes. Odorants in 10 uL volumes were then applied to the tip of a cotton swab and inserted into one end of the cage. Mouse investigatory behavior such as rearing within 2–3 cm of a presented cotton swab is recorded as investigation time. Odorants were presented for one minute, with two minutes between each presentation. Mice were given a total of four habituation odor presentations, and a final test odor presentation.

3.2.4 Automated Behavior

During all experiments, odor trials were 2 minutes and the inter-trial interval was 3 minutes. Mice were habituated to the test box for 10 minutes before the task began. Odor concentrations and the task setup varied depending on the task type (discrimination or detection threshold).

3.3 Olfactometer and Odor Delivery Setup

Automated experimentation was performed with a modified Vulintus behavioral olfactometer (Vulintus Inc. Dallas, Texas). This equipment is similar in design to the original liquid dilution, solenoid-based behavioral olfactometers first described in the 1970s (Slotnick 1974), and later revised in the early 2000s (Restrepo and Slotnick 2005). A simplified

representation of this equipment is depicted in Figure 5. An external master control board operated the olfactometer through direct input to our bay of solenoid valves, and output from the behavioral chamber IR sensor. The board was connected to a windows-based PC and controlled via a MATLAB-based graphical user interface. The behavioral chamber was housed within a sound isolation chamber (Lafayette Instrument: Lafayette, Indiana). External noise was further reduced by a noise-generating speaker placed next to the behavioral box. The speaker produced a constant 75dB brown noise within the sound isolation chamber, and was calibrated by an iPhone application (SPLnFFT: Kardous and Shaw 2014). Odorant delivery was accomplished using a pneumatic hose system with an air pump, charcoal filtration, and a flowmeter to control volume. A single line from the flowmeter was divided into eight individual lines, each passing through a solenoid valve, and into a corresponding 120 mL airtight vial. All vials have a second line for air output that connects to the multi airline input manifold. This manifold then attaches to the nose port of the behavioral chamber for odor presentation.

3.4 Odorants

Odorants used in this experiment were purchased from Sigma Aldrich at the highest available purity. Mineral Oil (MO) was used as both the habituation odorant and the diluent. Test odors were either amyl acetate (AA) or acetophenone (AP) and were serially diluted in mineral oil (v/v) to concentrations ranging from 1×10^{-3} to 1×10^{-9} , depending on experimental design. Dilutions were freshly prepared before each experiment, and test odor vials were remade for each animal.

3.5 Data Acquisition and Analysis

Animal behavior is measured as the overall animal investigation to a presented odor. One wall of the behavioral chamber contained an odor delivery nose port with a hidden infrared (IR) sensor. Animals interested in the presented odor insert their nose into the port, or “poke,” which breaks the IR beam. The breaking the IR beam was recorded as enter and exit times in a text (.txt) file by the controlling PC and allowed for calculation of time per nose poke. Post processing was then performed using an in-house MATLAB script to analyze text files (kindly provided by Dr. John Thompson). All sub-50 ms “pokes” were removed to control for background IR noise levels. Total “poke” time (ms) for each odor presentation was tabulated, normalized, and compiled per group in Excel. Due to the variable nature of curiosity between different animals, investigation times for each trial were normalized to the animal’s baseline level of activity. Normalized port investigation (NPI) was calculated as follows:

$$\text{NPI} = \text{Trial } x / \text{average investigation of habituation trial}$$

This allowed for better comparison of animals within group. The method used here was previously detailed in PROBES (Qiu et al. 2014). Student T-tests were performed on the final habituation trial and the test trial to determine significance of dishabituation. Significant p-values (> 0.05) denote successful odor detection or discrimination. All error bars denote standard error of the mean (SEM).

3.6 Anosmia Agent: Triton-100x and Delivery

Triton is a detergent capable of depriving sensory input to the olfactory bulb and thus inducing anosmia. Triton was diluted in phosphate buffered saline (PBS) to 0.1%. Mice were treated using intranasal irrigation with 10 uL of Triton per nare using a micropipette. Mice received three treatments spread across five days (Days 1, 3, and 5), for a total of 30 uL of Triton per nare. Control mice were treated with unaltered PBS using the same methods.

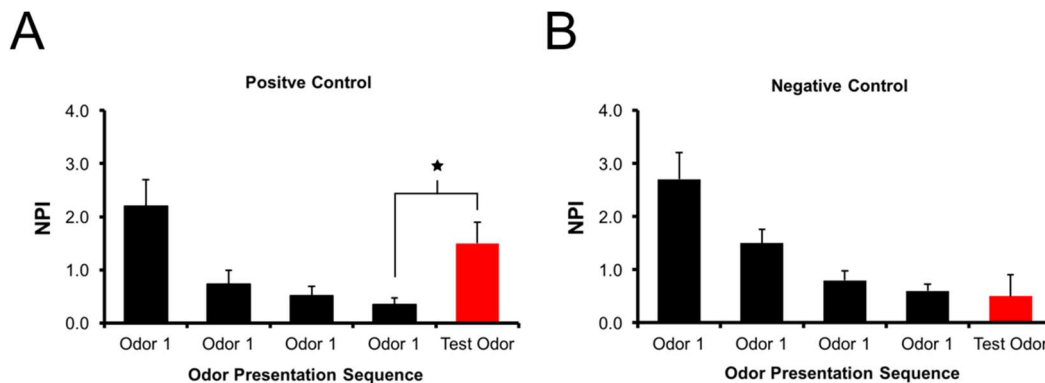


Figure 6: Examples of five-trial positive and negative control discrimination tasks. **A.** Five-trial positive control discrimination task. Odor 1 was presented four consecutive times, and a novel odor was presented in the test trial. Mice habituated or decreased their investigation across repeated trials of Odor 1. When presented a novel odor for a test trial, mice increased their olfactory investigation, and dishabituated. In positive control experiments, mice are expected to dishabituate to a test odor when it is different from the habituation odor. * Indicates significant difference in investigatory behavior between the test trial and the preceding MO trial. **B.** Five-trial negative control discrimination task. Odor 1 was presented five times, including the test trial. Mice again habituated across trials 1–4. When presented the same odorant for the test trial, mice are expected to remain habituated and not increase investigation

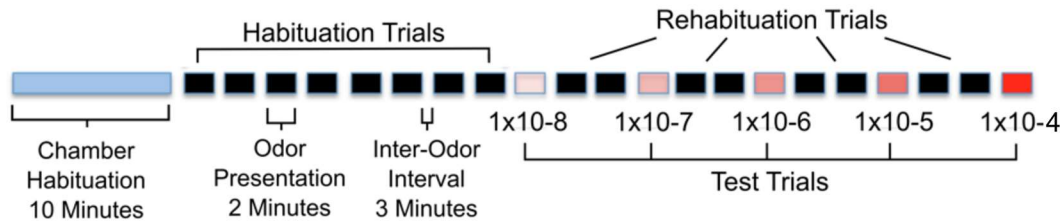


Figure 7: Odor detection threshold task. Threshold tasks are extended discrimination tasks using multiple test trials. Test trials gradually increase the concentration of the test odor. Each short black or red tile represents an odor presentation. Each odor is presented for two minutes. In-between each odor presentation are inter-odor-intervals. These are periods of non-stimulus presentation for three minutes. Mice are expected to initially habituate and remain habituated to all succeeding test trials until reaching the concentration needed for detection. Two mineral oil trials are presented between each odor to re-habituate animal behavior.

Chapter 4: Results

4.1 Preliminary Experiment Results

Preliminary experiments tested Triton treatment effectiveness in mice on manual (non-olfactometer based) discrimination tasks. Eighteen phosphate buffered saline (PBS) and Triton treated mice underwent five-trial acetophenone discrimination testing on either the day following treatment (Day 6), or with one day of recovery time (Day 7) (Figure 8). Mice were presented four mineral oil habituation trials, and a single 1:1000 acetophenone test trial. Both PBS control and Day 7 Triton mice habituated across the mineral oil trials, and significantly dishabituated to the 1:1000 acetophenone test trial (PBS: $p < 0.05$, $n = 6$; Day 7 $p < 0.05$, $n = 6$), indicating successful discrimination between mineral oil and acetophenone. Day 6 Triton treated mice, however, habituated to across the mineral oil trials, and failed to dishabituate to the acetophenone test trial ($p > 0.05$, $n = 6$), indicating impaired olfactory function. The contrast in successful discrimination behavior between Day 6 and Day 7 Triton mice offers initial supporting evidence for using Triton as a recoverable anosmia inducing agent.

4.2 Non-Motivated Behavior with Modified Commercial Equipment

4.2.1 Experiment 1: Validating Non-Motivated Behavior

We next set out to validate the use of non-motivated animal methodology in automated equipment. Specifically, we wanted to know if non-motivated behavior was capable of producing amyl-acetate detection thresholds in modified commercial behavioral olfactometers. Eight mice underwent an amyl acetate detection threshold curve. This test used amyl acetate concentrations of 1×10^{-8} , 1×10^{-7} , 1×10^{-6} , 1×10^{-5} , and 1×10^{-4} diluted in mineral oil (v/v). All eight mice

habituated to the mineral oil trials, and significantly increased their investigation on the first amyl acetate test trial at 1×10^{-8} in concentration (Figure 9A; $p < 0.05$, $n = 8$). However, this result was unexpected as the reported concentration needed for amyl acetate detection is approximately 2×10^{-6} . The mice investigated an odor at two orders of magnitude lower in concentration than the minimum reported in literature (Clevenger and Restrepo 2006; Qiu et al. 2014). Following this observation, we sought to validate this observed “investigation” of 1×10^{-8} amyl acetate (AA).

Mice were next tested on a five-trial amyl acetate discrimination tasks consisting of four mineral oil habituation trials, and a single test odor of 1×10^{-8} amyl acetate. A shorter discrimination task was employed to reduce the time necessary to test discrimination of individual concentrations of odorants. Five mice again habituated across the mineral oil trials, and significantly dishabituated to the 1×10^{-8} amyl acetate the test trial (Figure 9B; $p < 0.05$, $n = 5$). This result indicated, with our behavioral olfactometer, that mice can detect 1×10^{-8} amyl acetate. We did not believe this represented true detection. Experimentation went one step further, and tested mice discrimination of 1×10^{-9} amyl acetate. All mice habituated across the mineral oil trials, and significantly dishabituated to the 1×10^{-9} amyl acetate test trial (Figure 9C; $p < 0.05$, $n = 11$), indicating detection of amyl acetate three orders of magnitude below literature reported values (2×10^{-6}). These results elicited two possibilities: either our modified commercial apparatus was much more sensitive than equipment used previously, or there existed some extra-target stimulus that our animals cued to during behavioral experimentation.

4.2.2 Experiment 2: Negative Control Testing

We next began negative control testing over concern for possible presence of non-target cues such as external noise cues, additional odor cues from poorly cleaned hosing, or subtle differences in prepared odors. Twelve mice were tested in a negative control discrimination task with mineral oil as both the habituation odor, and the test odor. All twelve mice habituated to the mineral oil trials as expected, and statistically remain habituated to the mineral oil test trial (Figure 10A; $p < 0.05$, $n = 12$). At the group level, mice did increase test trial investigation though not significantly. Further analysis of these mice individually, however, revealed polarized test trial investigation. Four of 12 mice falsely dishabituated to the test odor (Figure 10B), denoting discrimination of some difference. The etiology of this polarization remained unclear, and substantial changes to the behavioral olfactometer were then made.

4.2.3 Olfactometer Modifications

To identify the cause behind the erratic dishabituation in the threshold and discrimination testing, multiple options were considered concerning proper stimulus control. Were the mice investigating the nose port solely due to the odor stimulus presented to them, or was some additional variable present? First, we considered the role of sound, such as the clicking noise of our valves, or other distracting ambient noise. To account for this, the valve bay and olfactory chamber were housed within individual sound isolation chambers. Foam window tape was added to all spaces of these isolation chambers for improved sound control, and a speaker playing a constant, 75 dB brown noise was added.

Furthermore, better airflow in the behavioral chamber was considered. Perhaps odorants were aggregating in the behavioral chamber and preventing the detection of new odorant stimuli. To correct for this, corrugated tubing was placed near an outport in the behavioral chamber wall,

opposite the nose port. This tubing was connected to a ventilation fan attached to the outside of the sound isolation chamber. These equipment changes were performed at different time points, and further negative control experiments were conducted. Unfortunately, all experiments produced the same, heterogeneous outcome in negative control testing—mice were not under stimulus control. For the sake of complexity and brevity, these experiments are not included here. Seemingly out of options, one last variable was considered—the odorants themselves.

Interestingly, it has been shown that some trained rodents can detect and discriminate between minor differences in different brands of mineral oil (Gamble and Smith 2009). This is important when considering the odor vial setup used in our equipment and experiments. Discrimination and detection threshold tasks to this point have relied on a two-vial setup (Figure 11A), where one vial supplies all the presentations for the habituation odorant, and a second vial supplies the test odorant. It is plausible that repeated use of the same vial of odorant for subsequent presentations may cause minor changes in the characteristics of that odor. These characteristic changes may produce detectable differences to mice for an otherwise familiar odor. To account for these possible odor changes from repeated vial use, we consider an alternative, individual odor vial setup (Figure 11B).

4.2.4 Experiment 3: Strong Stimulus Vial-Setup Testing

To determine if odor vial setup may prevent proper stimulus control of our mice, two negative-control experiments were considered. The first negative control experiment tested discrimination of a strong stimulus (amyl acetate). Twelve mice were split into two groups, consisting of two-vial (control) and five-vial (individual vial) setups. The two-vial mice groups habituated and significantly dishabituated to the amyl acetate test odor. In contrast, the five-vial

mice habituated and failed to dishabituate to the test odor (Figure 12; two-vial: $p < 0.05$, $n = 6$ five-vial: $p > 0.05$, $n = 6$). Furthermore, there was a significant difference in average test odor investigation between the two-vial and five-vial set up groups ($p < 0.05$, $n = 12$). This data demonstrated clear behavioral differences when odor presentations are sourced from multiple use vial setups (two-vial). With a strong stimulus, a five-vial (individual vial) odor setup maintained improved stimulus control over a two-vial setup. Unfortunately, not all experiments will use a strong stimulus such as amyl acetate. We also considered a weak stimulus, such as mineral oil, to determine if more subtle differences in behavior will result.

4.2.5 Experiment 4: Weak Stimulus Vial-Setup Testing

To further test odor vial setup, a second negative control experiment tested discrimination of a weak stimulus (mineral oil). Twelve mice were again split into two groups, consisting of two-vial (control) and five-vial (individual vial) setups. Interestingly, at the group level both two- and five-vial groups habituated, and remained habituated to the mineral oil test odor (Figure 13A: $p > 0.05$, $n = 6$). That is, mice in both vial set up groups appeared to remain under stimulus control. It is important to note, however, that the two-vial setup mice did increase their investigation of the test odor, though not significantly. Further analysis of the mice in the two-vial group again revealed polarization of investigation (Figure 13B) similar to mice from the earlier control experiments (see: Figure 10B). Three out of 6 mice incorrectly dishabituated to the test odor. Thus, about 50% animals were not under stimulus control in the two-vial set up. Why this polarization again occurred remains unknown however, using individual vials for each odor presentation clearly maintained better stimulus control in the mice, and resulted in more accurate behavioral data (Figure 12, 13A). Moving forward, we applied the individual odor vial

set ups to replicate detection threshold experiments. We hoped to find a more accurate detection threshold of amyl acetate that compares with empirically established values.

4.2.6 Experiment 5: Repeat Threshold Testing with Individual Vials

We returned to amyl acetate detection threshold testing (Figure 9) with an individual odor vial setup. Separate positive and negative control experiments were performed. The first experiment tested mice using amyl acetate concentrations of 1×10^{-8} , 1×10^{-7} , and 1×10^{-6} , all of which fall below the reported detection value ($\sim 2 \times 10^{-6}$; Qiu et al. 2014; Clevenger and Restrepo 2006). With the individual-vial set up, mice remained habituated across all trials (Figure 14A; $p > 0.05$, $n = 6$). Next, we tested a positive control detection threshold with two amyl acetate concentrations below threshold (1×10^{-7} , 1×10^{-6}), and one above (1×10^{-5}). Mice again habituated across all mineral oil habituation trials and to amyl acetate concentrations below detection threshold. Importantly, the mice significantly dishabituated to amyl acetate at a concentration of 1×10^{-5} (Figure 14B; $p < 0.05$, $n = 5$). This result demonstrates an amyl acetate detection threshold between 1×10^{-6} and 1×10^{-5} , which falls within the detection thresholds previously reported in literature ($\sim 2 \times 10^{-6}$; Clevenger and Restrepo 2006; Qiu et al. 2014).

4.3 Automated Testing of Triton Treated Mice

With evidence of proper equipment setup and stimulus control of mice, we returned to testing Triton treatment on mice. Measurement of non-motivated behavior of biologically altered mice must be possible using our automated equipment. Twenty-three mice underwent a five-trial acetophenone discrimination task identical to preliminary experiments. Mice were divided first between treatment groups. Half of the mice received 0.1% Triton, and the other half received

phosphate buffered saline (PBS) as a control. All mice groups received three treatments across five days (Day 1, 3, and 5). Mice were then further divided into treatment days, with half of Triton and PBS treated mice undergoing behavior on Day 6, and the other half on Day 7. Day 7 mice were thus allowed one day of treatment recovery time. Day 6 Triton mice habituated and failed to dishabituate to the test trial (Figure 15; $p > 0.05$, $n = 6$). This result indicated failed discrimination and impaired olfactory epithelium function. All mice in the PBS Day 6, PBS Day 7, and Triton Day 7 groups habituated to mineral oil, and significantly dishabituated to the 1:1000 acetophenone test trial (Figure 15; PBS Day 6: $p < 0.05$, $n = 6$, PBS Day 7: $p < 0.05$, $n = 6$, Triton Day 7: $p < 0.05$, $n = 5$), indicating both successful discrimination, and recovered olfaction in Day 7 Triton mice. This outcome met expectations, and the results were comparable with manual Triton testing outcomes (Figure 9). These results further established low-concentration Triton as an effective treatment for inducing recoverable anosmia. Lastly, these results also demonstrate that our modified behavioral olfactometer was capable of discerning changes in non-motivated olfactory behavior associated with Triton treatment.

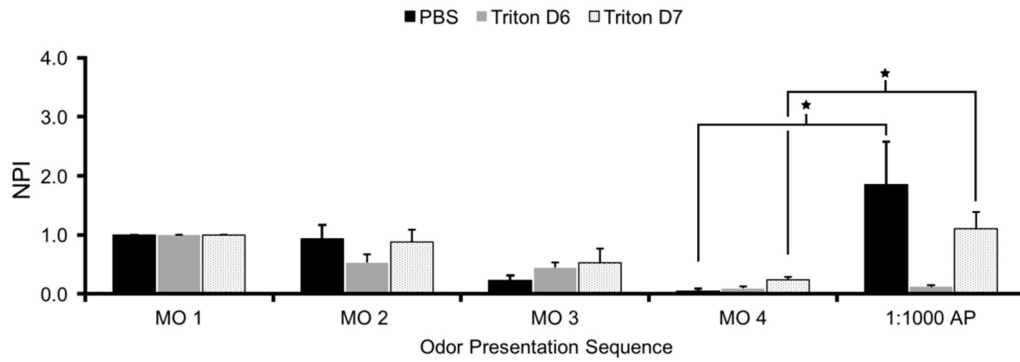


Figure 8: Manual odor discrimination testing of Triton treated mice. Day 6 Triton mice habituated across the mineral oil trials, and failed to significantly dishabituate to 1:1000 acetophenone test trial (Day 7: $n = 6$, $p > 0.05$). Both Day 7 Triton mice and PBS control mice habituated to the mineral oil test trials, and successfully dishabituated to the 1:1000 acetophenone test trial (Day 6: $p > 0.05$, $n = 6$; Day 7: $p < 0.05$, $n = 6$; PBS: $p < 0.05$, $n = 6$). * Indicates significant difference in investigatory behavior between the test trial and the preceding MO trial.

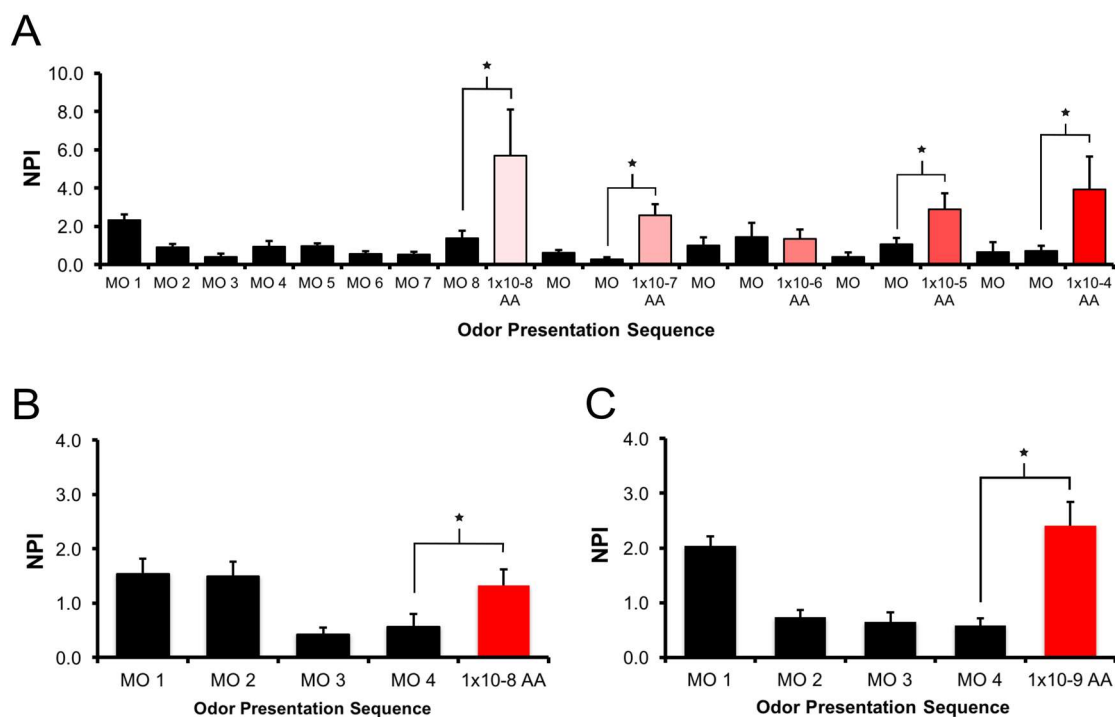


Figure 9: Odor discrimination and detection threshold tasks for amyl acetate. **A.** Normalized average of mouse investigatory behavior across eight habituation trials (MO), and subsequent odor test trials at varying concentrations of Amyl Acetate. * Indicates significant difference in investigatory behavior between the test trial and the preceding MO trial ($p < 0.05$, $n = 8$). **B & C.** Supplemental discrimination testing validating mouse detection of amyl acetate at 1×10^{-8} , as well as at 1×10^{-9} . This graph depicts normalized mouse investigatory behavior in a simplified discrimination assay with four MO habituation trials and a single test trial. * Indicates significant difference in investigatory behavior between the test trial and the preceding MO trial (**B:** $p < 0.05$, $n = 5$. **C:** $p < 0.05$, $n = 12$). * Indicates significant difference in investigatory behavior between the test trial and the preceding MO trial.

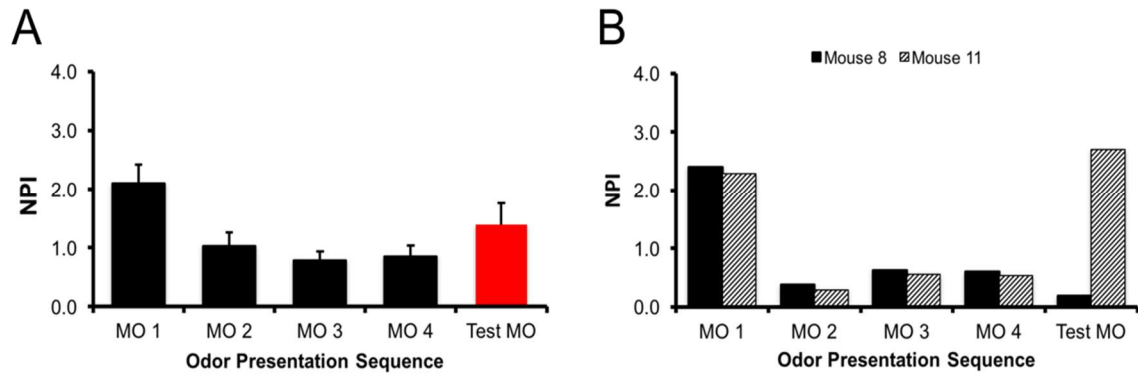


Figure 10: Negative control odor discrimination task for equipment testing. **A.** Normalized average of mouse investigatory behavior in response to four MO habituation trials and a 5th MO test trial presentation. No significant difference is observed between the test trial and the preceding MO trial. ($n = 12$, $p > 0.05$). **B.** Normalized investigatory behavior of individual mice in the habituation/dishabituation negative control assay. This graph shows the behavioral response of two individual mice from Figure 10A. Mouse 8 represented in black, demonstrated the desired behavior, depicting an animal under stimulus control for all trials. Mouse 11 demonstrated the undesired behavior of dishabituation to a non-novel test odor. Mice with behavior similar to mouse 11 did not appear under stimulus control. All mice in the sample demonstrated this polarized behavior.

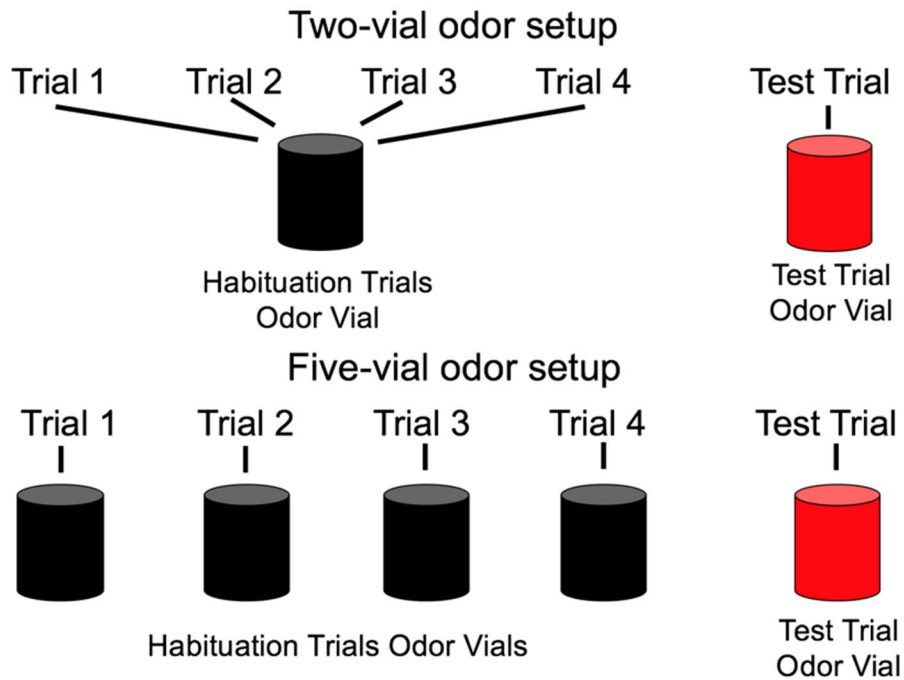


Figure 11: Competing odor vial setups for discrimination tasks. **A.** All preceding discrimination tasks used an odor vial for multiple odor presentations. For example, a discrimination task with five odor presentations would use only two vials of odorant. Multiple odor presentations from a single odor vial may cause discernible changes in odorant characteristics. These minor changes may prevent the proper stimulus control of mice during experimentation **B.** We next considered using individual odor vials per each odor presentation. With individual vials, five-trial discrimination tasks used five-vials of odorant; one vial per odor presentation.

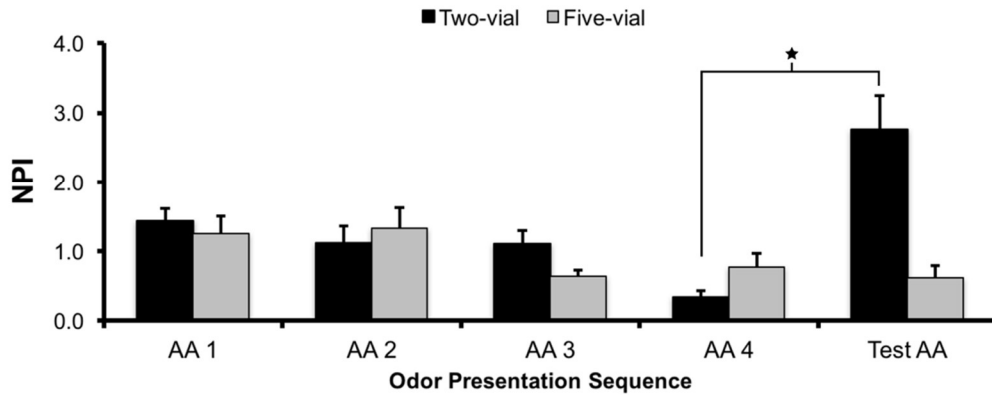


Figure 12: Strong stimulus negative control test with two different odor vial setups. A two-vial setup, with one vial for habituation trials and a 2nd vial for the test trial, was compared a five-vial setup. Each odor presentation in the five-vial setup used an individual odor vial. Mice in the two-vial setup dishabituated significantly to the amyl acetate 1×10^{-4} test trial, while the five-vial mice group remained habituated. The five-vial setup appears to abolish this significant difference, as five-vial mice remain habituated to the test odor (two-vial: $n=6$, $p < 0.05$, five-vial: $n=6$, $p > 0.5$). * Indicates significant difference in investigatory behavior between the test trial and the preceding MO trial.

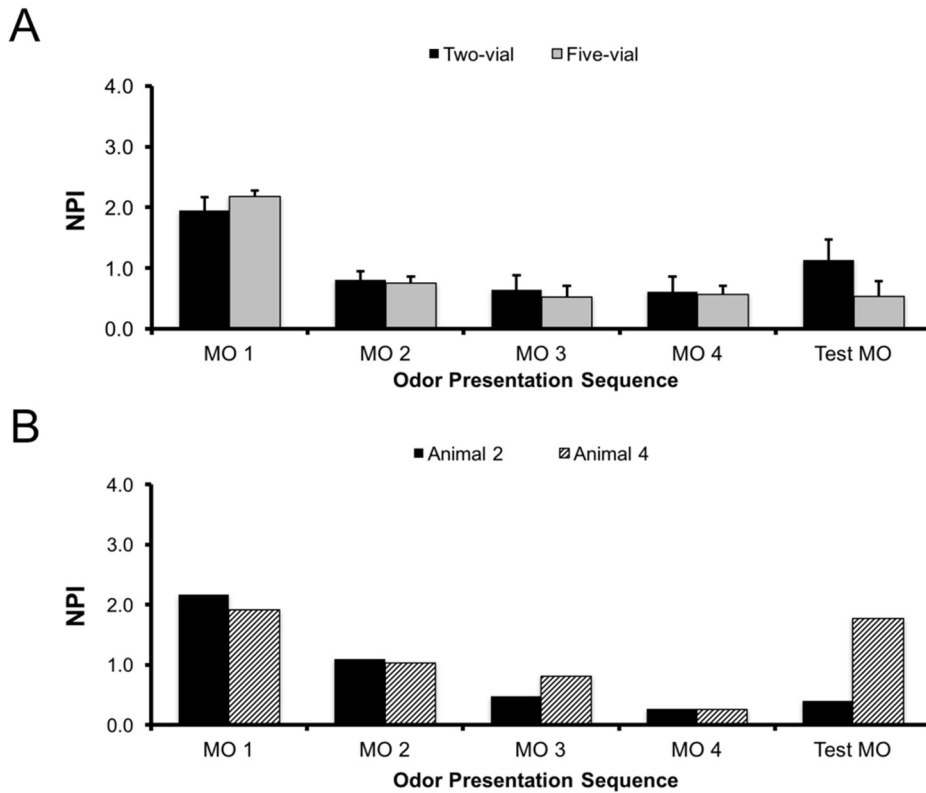


Figure 13: Weak stimulus negative control test with two different odor vial setups. **A.** Normalized mouse investigatory behavior comparing the two-vial and five-vial odor vial setups as depicted in Figure 11 with mineral oil. Both five-vial and two-vial mice habituated and remained habituated across all odor presentations (two-vial: $p > 0.05$, $n=6$, five-vial: $p > 0.05$, $n = 6$). Two-vial mice did increase investigation towards the test trial, though this was not significant. **B.** Individual investigation of animals 2 and 4 from the two-vial mice group in Figure 3A. Two-vial mice group response is polarized. Half of mice habituated and remain habituated similarly to Animal 2. The second half of two-vial mice habituated and dishabituated to the test odor similarly to Animal 4. These mice do not appear under stimulus control.

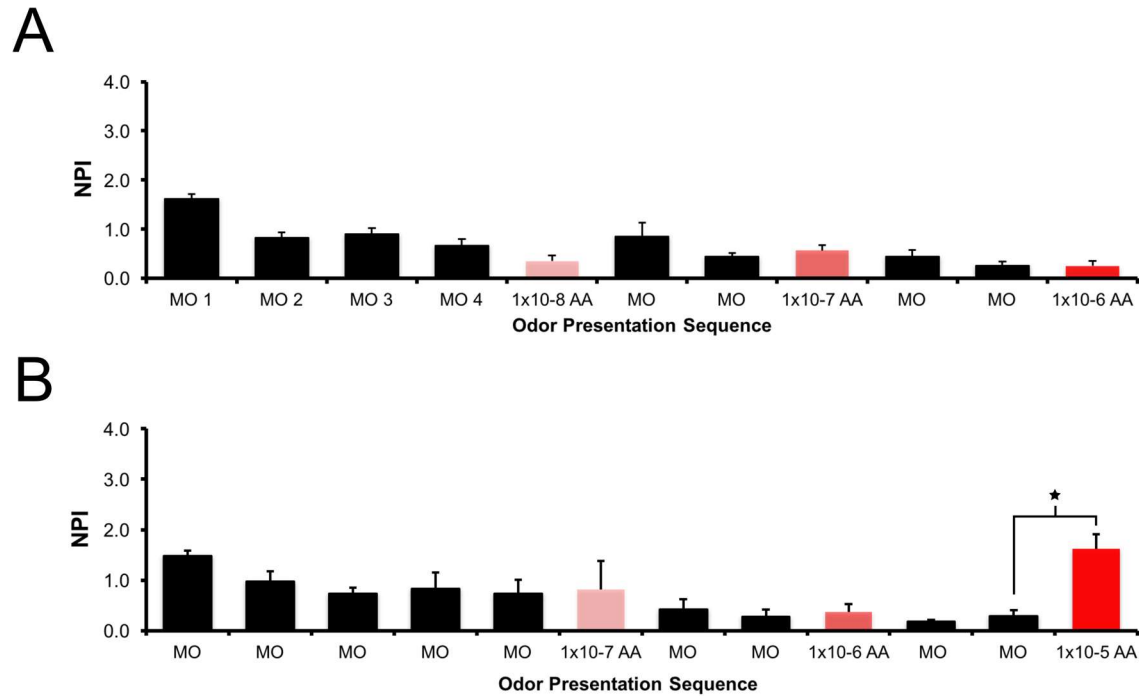


Figure 14: Repeat detection threshold testing for amyl acetate with individual vials. **A.** Amyl acetate detection threshold using concentrations of 1×10^{-8} , 1×10^{-7} , and 1×10^{-6} . 1×10^{-8} . All mice habituated and remained habituated across all amyl acetate test trials (1×10^{-8} , 1×10^{-7} , 1×10^{-6} : $p > 0.05$, $n = 6$). **B.** Amyl acetate detection threshold using concentrations of 1×10^{-7} , 1×10^{-6} , and 1×10^{-5} . All mice habituated and remained habituated through the amyl acetate test trial at a concentration of 1×10^{-6} . All mice then dishabituated to amyl acetate at a concentration of 1×10^{-5} ($p < 0.05$, $n = 5$). This result approximated the detection threshold of amyl acetate reported in the literature. * Indicates significant difference in investigatory behavior between the test trial and the preceding MO trial.

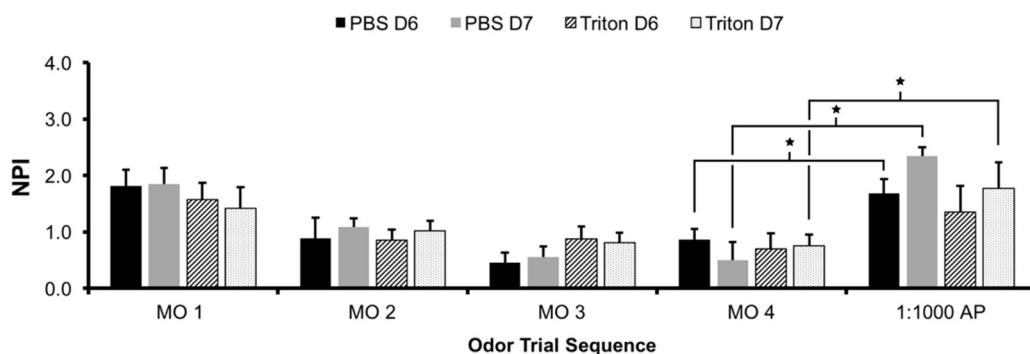


Figure 15: Automated odor discrimination testing of Triton treated mice. Mice underwent mineral oil-acetophenone discrimination testing on either Day 6 or Day 7 in the modified commercial behavioral olfactometer. Day 6 Triton mice habituated across the mineral oil trials, and failed to significantly dishabituate to the 1:1000 acetophenone test trial (Day 7: $n = 6$, $p > 0.05$). Day 7 Triton mice, as well as Day 6 and 7 PBS control mice, all habituated to the mineral oil test trials, and successfully dishabituated to the 1:1000 acetophenone test trial (Day 6: $n = 6$, $p > 0.05$, Day 7: $n = 6$, $p < 0.05$, PBS: $n = 6$, $p < 0.05$). * Indicates significant difference in investigatory behavior between the test trial and the preceding MO trial.

Chapter 5: Designing and Building a Behavioral Olfactometer

5.1 Introduction

The analysis of behavior is a powerful tool employed to study nervous-system function. Changes in neuronal activity, induced or not, can have profound effects on behavioral output (Banerjee et al. 2014; Escanilla et al. 2009). Knowing these implications, the fields of behavioral neuroscience and psychophysics have long studied the effects of stimuli on perception, and the resulting behavioral response (Nevin 1969). Methods and techniques in these fields are taught in many undergraduate institutions however, the opportunities for hands-on practice with behavioral equipment is limited in the classroom setting. Experiential learning has continuously been shown to result in better learning outcomes in students (Kirschner et al. 2006; Yardley et al. Dornan 2012). As students apply theory to practice, they are more likely to recognize their shortcomings, leading to better material retention and understanding (Rogers 1969; Kolb 1984). Learning behavioral methods in the classroom will also introduce equipment and techniques students may use in future advanced education, research, or commercial work.

Unfortunately, much of the equipment used in the behavioral and psychophysical methodology is expensive, and their operation can be quite complex. Equipment complexity prevents the use of experimental equipment in the classroom setting, in part due to faculty worry over the improper handling of their equipment. Additionally, the time necessary to properly train undergraduates in sophisticated equipment operation is prohibitory in an educational lab setting (Stevens 2004). To overcome cost, some commercial companies have developed behavioral equipment with expense in mind. However, these “inexpensive” behavioral olfactometers can cost upwards of \$3,000–4,000. As multiple sets of equipment would be required, these prices

prevent application to experiential learning in all but well-funded institutions. Furthermore, the “inexpensive” equipment still requires end user programming for operation. For non-computer science minded students, managing and operating this equipment may prove complex and time intensive.

In the present study, we aimed to develop inexpensive and easy to operate olfactory-behavior equipment for both teaching and research purposes. This equipment, known as an olfactometer, are experimental machines that deliver odorized air while carefully controlling physical and temporal delivery of odorants. The animal’s corresponding behavioral response to these stimuli are then recorded (Restrepo and Slotnick 2005). Our olfactometer was built from the ground up using Arduino microcontrollers, the python programming language, and Do-it-yourself (DIY) tools and materials. There are instances of behavioral equipment using Arduino components (Devarakonda et al. 2016; Pineño 2014), but none are designed for olfactory behavior research or experiential teaching. The modularity of our design also allows the equipment to change over time based on the needs of the researcher or classroom.

Neuroscience is an integrated, and rapidly growing field and major. This necessitates equally adaptive and broad functioning equipment. Arduino-based research equipment is perfectly suitable for such a need due to the limitless potential in design, modification, and application. Here, we demonstrate viable Arduino application to olfactory behavior equipment in commonly used olfactory tasks. Some experiments were performed by a newly trained undergraduate research assistant to demonstrate equipment simplicity. On our laboratory website, we shall provide a necessary parts manifest and source programming for the current iteration of our olfactometer, as well as provide a design schematic for DIY construction.

5.2 Methods:

5.2.1 Foreword

Much of the materials and methods used in the production of our apparatus is identical to those used for experimentation with commercial equipment. For the sake of brevity, Chapter 3 of this thesis should be referenced for animal use, behavioral tasks, odorants, and data analysis. The methods and experimental design detailed here are specific to building and testing the DIY behavioral olfactometer.

5.2.2 Olfactometer Hardware Design

The DIY behavioral olfactometer is a liquid dilution, solenoid based behavioral olfactometer. This design incorporates a pneumatic hose system for odor delivery. Air is pushed through silicone tubing using an air pump into 120 mL airtight vials where it is mixed with liquid odorant. From the inherent pressure, the mixed air is then pushed through a second line towards the operant wall and directly into the behavioral chamber. The clean air is scrubbed using an inline activated charcoal filter, and volume is controlled using a flowmeter before arriving in the vials. The silicone tubing is fed through solenoid valves, which permits control of odor delivery. A simplified representation of a behavioral olfactometer is depicted in Figure 5.

Our behavioral olfactometer is constructed using easy to obtain hardware and electronics store components. The operant chamber is constructed from bonded acrylic sheets with holes drilled for odor delivery and air filtration ports. To collect data, an infrared sensor is placed in the nose port that records nose entrance and exit times for investigation behavior. A house light is placed on the top of the behavioral chamber, and is constructed from a single LED high intensity bulb. The behavioral chamber is then housed within a sound isolation chamber to

account for environmental noise, and contains a 75-dB brown noise-generating speaker for additional external noise control. An air output tube is placed adjacent to the behavioral chamber, which provides air circulation via an externally mounted fan system on the isolation chamber. The behavior chamber and associated equipment can be seen in Figure 16.

The house light, solenoid valves, and infrared (IR) sensor are controlled by a central Arduino microcontroller. The logic board, consisting of the Arduino microcontroller and associated relay and control boards, can be seen in Figure 17. This equipment employs a central Windows 7 PC installed with the open source Spyder integrated development environment (IDE) for programming in Python. Many different IDE's exist, and any can be used. Spyder is used to operate the behavioral olfactometer with in-house composed Python programs.

5.2.3 Olfactometer Software Design

Operation of the DIY behavioral olfactometer is done within Spyder using two Python programs, which allow for direct communication and command of the Arduino logic board. The command program is functionally comparable to a graphical user interface (GUI), and permits the researcher to control odor presentation order, timing, and duration. In addition, this command software also monitors the enter and exit times of mouse nose port investigation, and records the data to a text (.txt) file for later analysis.

5.2.4 Experimental Design: Discrimination Testing

For odor discrimination testing, thirty-six mice were divided into three groups and underwent odor discrimination testing. The groups of twelve mice were categorized into either mineral oil to mineral oil (MO-MO), amyl acetate to amyl acetate (AA-AA), or amyl acetate to

acetophenone (AA-AP) tasks. Each task consisted of four habituation trials, and a fifth test odor trial. These groups were further divided, with six mice undergoing the task in the DIY behavioral olfactometer, and the other six undergoing the task in a modified commercial behavioral olfactometer for control. Examples of these tasks can be seen in Figure 6, A and B. Investigatory behavior was compared between mice tested in the DIY and commercial behavioral olfactometers.

5.3 Results

5.3.1 Discrimination Experiments

We tested our DIY behavioral olfactometer through comparison of behavioral data from a commercial behavioral olfactometer (Vulintus Inc., Dallas, Texas). Thirty-six mice underwent a series of three, five-trial positive and negative control discrimination tasks. The first experiment tested twelve mice in a five-trial mineral oil discrimination task. All mice in both DIY and Commercial olfactometer groups habituated to the mineral oil trials, and remained habituated to the mineral oil test trial (Figure 18; $p > 0.05$, $n = 12$), indicating successful non-discrimination. These results demonstrate the DIY olfactometer maintained stimulus control of mice using a weak stimulus (mineral oil).

The second experiment tested mice capability in a strong stimulus, five-trial discrimination task. Mice were presented four habituation trials of 1×10^{-4} amyl acetate diluted in mineral oil, and a fifth, 1×10^{-4} amyl acetate test trial. Again, all mice in both the DIY and Commercial olfactometer groups habituated the amyl acetate habituation trials, and remained habituated to the amyl acetate test trial (Figure 19; DIY: $p > 0.05$, $n = 6$; Commercial: $p > 0.05$, $n = 6$). This evidence demonstrated proper stimulus control of mice in the DIY olfactometer using

a strong stimulus. With successful negative control testing with weak and strong stimuli, we next sought a positive control.

The final experiment tested the remaining twelve mice in a five-trial positive control discrimination task. The twelve mice were presented four habituation trials of 1×10^{-4} amyl acetate, and a fifth, 1×10^{-3} acetophenone test trial. All mice in both DIY and Commercial olfactometer groups habituated across the amyl acetate habituation trials, and significantly dishabituated to the 1×10^{-3} acetophenone test trial (Figure 20; DIY: $n = 6$, $p < 0.05$; Commercial: $n = 6$, $p < 0.05$), indicating successful discrimination. Collectively, the results from all three discrimination experiments demonstrate proper design and stimulus control of the apparatus.

5.3.2 Undergraduate Researcher Assistant

As we hope our DY behavioral olfactometer will be used in experiential learning of psychophysical techniques, it is important to incorporate undergraduate research assistants with no previous behavior experience in the equipment testing process. Our undergraduate received 2–3 miniature lectures on behavioral olfactometry methods, and one morning of direct equipment training. They then successfully conducted the mineral oil discrimination assay (Figure 18), demonstrating the minimal training needed to operate the apparatus. With minimal lecture and training time necessary, the DIY behavioral olfactometer appears ideal for a classroom setting.

5.4 Discussion

The DIY behavioral olfactometer (box) is an easy to use apparatus designed around Arduino microcontrollers for both basic-olfactory science and the experiential teaching of

psychophysical techniques. Our apparatus's main appeal rests in cost, ease of use, and modularity. Current commercial behavioral olfactometers can cost upwards of \$30,000 for fully programmed and functioning equipment, and even "inexpensive" equipment can still \$3–4,000. The sheer cost of equipment alone could make any investigator weary of undergraduate or untrained equipment use. Fully assembled, our apparatus costs approximately \$750, and Arduino components and parts used can be found at most major hardware and electronics stores. One box can be constructed in 2–3 days depending on experience and available time, and the operating software will be released open source enabling near-immediate use

Psychophysical experiments are often not incorporated into the undergraduate classroom. One reason is that significant time investment is necessary to properly train new operators. To use our DIY behavioral olfactometer, our undergraduate researcher required 1–2 days of discussion and literature review on olfactory behavior methods, and one morning of equipment training. We affirm our equipment training regimen is accurate and appropriate for use in the classroom setting.

Finally, modularity is uncommon in commercial equipment. Behavioral equipment designed for one experiment cannot be easily restructured for use in another. Our box is designed from the ground up to be modular through the incorporation of the Python programming language and Arduino microcontrollers. Python is an open source, easy to learn, and easily modifiable. For these reasons, python is now commonly the first programming language computer science students learn (Radenski 2006). Arduino's can be programmed using python (Koenka et al. 2014) for a multitude of home (Klosowski 2015) and research uses (Anzalone et al. 2013; Barroca et al. 2013; Sáiz et al. 2013). The olfactometer can be expanded using Arduino "shields," which are plug and play expansion boards that add hardware (i.e. LEDs, sensors, and

motors) (Devarakonda et al. 2016). Thus, the plug and play combination of Arduino and Python is limitless, and should allow easy transition of the apparatus between different olfactory behavior experiments.

The DIY behavioral olfactometer is not without limitations. Assembly of the apparatus will require soldering, as well as other hardware fabrication methods such as sawing and drilling. In addition, the apparatus will require setup and installation of a python independent development environment (IDE) and subsequent olfactometer control programs. Assembly may prove difficult for those less tech savvy. The availability of tools necessary for equipment production may also be limited and prevent easy assembly.

In conclusion, we have demonstrated that self-built (DIY) olfactory behavior equipment made from Arduino components is a viable alternative to purchasing commercial equipment. We produced a DIY behavioral olfactometer, at a fraction of the cost of commercial equipment, that produces comparable behavioral data in a common olfactory assay. Our apparatus is highly modular by design and easy to learn, making it appropriate for experiential learning of various psychophysical techniques in the classroom setting.

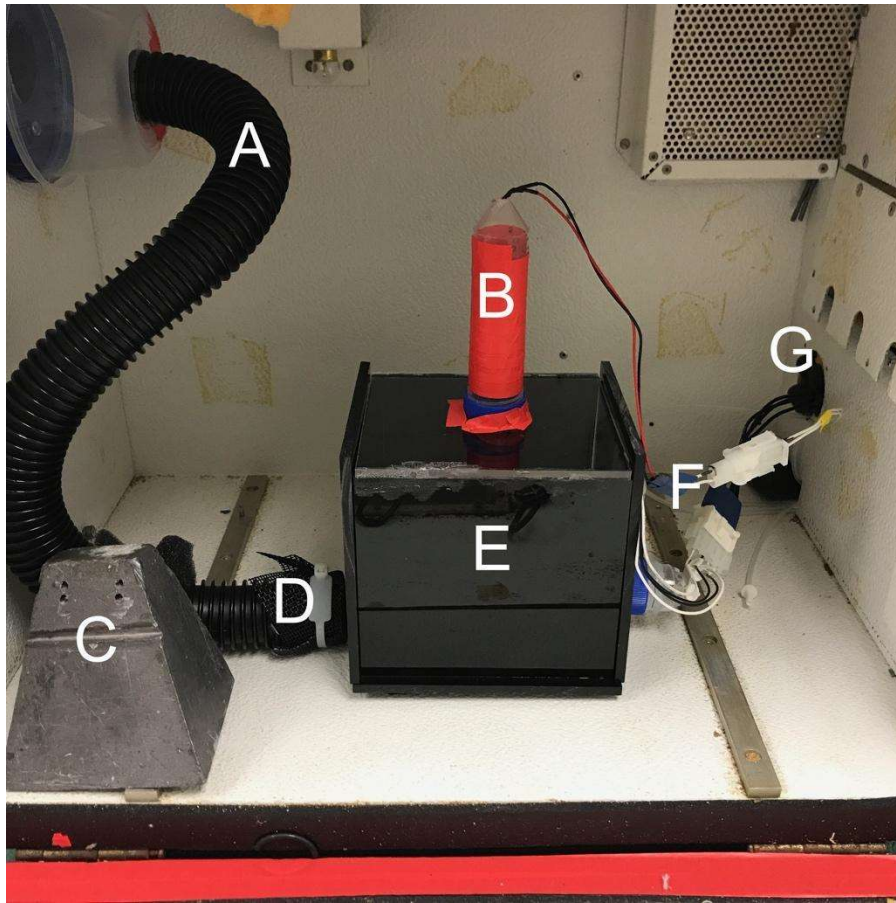


Figure 16: The DIY behavioral chamber and peripheral equipment. **A.** Exhaust fan and ventilation hose attachment. **B.** House light tube with LED. **C.** Weight for easy hose positioning. **D.** Exhaust port and ventilation hose attachment. **E.** Behavior chamber. **F.** IR sensor wiring and odor tube manifold. **G.** Not pictured: Brown noise generator.

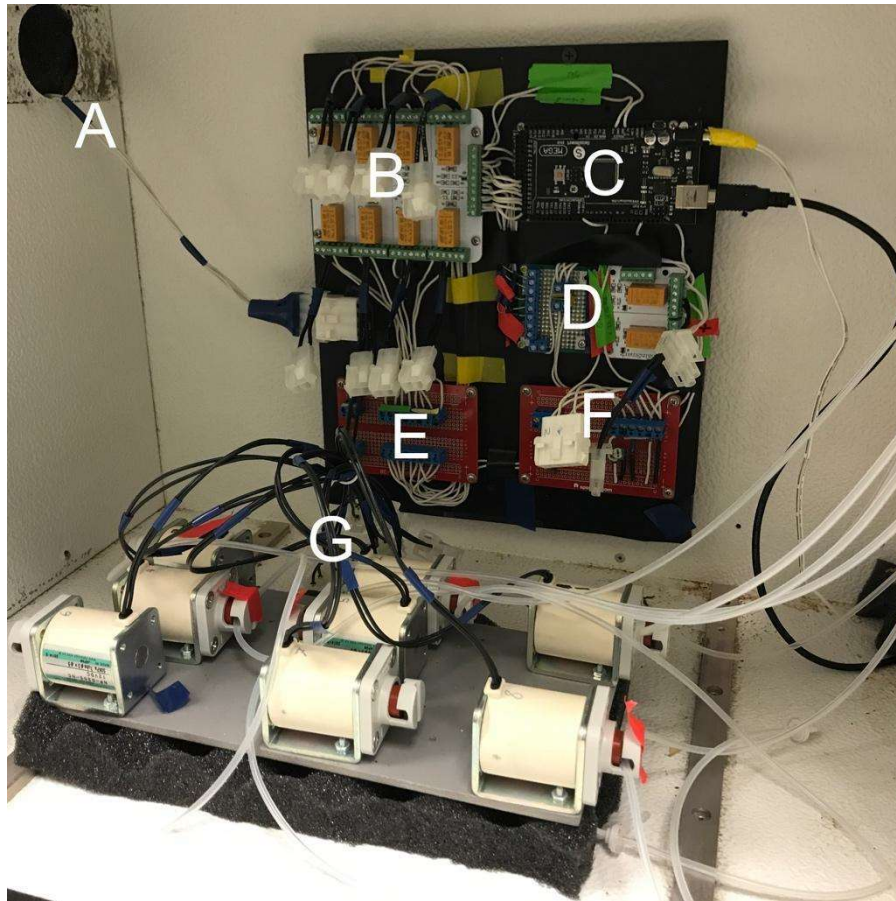


Figure 17: The Arduino logic board and solenoid valve bay. **A.** Power cable from external computer class power supply unit. **B.** Sainsmart 8-Channel relay board for individual control of solenoid valves. **C.** Arduino MEGA 2650 R3 controller board. **D.** Sainsmart 2-channel relay board with associated breadboard for distractor valve control (not pictured). **E.** Power Input breakout board for solenoid valves. **F.** In-house Infrared sensor breakout board. **G.** Solenoid valves with silicone tubing.

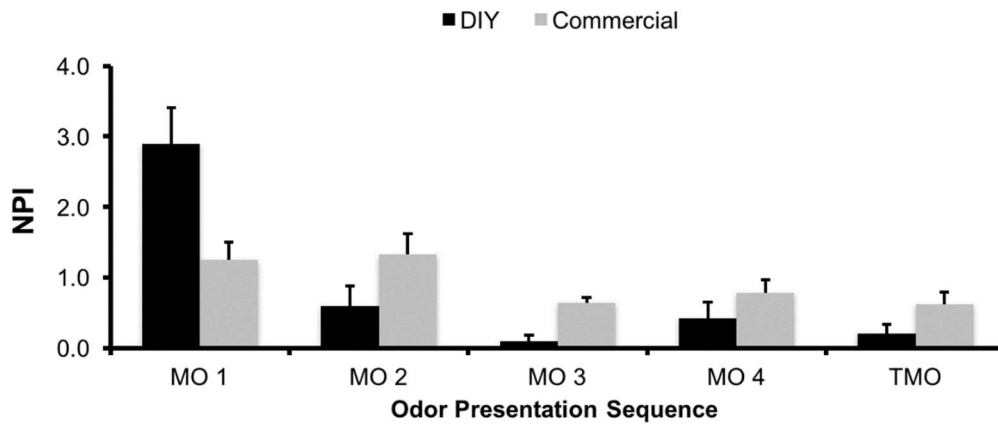


Figure 18: DIY weak stimulus five-trial odor discrimination task. Normalized average of mouse investigatory behavior in response to four mineral oil habituation trials and a fifth mineral oil test trial. Both DIY and Commercial mice groups habituate and remain habituated to the mineral oil test trial. No significant difference is observed between the test trial and the preceding MO trial for DIY or Commercial mice groups (DIY: $p > 0.05$, $n = 6$; Commercial: $p > 0.05$, $n = 6$).

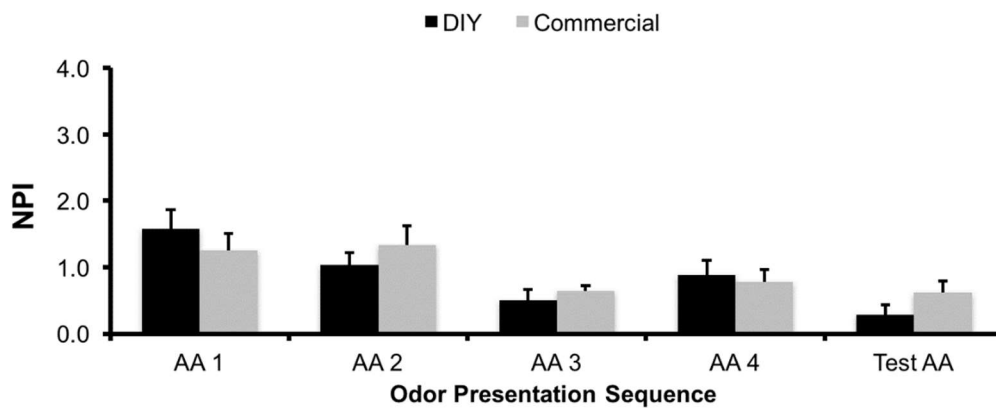


Figure 19: DIY strong stimulus five-trial odor discrimination task. Normalized average of mouse investigatory behavior in response to four amyl acetate habituation trials and a fifth amyl acetate test trial. Both DIY and Commercial mice groups habituate and remain habituated to the amyl acetate test trial. No significant difference is observed between the test trial and preceding MO trial investigation for either DIY or Commercial mice groups (DIY: $p > 0.05$, $n = 6$; Commercial: $p > 0.05$, $n = 6$).

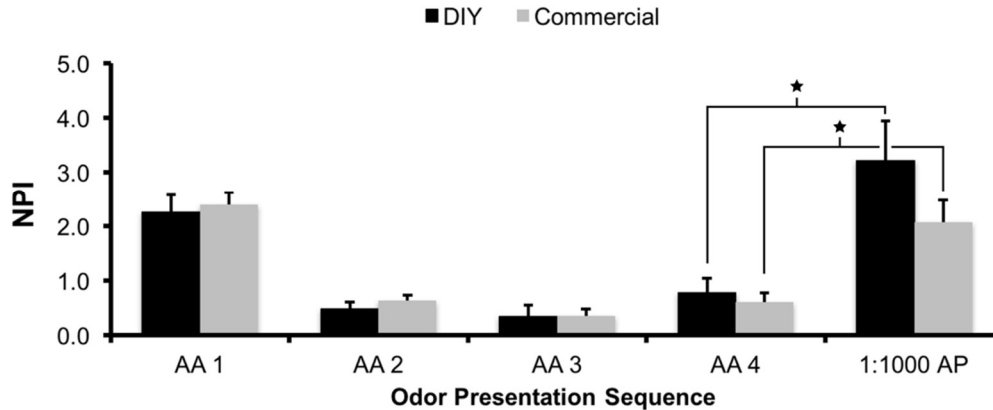


Figure 20: DIY Positive control five-trial odor discrimination task. Normalized average of mouse investigatory behavior in response to four 1×10^{-4} amyl acetate habituation trials and a fifth 1:1000 acetophenone test trial. Both DIY and Commercial mice groups habituated across the amyl acetate trials, and significantly dishabituated to the acetophenone test trial (DIY: $p < 0.05$, $n = 12$; Commercial: $p < 0.05$, $n = 6$). * Indicates significant difference in investigatory behavior between the test trial and the preceding MO trial.

Chapter 6: Summary, Conclusions, and Inferences

6.1 Modifying and Validating Existing Behavioral Equipment

Our first goal was to determine if a non-motivated behavioral model could produce robust and effective data for olfactory research. Non-motivated behavior was first proposed in 2014, and has received little to no attention since initial publication (Qiu et al. 2014). Non-motivated behavior relies on the innate curiosity of animals to drive investigatory behavior in olfactory discrimination and detection threshold tasks. One benefit of the non-motivated behavior model is a decrease in cost and time associated with training animals for traditional operant conditioning tasks (i.e., Go/No-Go and Two-Alternative Forced Choice). Furthermore, non-motivated behavior does not rely on the reward pathway for incentive, allowing isolated study of the olfactory circuits. We sought to further validate the non-motivated model as no replicated study of effectiveness exists. In addition, we sought to modify existing behavioral olfactometers for non-motivated behavior to lower costs.

In conclusion, we found that non-motivated methods are an effective means of measuring mouse olfactory behavior, though with some caveats. When using modified liquid dilution behavioral olfactometers, careful attention must be given to equipment testing to ensure mice remain under stimulus control (i.e., mice must only respond to the odor stimulus presented to them). Our modified commercial olfactometer uses liquid dilution of odorants, a less effective means of odor deliver when compared to mass flow meters (Qiu et al. 2014). As air-diluted concentrations of odor were likely not maintained, abnormal mice detection and discrimination behavior was observed. Mice detected amyl acetate at concentrations two and three orders of magnitude lower than the reported detection threshold in literature (Figure 9A, B, and C)

(Clevenger and Restrepo 2006; Qiu et al. 2014), and were discerning differences between same odorants (Figure 10A; Figure 13A). After multiple attempts at improving stimulus control, we ultimately determined that odor vial setup played a key role.

Until consideration of odor vial setup, all experiments utilized a single odor vial for multiple odor presentations (Figure 11A). Specifically, for a five-presentation discrimination task, one vial provided odorant for the first four odor presentations, while the test odor was provided by a second odor vial. An individual odor vial setup was then considered (Figure 11B), and appeared to solve the inconsistent stimulus control; five-vial setups produced more accurate and consistent behavioral data than the two-vial setups (Figures 12 and 13AB). We then repeated the amyl acetate detection threshold using individual vials for each odor presentation. Mice no longer “detected” amyl acetate at subthreshold concentrations (Figure 14A), and only dishabituated to amyl acetate at concentrations near the reported detection threshold values (Figure 14B).

In conclusion, non-motivated behavioral olfactometry methods are effective. However, careful attention must be given to stimulus control when using liquid dilutions. There are expensive solutions to stimulus control, namely using mass flowmeters to regulate odor concentration. However, using individual odor vials for each odor presentation is inexpensive, and seemingly produces consistent mouse stimulus control. Why using individual vials improves stimulus control, however, remains unknown. We do hypothesize that repeated use of the same odor vial may decrease the headspace concentration of aerosolized odor. On each presentation thereafter, the relative concentration of odorized air is lower than the previous presentation. This relative difference is likely greatest when switching from an odor vial with multiple uses to a

fresh, never used odor vial. Further testing should utilize photoionization detector (PID) measurements to validate these predictions.

6.2 Triton Treatment and Mouse Olfactory Behavior

Our second aim was to determine effectiveness of Triton (0.1% at 10 ul/nare, intranasal irrigation) for recoverable anosmia induction in mice. Triton has been used throughout literature, but never at low concentrations, and never with the expectation of anosmic recovery. At low concentrations, Triton is believed to cleave the cilia of the olfactory sensory neurons (OSN) in the nasal epithelium, while leaving the OSN themselves intact. The clipping of cilia prevents OSN signal transduction, and induces anosmia through temporary sensory deprivation. Sensory deprivation is known to alter both main olfactory bulb (MOB) morphology in the glomerular layer, and decrease both tyrosine hydroxylase (TH) and glutamate decarboxylase 67 (GAD67) expression. Since future studies aim to study the role of TH+/GAD67+ (SA) cells in olfactory processing and behavior, an inexpensive, easy to administer, and recoverable anosmia treatment, such as Triton, would be of great benefit.

We have found that Triton meets these expectations through both manual and automated discrimination tasks using non-motivated behavior. All mice that underwent discrimination testing the day after receiving Triton treatment were unable to discriminate between mineral oil and 1×10^{-3} acetophenone (Day 6 Triton mice; Figure 8; Figure 15). This result indicated impaired olfactory function. However, Triton treated mice that underwent behavior two days following treatment (one day of recovery) were then able to discriminate between the mineral oil and acetophenone (Day 7 Triton mice; Figure 8; Figure 15), indicating return of olfactory function.

Collectively, these results demonstrated intranasal irrigation of low concentration (0.1%) Triton as an effective means of inducing recoverable anosmia in mice. We hope to expand on this work by studying further olfactory deficits in Triton treated mice.

6.3 Producing Inexpensive DIY Behavioral Olfactometers

Our final aim attempted to reproduce our modified commercial behavioral olfactometer inexpensively, but with comparable functionality in a do-it-yourself (DIY) manner. Arduino microcontrollers were used to accomplish this task. These readily available and inexpensive circuit boards are programmed using Python, a common open source language. Arduinos have been used globally to create every-day and advanced electronics, including outdoor thermometers, alarm clocks, and even lab grade research equipment (Anzalone et al. 2013; Barroca et al. 2013, Klosowski 2015; Sáiz et al. 2013; Scheltema and Bunker 2015). The highly modular nature of Arduino's, the inexpensive to obtain components, and the open source nature of Python all lend to future widespread incorporation in basic science research. The rest of our olfactometer, including the behavioral chamber, isolation chamber, pneumatic air system, and ventilation system, was constructed using materials commonly found online, and at large hardware and electronic stores around the United States.

As our behavioral olfactometer is liquid dilution based, it was important to ensure mouse stimulus control was maintained. This was done through a series of positive and negative control discrimination tasks. These tasks included testing mouse discrimination of mineral oil (MO), amyl acetate (AA), and acetophenone (AP). These experiments validate our DIY behavioral olfactometer for use in common olfactory behavior tasks. The mice were tested in either the DIY equipment, or a commercial behavioral olfactometer for control. In all three discrimination

tasks, DIY group mice performed comparably to mice tested in commercial equipment (Figures 16, 17, and 18), indicating proper stimulus control. All mice habituated and appropriately dishabituated or failed to dishabituate to all test trials.

As we designed the DIY equipment with experiential learning in mind, we found it important to incorporate an undergraduate research student in the equipment validation process. After approximately 1–2 days of equipment and psychophysics method instruction, our supervised undergraduate researcher successfully completed the mineral oil discrimination task. This undergraduate student had never partaken in previous animal behavior research. The student's success with little previous training demonstrates the simplicity of our equipment, which we hope will extend to a classroom setting.

The instructions and parts list for building our DIY behavioral olfactometer, in addition to the python code needed for operation, will be released open source for those who wish to reproduce this equipment.

Chapter 7: Future Directions

7.1 Review of Early Glomerular Sensory Processing

Our primary goal following method development has been to study the interneuron networks of the main olfactory bulb (MOB). Specifically, we hope to investigate how specific interneurons in the glomerular layer play a role in olfactory sensory processing, and how changes in sensory processing networks may alter olfactory behavior. Some evidence points to specific interneurons at play in the modulatory network. It has been shown that mice undergoing sensory deprivation have significant depression in MOB tyrosine hydroxylase (TH) and glutamate decarboxylase (GAD) 67 (Baker et al. 1983; Parrish-Aungst et al. 2011), as well as a diminished glomerular layer (Baker et al. 1983). As the rate limiting enzymes of dopamine and gamma-Aminobutyric acid (GABA), respectively, glomerular TH+/GAD67+ short axon (SA) cells likely play an active role in sensory processing and modulation. In fact, recently published data has shown that short-axon (SA) cells directly synapse with and inhibit mitral and tufted (M/T) cells in both proximal and distal glomeruli (Banerjee et al. 2014, Vaaga et al. 2017). While these studies have determined a definite function of SA cells, none have determined how changes in TH+/GAD67+ SA cells may influence olfactory processing and behavior. However, it has been suggested that these cells may function through gain control and center surround inhibition mechanisms (Aungst et al. 2003; Banerjee et al. 2014; Ennis et al. 2001; Vaaga et al. 2017).

7.2 Proposed Future TH and Dopamine Behavior Experiments

In our future experiments, we hope to impair the SA cell network and observe the consequences in olfactory behavior. Here, we present two such methods.

1. **Triton:** We have demonstrated the effectiveness of Triton as a recoverable anosmia inducing agent. From internal testing, we also know that Triton is capable at decreasing TH concentration within the olfactory bulb. We hope to treat mice with Triton and observe the subsequent olfactory behavior.
2. **Optogenetics:** Recent publications have demonstrated inhibitory function of SA cells on M/T cell output using optogenetic techniques (Vaaga et al. 2017). Now that we have inexpensive, and reliable means of performing olfactory behavior experiments, we would like to combine the use of non-motivated animal models with optogenetics techniques to more carefully drive or inhibit SA cells to study the resulting olfactory behavior.

In either proposed experiments, mice would undergo odor detection threshold assays, as well as discrimination assays with increased complexity. We hope these future experiments will increase understanding of the SA cell network, the sensory processing and modulation within the glomerular layer, and the MOB overall.

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