ACTIVE OLFACTOMOTOR RESPONSES IN HEAD-FIXED MICE

by

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A THESIS

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Autism spectrum disorder (ASD or autism) is a neurodevelopmental condition characterized by deficits in verbal and non-verbal communication skills, narrowed interests, and repetitive behaviors. Altered sensory behaviors, such as abnormal eye tracking, temperature insensitivity, and excessive sniffing, which we will refer to as "olfactomotor" behaviors, have been identified as a common symptom in individuals with autism. Olfactomotor responses, such as sniffing, are respiratory, orofacial, and locomotive movements that allow an organism to sample and react to odors (Esquivelzeta Rabell et al., 2017; Findley et al., 2021; Johnson et al., 2003a; Jones & Urban, 2018; Kurnikova et al., 2019; Wesson et al., 2008). Neurotypical individuals modulate their sniffing behavior when presented with aversive odors, but those with ASD do not despite identifying the odors as unpleasant, suggesting an altered unconscious motor response (Rozenkrantz et al. 2015). To investigate the neural mechanisms underlying olfactomotor sampling, we investigated respiratory and orofacial responses to odor using wildtype mice. Wildtype mice were exposed to 2-phenylethanol (attractive odor), 2-methylbutyric acid (aversive odor), alpha-pinene (neutral odor), or clean air over the course of a behavioral session. We

recorded respiration with an intranasal thermistor and track orofacial movements using DeepLabCut. Our preliminary results in wildtype mice (n=3) suggest that mice alter their sniffing and nose movement in response to odor stimuli. This work will shed light on active olfaction and establish the framework for testing autism model mice in the future.

Acknowledgements

I was curious about research when I came into college, but never in my wildest dreams did I think I would be where I am today: graduating with a BS in Neuroscience with several research accolades, extensive research experience including a publication and conferences, and an acceptance to a PhD program. I could not be where I am today without the support of my thesis committee, mentors, friends, and family.

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Table of Contents

List of Figures

List of Tables

List of Accompanying Materials

1. Olfactomotor Analysis and Graph MATLAB Code

BACKGROUND

Personal Connection to Thesis Research

In my family, I am the oldest child of four. It is me, my brother Davis, sister Lily, and youngest brother Ford. Davis was diagnosed with Asperger's syndrome, now called autism spectrum disorder (ASD), at 2 years old. Although we are only a year and a half apart in age, I understood that his autism made him different, but did not learn what that meant until we were older. He screamed at the sound of fireworks, flapped his hands when he was anxious, spoke (and still speaks) in a British accent, avoided eye contact during conversations, repeated my last words after conversations ended, and experienced narrow, but deep interests in art, animation, movies, and international airports. From a young age, I was always curious what in his brain was different than mine. I continually found myself applying my science class materials to this question and still have as an undergraduate student. I dedicate my thesis to my goofy, sassy, brilliant brother, and I hope that this work along with my future work can help make the world friendlier for you and others with ASD.

Autism Spectrum Disorder

Autism spectrum disorder (ASD or autism) is a neurodevelopmental condition often characterized by deficits in verbal and non-verbal communication skills, altered sensory perception, narrowed interests, and repetitive behaviors. Autism was first described by Leo Kanner in his 1943 paper, "Autistic Disturbances of Affective Contact"(Kanner, n.d.). He described the first case studies of 11 children from the Baltimore area using anecdotal quotes from parents, written letters, and in-clinic

evaluations (Harris, 2018; Kanner, n.d.). The number of autistic children has risen exponentially since 1943 as medical doctors have made monumental strides in characterizing the variable symptoms of autism and standardizing diagnostic tests. Today, 1 in 44 children receive an autism diagnosis (Center for Disease Control, 2022; Screening and Diagnosis of Autism Spectrum Disorder | CDC, n.d.). (Table 1)

Identified Prevalence of Autism Spectrum Disorder
ADDM Network 2000-2018 Combining Data from All Sites

Surveillance Year	Birth Year	Number of ADDM Sites Reporting	Combined Prevalence per 1,000 Children (Range Across ADDM Sites)	This is about 1 in X children…
2000	1992	6	6.7 (4.5-9.9)	1 in 150
2002	1994	14	6.6 (3.3-10.6)	1 in 150
2004	1996	8	8.0 (4.6-9.8)	1 in 125
2006	1998	11	9.0 (4.2-12.1)	1 in 110
2008	2000	14	11.3 (4.8-21.2)	1 in 88
2010	2002	11	14.7 (5.7-21.9)	1 in 68
2012	2004	11	14.5 (8.2-24.6)	1 in 69
2014	2006	11	16.8 (13.1-29.3)	1 in 59
2016	2008	11	18.5 (18.0-19.1)	1 in 54
2018	2010	11	23.0 (16.5-38.9)	1 in 44

Table 1: Autism spectrum disorder (ASD) Prevalence Rates 2000-2018

Taken from (Center for Disease Control, 2022; Maenner et al., 2021)

To be diagnosed with autism, one must display two core symptoms: deficits in social communication skills (verbal and non-verbal) and display restricted or repetitive

behaviors, interests, or activities (Psychiatry Online | DSM Library, n.d.).However, these two core symptoms' presentations vary greatly between individuals. For example, repetitive behaviors may include physical body movements such as flapping hands or jumping, actions such as moving a toy, or behaviors such as repetitive words after spoken (echolalia). Thus, the term autism spectrum was coined to capture the disorder's variability in symptom severity and presence. Despite our increased knowledge and cultural awareness of autism since Kanner's day, our diagnostic process has not changed much since the 1940s and is still heavily reliant on historical behavioral reports. If a child is suspected to be autistic, a doctor or trained psychologist uses a behavioral battery of tests, called the Autism Diagnostic Observation Schedule (ADOS), to examine a child's behavior, communication, and social skills through an interactive play task (*Screening and Diagnosis of Autism Spectrum Disorder | CDC*, n.d.; *Screening and Diagnosis of Autism Spectrum Disorder for Healthcare Providers | CDC*, n.d.). In addition to ADOS, parents, caregivers, or other related adults complete several interviews and questionaries that allow the doctors to gain insight into the child's behaviors and symptoms. While these tests do have strong validity and replicability for diagnosing autism, the child's symptoms must first be noticed by caretakers before the tests are initiated. Unfortunately, not all parents are able to identify symptoms early enough in development, which often results in the child having delayed access to educational and behavioral interventions.

With an inadequate diagnostic infrastructure and steadily increasing prevalence of ASD, it has become imperative to investigate biomarkers other than social behavior to diagnose ASD more efficiently and earlier in development (Hammock et al., 2012;

Masi et al., 2017; Mosconi & Sweeney, 2015). An earlier diagnosis means earlier access to services such as academic accommodations, social skills classes, physical, desensitization, occupational, and speech therapies along with other interventions (Blanc et al., 2021; Fountain et al., 2012; Landa, 2018; Vohra et al., 2014). By identifying relevant autism biomarkers that occur early in development, we may streamline autism diagnosis in young children, resulting better interventions and, consequently, better long-term outcomes (Estes et al., 2015).

Biomarkers of Autism

In recent years, scientists and medical professionals have shifted focus from examining symptoms in isolation to determining if there are biological signs predating the onset of illness called biomarkers. Biomarkers are physiology-based indicators, such as altered motor function, cell markers, or other altered physiological signs that can predate the onset of traditional symptoms (Strimbu & Tavel, 2010). Olfactory dysfunction (reduction or loss of smell) has been used as a predictive biomarker in several different neurodegenerative and psychiatric conditions such as Parkinson's disease, schizophrenia, and Alzheimer's (Beach et al., 2020; Brewer & Pantelis, 2010; Doty, 2012; Doty et al., 1989; Haehner et al., 2011; Jankovic et al., 2021; Kumaresan & Khan, 2021; Moberg et al., 2014; Morley & Duda, 2010; Roos et al., 2019; Walker et al., 2021a, 2021b; White et al., 2016). With the increasing prevalence of autism diagnoses, scientists are trying to find biomarkers that may help with early identification. Some examples of proposed biomarkers for ASD include immunological cells present in blood, oxytocin and serotonin levels, and olfactory behaviors (Bridgemohan et al., 2019; Hammock et al., 2012; Masi et al., 2017; Rozenkrantz et al.,

2015). While potential biomarkers have been examined, there are currently no validated biomarkers in use for diagnosing autism.

INTRODUCTION TO OLFACTION

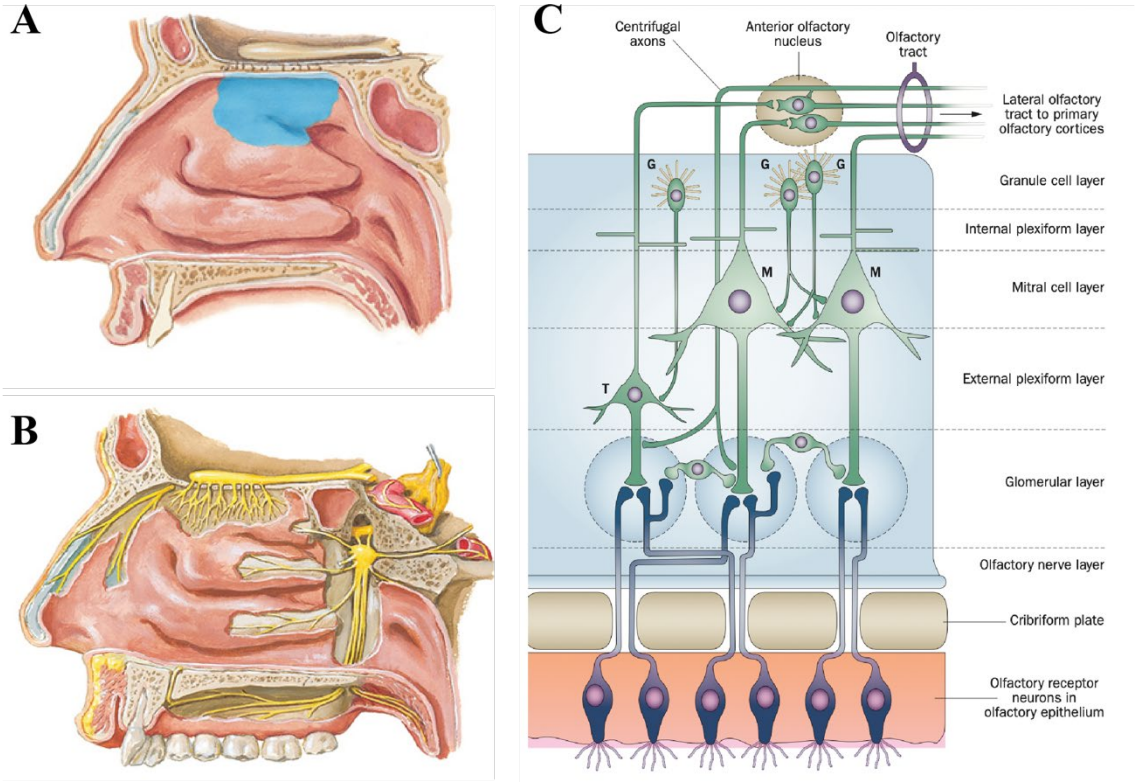

Figure 1: Anatomy of the nose and olfactory bulb (Taken from Frank Netter Anatomy and Doty 2012)

A) Image inside lateral nasal cavity. Blue denotes olfactory epithelium within nasal cavity B) Cross-section of nerves within the nasal cavity. Olfactory bulb's olfactory sensory neurons project down into superior region of the nasal cavity C) Anatomy of olfactory bulb

Our sense of smell, or olfaction, is the detection and perception of volatile odorant molecules. Olfaction is an indispensable tool in our everyday sensory arsenal. Olfaction, in humans, can be used to identify mates, locate food sources, modulate internal states, detect dangerous chemicals, and strengthen mother-infant connections

(Herz & Inzlicht, 2002; Hofer et al., 2018; Keaveny & Mahmut, 2021; Miller & Maner, 2010; Morquecho-Campos et al., 2020; Porter, 1998; Sarafoleanu et al., 2009; Sarkar et al., 2019; Wedekind et al., 1995).

Olfaction was one of the first sensory systems to emerge in organisms and is highly conserved across phylogeny (Eisthen, 1992, 1997; Hosek & Freeman, 2001). Bacteria perform a similar homolog to olfaction called chemotaxis. Using transmembrane chemoreceptors to sample their environment, bacteria can move and orient towards beneficial chemicals in high concentration or away from harmful chemicals via intracellular signaling pathways (Wadhams & Armitage, 2004).

Among larger organisms, insects have a strikingly similar olfactory system to vertebrates and are often used as a model organism for olfactory studies. For insects such as moths, ants, and flies, antennae operate as an olfactory organ similar to a mammalian nose. Insect antennae are lined with hair-like projections called sensilla which contain olfactory receptors to which odorant molecules bind. (Carey & Carlson, 2011; Hansson & Stensmyr, 2011; Klinner et al., 2016). Insects actively move these antennas to sample their environment for olfactory, gustatory, and mechanical cues (Birgiolas et al., 2017; Hansson & Stensmyr, 2011).

Olfaction, however, poses a unique problem for humans in comparison to other sensory modalities. Mammals, unlike bacteria and insects, do not have odor sensing organelles on the surface of their bodies and do not passively receive odor. Odors, such as the smell of a delicious muffin or a fragrant flower, most commonly distribute as a chemical gradient through airborne diffusion. Airflow changes, such as the day's breeze or the simple movement of a nearby person or object, disrupt this even diffusion

creating noisy, turbulent odor gradients that make the localization of odorant sources difficult. Despite the challenge of tracking airborne odor gradients, over hundreds of millions of years, mammals have developed highly effective olfactory search strategies to solve this problem by coupling movements of the nose, head, and body to sample sensory stimuli, which I will here on out refer to as "olfactomotor" movements.

Olfactomotor responses are respiratory, orofacial, and locomotive movements that allow an organism to sample and react to odors (Esquivelzeta Rabell et al., 2017; Findley et al., 2021; Johnson et al., 2003a; Jones & Urban, 2018; Kurnikova et al., 2019; Wesson et al., 2008). The most common example of an olfactomotor behavior is "sniffing", which is the contraction of orofacial muscles to modulate and control airflow and produce inhalations and exhalations. Olfaction in mammals begins by contracting the diaphragm, which expands the lungs and pulls in air through the naris vestibuli. The air passes through the nasal cavity to a sensory surface called the olfactory epithelium along the superior nasal cavity. The olfactory epithelium contains specialized neurons called olfactory sensory neurons (OSNs) that convert receptor binding events to electrical signals that can be routed into the brain (*Netter Atlas-7e*, n.d.; *Olfactory Pathway and Nerve: Anatomy | Kenhub*, n.d.). OSNs have small cilia that project into the nasal cavity and allow odorant molecules to bind to olfactory receptors (ORs) that line their endings. A single OR can have multiple types of molecules bind to it. This contrasts to other sensory systems in which sensory cells are specifically tuned to a single stimulus, like a Meissner's corpuscle, a receptor that responds only to light touch in the somatosensatory system (Malnic et al., 1999; Piccinin et al., 2021).

The mapping between receptors and odorants is not one-to-one. Odor perception can be triggered by a single chemical, or by complex mixtures of molecules, like a perfume. When odorant molecules bind to an OR, they cause either an excitatory or inhibitory response in the OSNs, depending on the molecular make-up of the stimulus. ORs can bind multiple odorant molecules, so odor identity is coded in the pattern of activation across the population of OSNs, which is known as combinatorial coding (Genva et al., 2019; Malnic et al., 1999; Wilson & Mainen, 2006; Xu et al., 2020). After odorant binding, the resulting signal travels from the olfactory epithelium, through the cribriform plate, and terminate in the glomerular layer of the olfactory bulb (Zou et al., 2009). Here, OSNs communicate with mitral and tufted cells (MT cells) that project widely into other olfactory cortices and higher order areas including amygdala and piriform cortex (Doty, 2012; LaMantia, 2015; Margrie et al., 2001; Zou et al., 2009). The glomerular layer of the olfactory bulb has been shown to encode odor identity and is considered the site of first-order olfactory processing (Moran et al., 2021; Wilson & Mainen, 2006).

The Olfactory Basis of Autism

As the search to effective biomarker for autism continues, evidence has suggested that olfactomotor behaviors may be a viable biomarker. The latest version of the Diagnostic and Statistics Manual of Mental Disorders (DSM-5) includes sensory issues, specifically olfaction, as a primary symptom to diagnose ASD: "Hyper- or hypo-reactivity to sensory input or unusual interest in sensory aspects of the environment (e.g., apparent indifference to pain/temperature, adverse response to specific sounds or textures, *excessive smelling* or touching of objects, visual fascination

with lights or movement) [emphasis added]" (Psychiatry Online | DSM Library, n.d.). Since this addition to ASD's definition in 2013, olfactomotor behaviors have gained traction as a potential biomarker.

One of the primary papers informing this research into olfactomotor behaviors and autism comes from Noam Sobel's lab at the Weizmann Institute of Science. Previous research has shown that neurotypical adults inhale a larger volume when exposed to pleasant odors, and in contrast, inhale a smaller volume of air when presented with an unpleasant odor (Bensafi et al., 2003; Johnson et al., 2003b; Larsson et al., 2017). It has been suggested that autistic children have altered "internal action models" and that olfaction can be used as a mechanism to test this hypothesis. Sobel's group examined if "[children with ASD] will generate an improper sniff given a particular odor" (Rozenkratz et al. 2015). To test this, autistic and neurotypical children were exposed to attractive and aversive odors via a custom-designed nasal cannula that simultaneously recorded sniffing and dispersed odors while they watched cartoons. Odor stimuli were delivered at the onset of inhalation and were either attractive, such as roses or shampoo, or aversive, such as rotten milk. The researchers found that neurotypical children responded similarly to previous studies with higher inhalation volumes to pleasant odors and lower inhalation volumes to unpleasant odors. In contrast, children with ASD did not inhale the odorants differently. The autistic children could still verbally identify odors as pleasant or unpleasant, but did not modulate their sniffing behavior, suggesting an altered unconscious motor response rather than perceptual differences (Rozenkrantz et al., 2015).

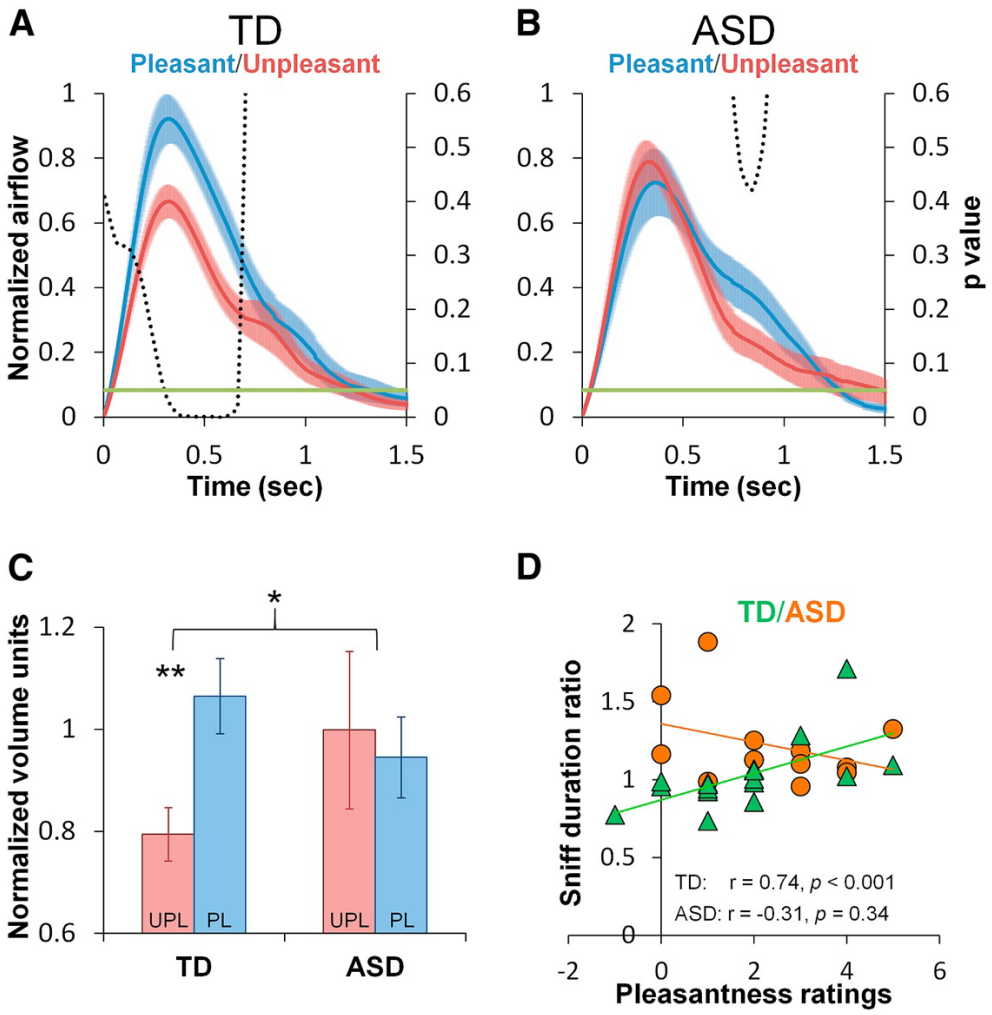

Figure 2: Difference in inhalation volume between autistic and neurotypical children

A) TD = typically developing (or neurotypical) children. Green line denotes 0.05 p-value significance, and dotted lines represents Bonferroni p-values from paired-t-test between pleasant and unpleasant odors B) Same graph as A for autistic children C) A and B graphs combined to demonstrate peak inhalation volume D) Pleasantness ratings for TD and ASD groups against pleasantness rating response (pleasant – unpleasant). Children with ASD could rate odors as pleasant or unpleasant but did not change their sniffing responses.

Several groups have studied ASD's altered olfactory responses prior and after the Rozenkratz et (2015) paper, but results have been conflicting. These mixed results are likely due to variability in study methodology for human subjects (Addo et al., 2017; Ashwin et al., 2014; Dudova et al., 2011; Kumazaki et al., 2016; Larsson et al.,

2017; Tavassoli & Baron-Cohen, 2012). Despite variable results, an altered sensory phenotype, whether hyper-reactive or hypo-reactive, appears to be common amongst autistic individuals. The causes of this altered olfactomotor phenotype, however, remain unknown. While we cannot evaluate the underlying neurobiology in human subjects, we can use genetically modified mouse models and cutting-edge neuroscience techniques to examine altered neural mechanisms. The purpose of this research is to replicate Rozenkratz et al. (2015) using wildtype and autism model mice to investigate the neurological mechanisms underlying altered sniff sampling behavior observed in children with ASD.

Research Justifications

For our research, we used a genotypic model for autism through genome editing to "knock out"," or remove the specific gene that is implicated in human autism. Through genome editing, mice can be engineered to parallel humans with non-functioning copies of the same genes. The genetically modified mice demonstrate behaviors characteristic that are consistent of those in humans with ASD. Examples of validated behaviors displayed in autism model mice include: decreased ultrasonic vocalizations, decreased sociability with other mice, increased repetitive grooming or motor patterns, and cognitive deficits in memory tasks, like the Morris water-maze or other cognitive tasks (Delorey et al., 2008; Dickson et al., 2013; Geramita et al., 2020; Greco et al., 2013; Greene-Colozzi et al., 2014; Kwon et al., 2006; Nakatani et al., n.d.; Pasciuto et al., 2015; Peça et al., 2011; Tsai et al., 2012; Won et al., 2012). Genome editing targets a selection of well-validated genes that encode scaffolding proteins in neuronal synapses (SHANK1, SHANK2, SHANK3, CNTNAP2, NLGN, and NRXN),

single chromosomal genes (15q11-q13, 22q11.21,NRXN1), or single gene alterations such as FRM1 or MECP2 (Brunner et al., 2015; Kabitzke et al., 2018; Kazdoba et al., 2016).

Our project will investigate altered olfactory behaviors observed in SHANK3 -/- mice. SHANK3 is a scaffolding protein commonly found in post-synaptic terminals and is essential for connecting neurotransmitter receptors, ion channels, and other terminal proteins to the post-synaptic terminal cell membrane along with dendritic spine maturation (Leblond et al., 2014; Monteiro & Feng, 2017; Sala et al., 2015; SFARI, n.d.). SHANK3 mutations, such as de novo mutations, chromosomal, exon, or amino acid deletions, have been found in many autistic individuals through broad genetic studies (Chen et al., n.d.; Delling & Boeckers, 2021; Kabitzke et al., 2018; Moessner et al., 2007; Monteiro & Feng, 2017; Peça et al., 2011; Zhou et al., n.d.). In addition, the SHANK3 gene is highly prevalent in the olfactory bulb, higher olfactory cortical areas, and many other sensory areas such as the cerebellum, which is important for controlling sniff responses (Figure 3B)(Allen Brain Institute, n.d.; Deschênes et al., 2016; McElvain et al., 2018). In addition to displaying autistic-like behaviors, SHANK3 -/- mice have been well-validated as a mouse model of ASD and exhibit decreased inhalation volume during odor presentation along with having altered synaptic connections between OSNs, tufted/mitral, and peri-glomerular cells (Figure 3A) (Geramita et al., 2020). As such, SHANK3 -/- are an idea candidate to study the potential differentiations in behavior.

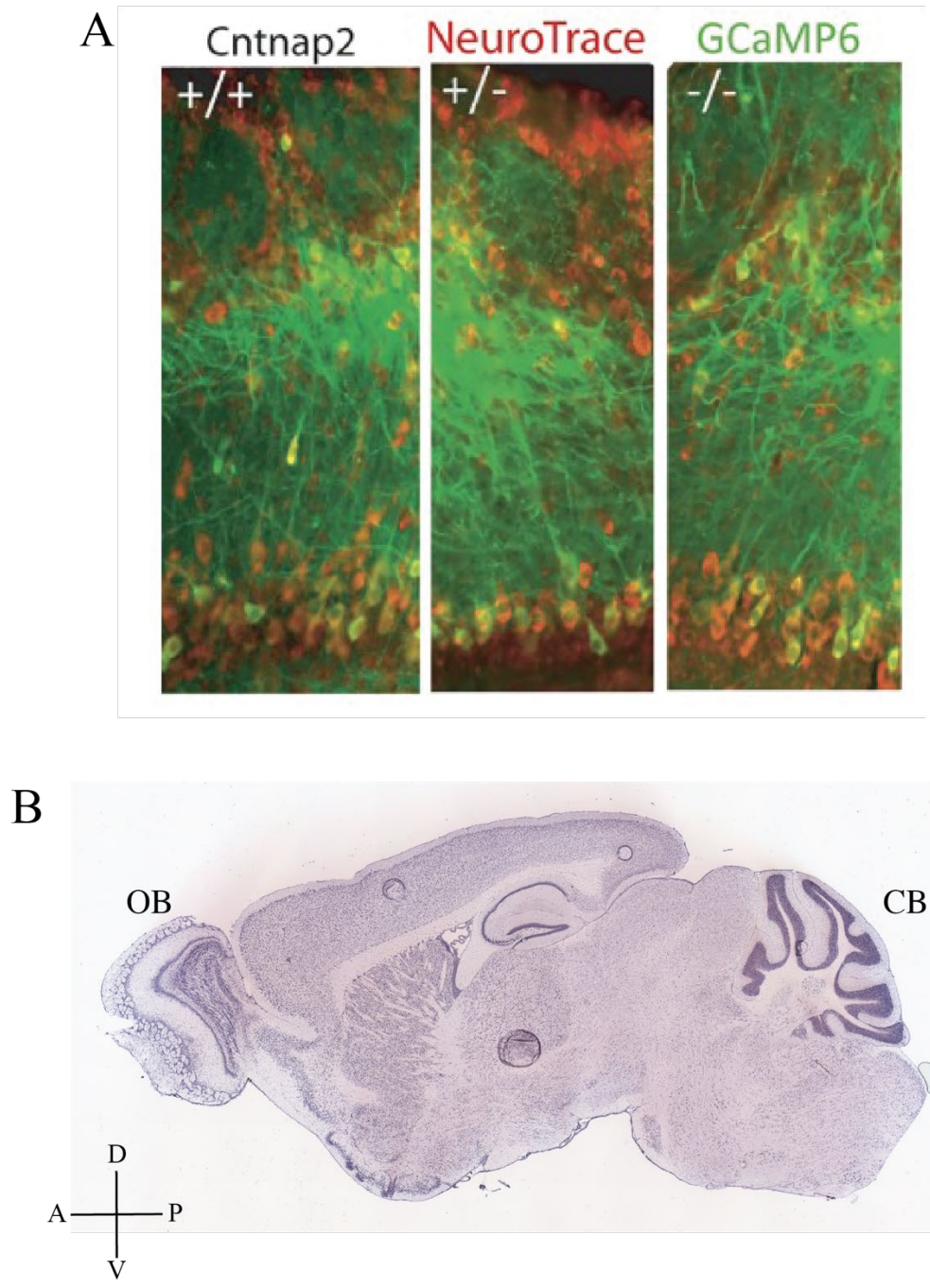

Figure 3: Presence of SHANK3 across mouse brain

A) Image taken from Geramita et al. 2020. Images of olfactory bulb cell layers stained with Neurotrace and GCaMP6 (stains cell body and glutamatergic cells) in CNTNAP2 mouse. SHANK3 mutants showed similar altered olfactory bulb anatomy to CNTNAP2. +/+ = Homozygote SHANK3 absent, +/-- = Heterozygote, -/- = WT Sham SHANK3 mouse B) Image taken from Allen Brain Institute. Purple represents tissue within brain with high concentrations of SHANK3 proteins. OB = Olfactory Bulb, CB = Cerebellum. Anatomical references were added using Adobe Illustrator

Hypotheses

Aim 1: Wildtype mice will exhibit a higher sniffing volume and/or rate when exposed to the attractive odorant, a decreased sniffing to the aversive odor than the control odor, and an elevated sniffing to the neutral odorant, but not higher than the attractive odorant.

Aim 2: SHANK3 -/- mice will show no difference in sniffing volume and/or rate when exposed to the attractive, aversive, neutral, and control odorants.

METHODOLOGY

Animal Subjects

The Institutional Animal Care and Use Committee (IACUC) at the University of Oregon approved all experimental procedures and are compliant with the National Institutes of Health Guide to the Care and Use of Laboratory Animals. I used 3 C57BL/6J mice (2 Males/1 Female, mean age at surgery = 5 months) from the Terrestrial Animal Care Services (TeACS) at the University of Oregon for behavioral experiments. I individually housed each mouse in a plastic cage with bedding and either a small plastic shelter or running wheel provided by TeACS. Mice ate standard rodent food and had unrestricted water. I performed daily health assessments before the animal went through experiments and placed the mouse on a "health check" if they appeared ill, injured, or lethargic. I provided supplementary NutriCal if the mouse was in recovery post-surgery or on "Health Check" status.

Surgical Procedure: Thermistor and Headbar Implantation

IACUC at the University of Oregon approved all surgical procedures and were compliant with the National Institutes of Health Guide to the Care and Use of Laboratory Animals. I performed all surgical procedures in our designated surgery suite in the University of Oregon's TeACS facility. Prior to anesthetizing the mouse, I calculated all drug dosages based on mouse's weight at time of surgery. I sanitized all hardware for at least 10 minutes prior to surgery using 10% hydrogen peroxide. I documented the mouse's weight before surgery in its designated surgery sheet in addition to other relevant details of surgery at a minimum of every 15 minutes

throughout the duration of the surgery. I anesthetized mice with 3% isoflurane and altered concentration of isoflurane based on animal's responsiveness to tail/toe pinch and breathing rates while under. The mouse's head was secured in a stereotaxic surgery frame using two ear bars. I cleared all hair from the surgery area using a combination of hair clippers and Nair hair removal cream. After, I cleaned the incision cite using Hibiclens and Betadine at least 3 times. Once the mouse was prepped, I injected the mouse with the calculated dosage (2 mg/mL) of buprenorphine subcutaneously (SQ) in the leg, 4 mg/mL of Meloxicam SR in the intraperitoneal (IP) cavity, and 0.03 mL (20 mg/mL) of lidocaine in the scalp SQ before making an incision from the midline of the skull to the middle of the nose. I cleared excess connective tissue and dried the surface of the skull using suction. Using the nasion skull sutures as a guide, I drilled a small hole into the nasal bone until nasal epithelium was visible in addition to creating a small groove for the thermistor wire to rest in. I inserted the thermistor bead into the nasal cavity through the drilled hole. After, I secured the thermistor in place using dental and super glue. I used coronal (bregma) and lambdoidal (lamda) sutures as guides to place and secure the head bar to the skull. Afterwards, I sealed all hardware and exposed skull with glue. All animal subjects recovered and were monitored for at a minimum of 3 days before participating in experiments.

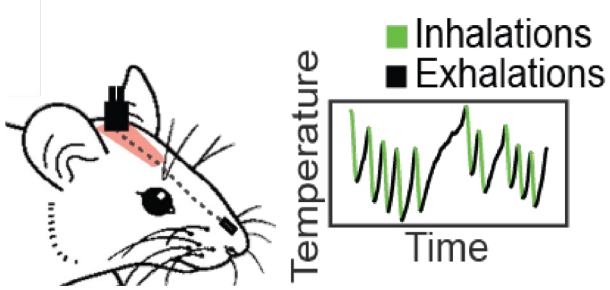

Figure 4: Image of Thermistor implantation into nasal cavity and associated "sniff" signal

Image adapted from Findley et al., 2021. Inhalations are marked by decreases in temperature, and inhalations as increases in temperature.

Odor Delivery

All odor deliveries were controlled and run with custom MATLAB code using a Sanworks BPOD State Machine r2, SanWorks Analog input module system, and 2 Sanworks valve module driver (Sanswork, Product ID: 1024, 1021, 1015). All odor samples were contained in 50 mL glass vials. Odors were as follows: 1) (α)-(–)-Pinene (Sigma-Aldrich P45702-250mL, 2mL with 1:100 dilution with caproic acid (Sigma-Aldrich 153745,1.8 mL)), 2) 2-Phenylethanol (Sigma-Aldrich 77861, 2mL with 1:100 dilution with DI Water), 3) DI Water, (2mL, 100%), 4) 2-Methylbutyric Acid (Sigma-Aldrich 49659-1mL, 2mL with 1:100 dilution with DI Water), and 5) Blank (Empty Vial) (Findley et al., 2021; Kobayakawa et al., 2007; Root et al., 2014; Rozenkrantz et al., 2015). All odorants and their classifications are listed in Table 3. Air and nitrogen systems were connected directly the source supply and fed into odor manifold. Air and Nitrogen calibrations were determined by feeling the odor released from the final valve and calibrating until minimal pressure disruption was felt. A manifold (NResearch Incorporated, #225T082) housed our odorant vials and solenoids to control odor presentation. To present an odor to the mouse, the BPOD analyzed the thermistor signal

using the analog input machine to find an upcoming inhalation. Once an inhalation was identified, the BPOD triggered the solenoids to open and close, which delivered a burst of air and nitrogen into the odorant vial to aerosolize the odor and push it through small Teflon tubing into the blank vial. After stabilizing in the blank vial for 5 seconds, the odorant was delivered to the mouse through the final valve for 0.5 seconds and resulting sniffing data was recorded after for 5 seconds.

Odorant Name	**Abbreviation**	**Classification**
2-Phenylethanol	2-PE	Attractive
2-Metylbutryic acid	2-MB	Aversive
α - (-) – Pinene	Pinene	Neutral
Deionized Water	Blank	Control

Table 2: Odorants Utilized and their Classification within the experiment

Experimental Paradigms

Animals were head fixed and restrained by a custom-3D-printed mount. After weight was recorded before the trial, mice were placed in-front of the odor delivery tube, head-fixed for the duration of the task, and had their sniffing responses recorded through the surgically implanted thermistor. For odor presentation, the trigger (the voltage that signals the end of an exhalation) and the reset value (voltage that signals the top of an inhalation) were set for each animal based on their present sniff signal range on the oscilloscope. When put in the BPOD code, it allowed the code to determine when the voltage is above or below their trigger and reset values to start or end a trial. No training was required for animal subjects as the experiment aimed to examine novel responses to the attractive, aversive, neutral, and control odors as

described in the Odorant Delivery section. Over the course of 1 session (~20 minutes), a mouse experienced 100 trials. The first 20 trials were blank odorant and for the remaining 80 trials, the BPOD code randomized the odorant display to the mouse.

Previous research has shown that both humans and mice's sniff response habituate after repeated exposure to an odorant (Kim et al., 2020). To control for habituation responses, I ran two mice (1052 and 2176) as close to every weekday as possible and one mouse (1051) once a week. A summary of trials and sessions per mouse is displayed in Table 4.

	Pinene Trials	**2-PE Trials**	**Blank Trials**	**2-MB Trials**	**Total Number of Sessions**	**Frequency of Sessions**
1051	70	47	70	53	4	Once a week
1052	287	301	280	332	16	Weekdays
2176	303	285	285	251	16	Weekdays

Table 3: Summary of Session and Odor Trial Distributions per mouse

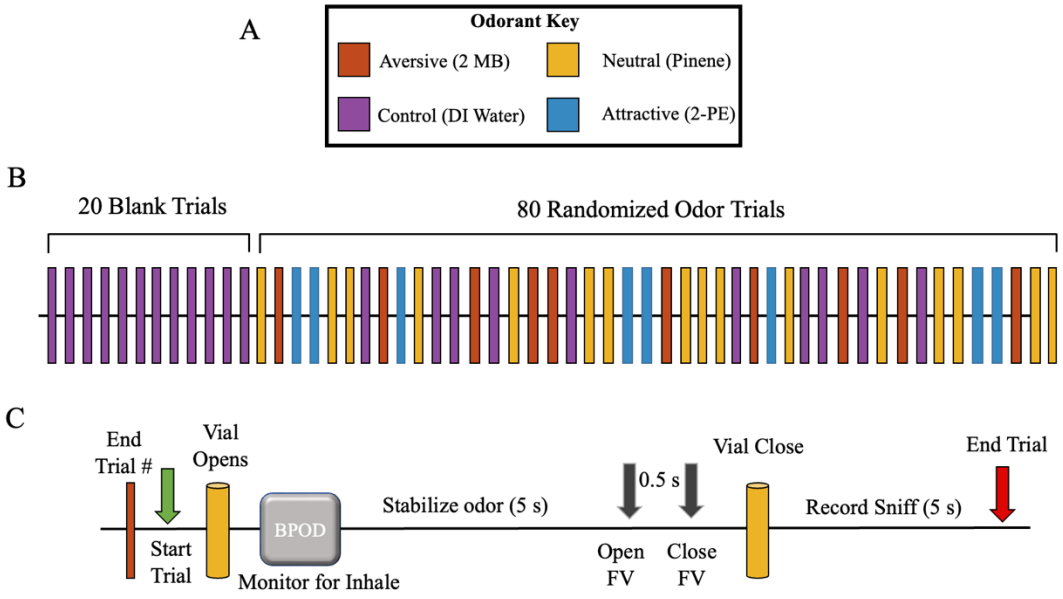

Figure 5: Summary of odorant valence, presentation, and event order during a typical session

A) Valence of odors used in experiment with color code used throughout thesis. B) Depiction of an average trial. C) Order of events within a single trial for odor presentation

ASD_FaceTracking and FaceTracking_TTL MATLAB Code

All code for the experimental paradigm was written by Dr. Avinash Singh Bala and can be requested by directly emailing him at avinash@uoregon.edu.

Orofacial And Olfactomotor Video Recording

In addition to odor delivery during the trial, a mouse had its mouth and facial (orofacial) and nose (olfactomotor) movements recorded. Videos were recorded using 2 Raspberry Pi 4 Computers (Model B with 8GB RAM) connected to Raspberry Pi NoIR Cameras (Module 2) mounted with Computar MegaPixel Vari Focal lenses (Computar, Product #AG4Z1214FCS-MPIR). One camera was mounted above the mouse to capture the nose's yaw (side to side movements) and another on the side for pitch (up and down

movements). The mouse was in darkness during the task, but an IR light illuminated the box to observe orofacial and olfactomotor movements in videos. As a secondary indicator for odor presentation, an IR light indicator was placed in view of the side camera and flashed when the final valve opened to deliver odor. After a session was completed, videos were converted from .h264 to .mp4 using VLC-Media Player.

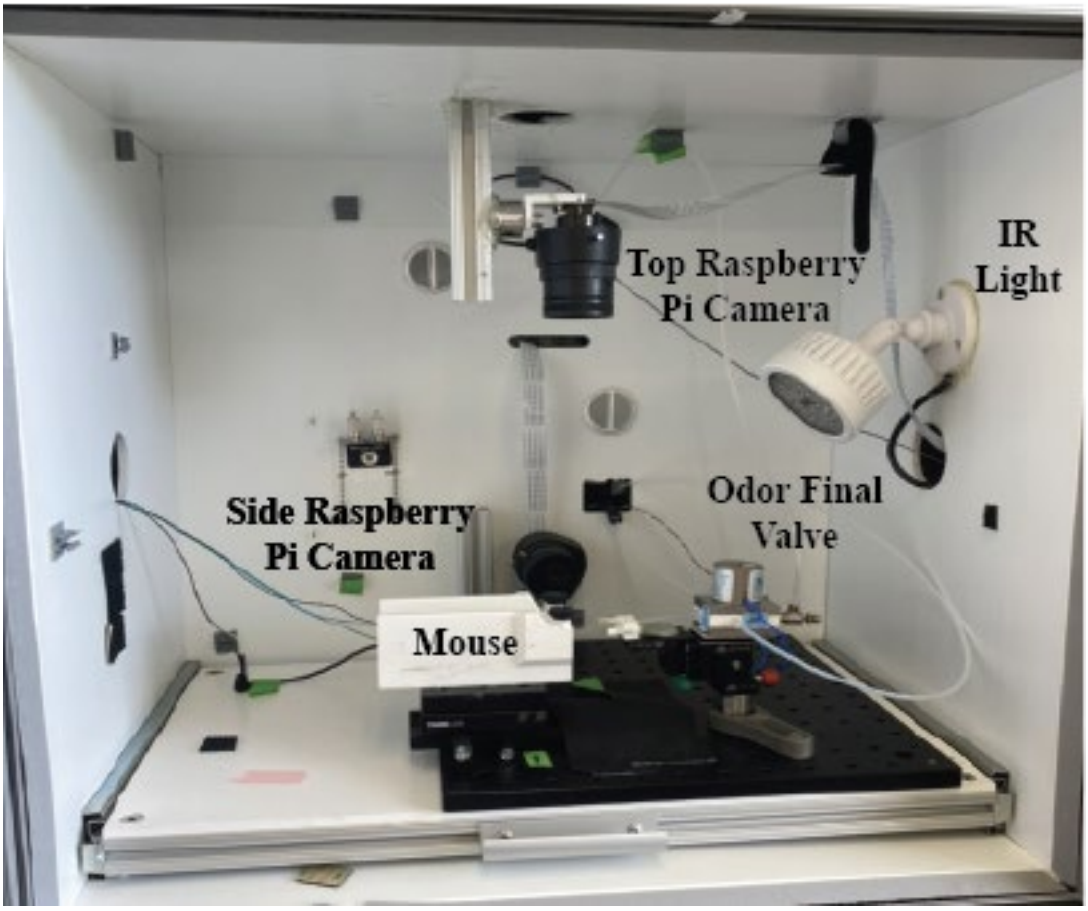

Figure 6: Labeled photo of inside olfactomotor rig

DeeLabCut and Olfactomotor/Orofacial Analysis

All top and side videos were evaluated using an open-source software called DeepLabCut, allowing users to estimate and track body positions within videos through

neural networks(DeepLabCut — The Mathis Lab of Adaptive Motor Control, n.d.; Mathis et al., 2018). Networks were trained to evaluate nose, jaw, and mouth movement within sessions for all the mice. Subsequent analysis and graphs were created by me, Dr. Teresa Findley, and Dr. Matt Smear in the Smear Lab.

MATLAB and Associated Statistical Analysis

I wrote all custom analysis code in collaboration with Dr. Matt Smear and Dr. Teresa Findley. Analysis code is available upon request.

RESULTS

Sniffing Responses to Attractive, Neutral, Aversive, And Control Odorants (2020 Data)

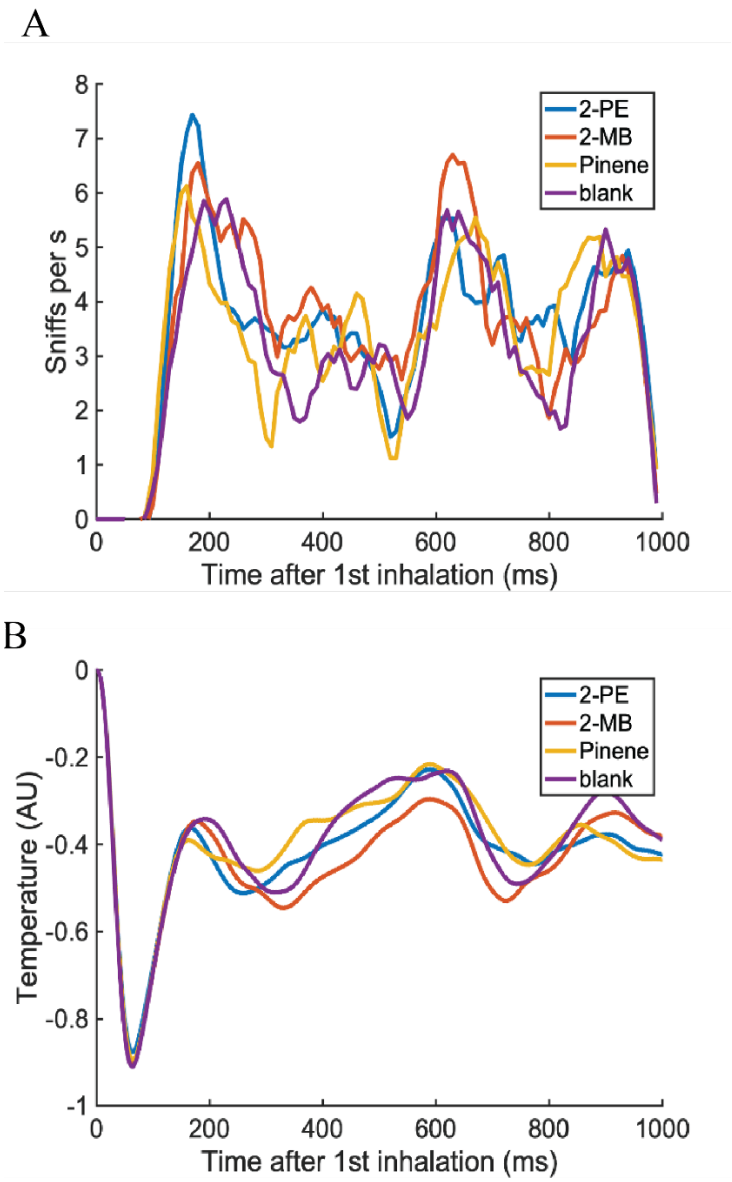

Figure 7: Inhalation Volume and Sniffing Speed Results from 2020

Change in temperature as a measure of average inhalation volume after odor onset

Average sniffing speed in a peri-stimulus time histogram (PSTH)

I began working on this project in July 2020, however, due to the pandemic, I worked remotely from New Hampshire. During this time, two members of our lab, Dr. Avinash Singh Bala and Dorian Yeh, started my project by building the original paradigm set-up, creating the code to run our experiment, and performing surgeries on mice. In this time, I conducted background research, identified viable mouse models, designed my experiments, oversaw preliminary data collection, and analyzed project data using Python and DeepLabCut.

The results above in Figure 7 were based on 2 wild-type mice with the same odorants listed in my methods, however, all were diluted with mineral oil instead of deionized water. Mice through this set of data did not differentiate their inhalation volume but did change their sniff speed after odor onset. (Figure 7A and 7B). Over the summer of 2021, our lab determined that mice could smell the mineral odor and that the observed results above were not reflective of the odorants themselves. Therefore, we decided to repeat our study using deionized water (2-MB and 2-PE) and caproic acid (Pinene) as our dilutants.

Sniffing Responses to Attractive, Neutral, Aversive, And Control Odorants (2022 Data)

When analyzing the inhalation volume of the odors across mice, the neutral odorant appears have a smaller inhalation volume than the attractive, aversive, and blank odorants. (Figure 8A-C) As I previously mentioned, two mice (1052/2176) were run as close to every weekday and 1051 was run once a week. When you separate these datasets by session frequency, we still see a similar pattern to Figure 8A and 8B, however, 1051 had a larger first inhalation across all odorants and 1052/2176 had a

smaller first inhalation across all odorants. Both 1051 and 1052/2176 all inhaled the neutral odorant the least and did not differentiate inhalation volume to the other odorants.

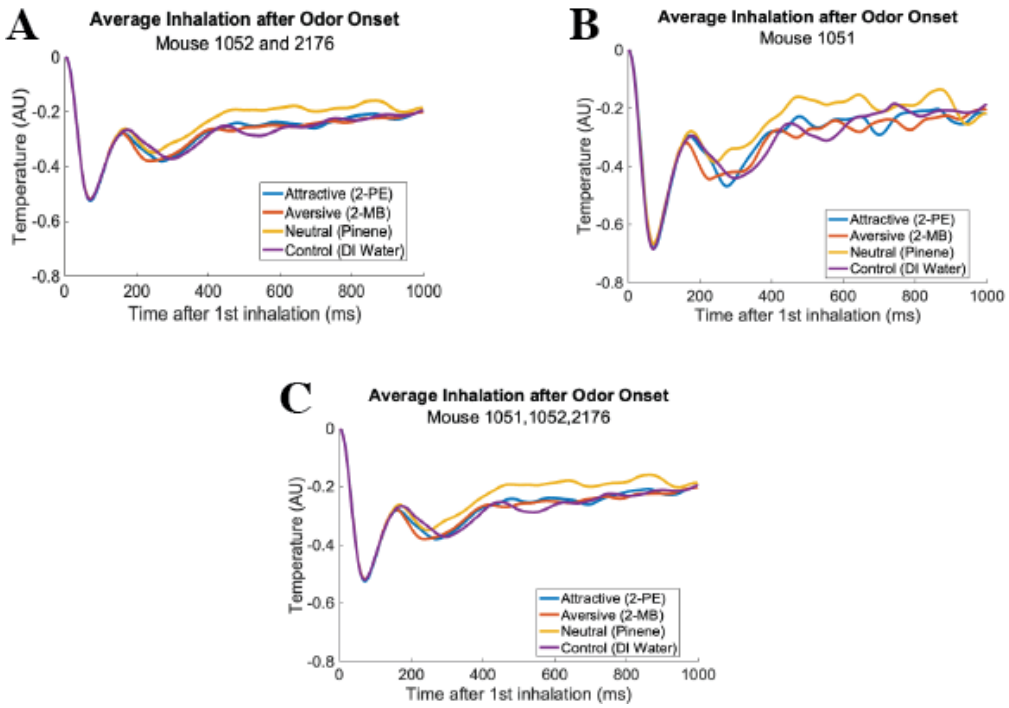

Figure 8: Change in temperature as a measure of average inhalation volume after odor onset

A) Mice ran several days a week B) Mouse ran once a week C) Average across all three mice

In addition to average inhalation volume, I examined if sniffing speed changed after odor onset. Data is displayed as a peri-stimulus time histogram (PSTH), which is a variation of a raster plot. When averaged across all three mice, the data suggests that the aversive odor is smelled the fastest after odor onset with the neutral and attractive following third (Figure 9C). Like in the average inhalation plots, I separated data by how frequently mice were run. It is evident that the neutral, attractive, and aversive odorants were smelled at similar speeds in the 1052/2176 mice (Neutral = 7.83 sniff/s,

Attractive = 7.3 sniffs/s, Aversive =7.89 sniffs/s) but not in 1051. In 1051, the attractive and aversive odorants were smelled at a higher sniffing rate (Attractive = 9.48 sniffs/s, Aversive = 9.54 sniffs/s) with the neutral odor with the next highest sniffing speed (Neutral = 7.83 sniffs/s) (Figure 9A, 9B). All mice sniffed odorant trials faster than the control odorant, suggesting that the mice did have increased sniffing responses to odorants. There is a large difference between the mice run more frequently and the mouse run once a week, suggesting that how close together sessions occur influences the resulting sniff response.

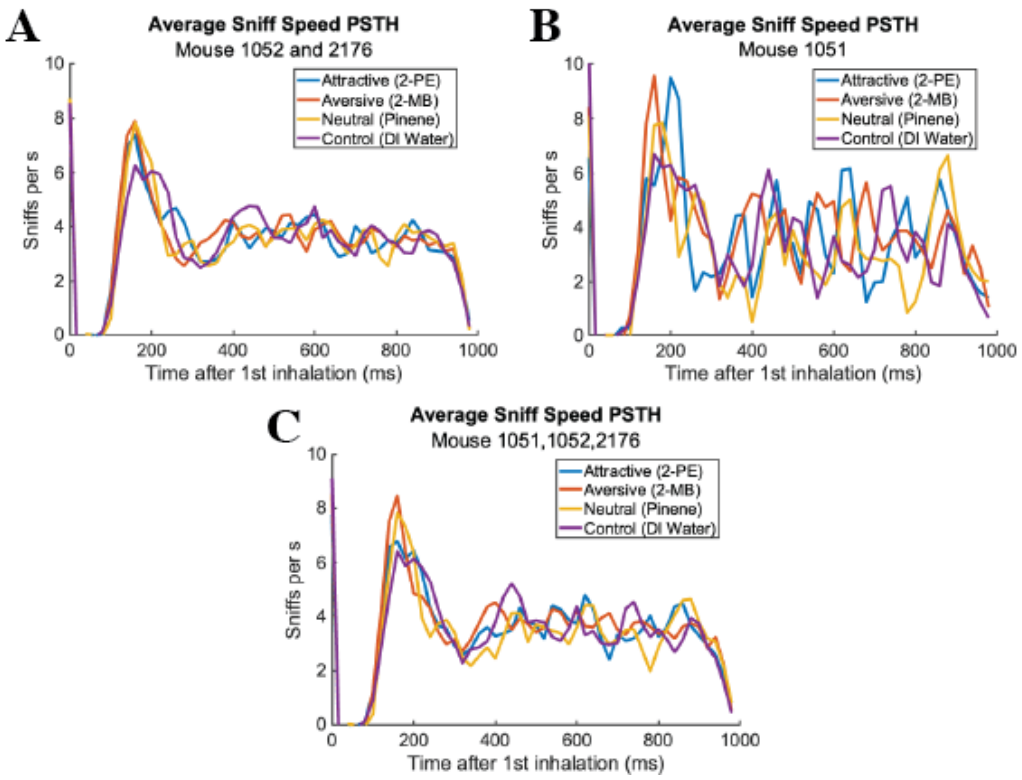

Figure 9: Average sniffing speed in a PSTH graph

A) Mice ran several days a week B) Mouse ran once a week C) Average across all three mice

Orofacial and Olfactomotor Responses to Odorants

Examples of the DeepLabCut tracking are shown in Figure 10A-D. I was not able to create plots to analyze the difference in nose movement in response to odorants in time, however, I was able to create plots to show overall movement of the nose based on tracking points (Figure 10B,10D). In examining Figure 10, we see that across a whole sessions the mouse's nose movement vary across the X axis (Figure 10B) and the Y axis (Figure 10D). Further analysis is needed to make supported claims about how olfactomotor and orofacial movements are altered in response to these odors.

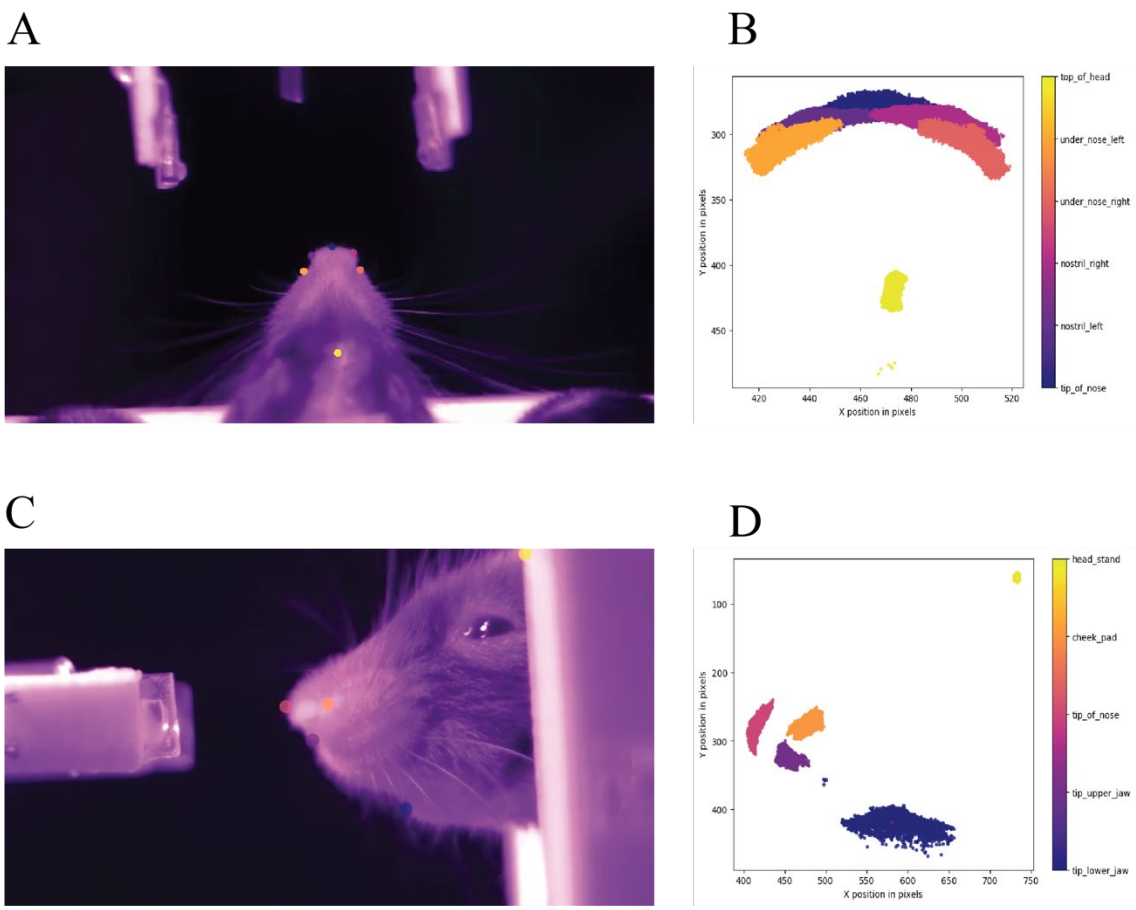

Figure 10: DeepLabCut analysis of olfactomotor and orofacial movements for side and top videos

A,C) Labeled analysis video of one session from above (A) and side (C). Dots represent points tracked across videos and color coded. B,D) Distribution of tracked points across one session. Each dot represents a part of the face, mouth, or nose we tracked from above (B) and the side (D). Dots are color coded using the color scale on the side.

DISCUSSION

This thesis aims to examine the neural mechanisms of the altered olfactomotor behaviors observed in Rozenkratz et al (2015). In this past year, I created my experimental rig, performed surgeries and daily data collection, and ran data analysis to determine if wild-type mice performed similar sniffing behaviors to neurotypical individuals' responses from Rozenkratz et al. 2015. My data, thus far, does not support my proposed hypothesis. Although my current data does not support my original hypothesis, there may be several experimental design flaws and other factors that contributed to produce my null results.

Potential Experimental Confounds

First, my data is based on a both a very small sample size of mice (n=3) and number of sessions ($n_{weekly} = 4$, $n_{daily} = 16$). Both a larger sample size of mice and number of conducted sessions is needed to determine if the observed differences between odorants are statistically significant.

In addition, the frequency at which mice are put through the experiment appears to influence the resulting sniffing response speed and inhalation volume. Such differences resulting from odor habituation are supported by both human and animal data (Chaudhury et al., 2010; Kim et al., 2020; Pellegrino et al., 2017). In future experiments, I believe we should only run mice one to two times a week to keep the odorants "novel" to the mouse for longer. Moreover, changing our session frequency will more closely mimic the Rozenkratz study where 30 participants participated in one session.

Another experimental design flaw may be some of the cleaning protocols in place. My experiment exposes mice to four different odors; however, these are all different types of chemicals (acids, oils, or water) and run through the same tubing to be dispersed through the final valve. Over time, odor residue may have built up in the tubing and altered the valence of odorants when presented. Currently, our protocol is to replace the tubing in the rig every 3 months and may need be replaced more frequently.

In addition to experimental design flaws, several equipment issues may have contributed to our null results, such as unbalanced air pressure upon odor onset and oscilloscope trigger levels. First, our system's air and nitrogen come directly from the source tanks and was fed through a series of tubes into the rig's final valve where odor is dispersed. Since air and nitrogen levels are not regulated by a mass flow controller, any pressure change in the tubing may have resulted in our air and nitrogen levels shifting unexpectedly. I noticed a few weeks after calibrating our air and nitrogen levels that when the final valve was opened, there was a large change in air pressure before odor onset and during odor presentation. I recalibrated the air and nitrogen levels after I noticed the issue, but I do believe this may have created artificial olfactomotor or orofacial responses. Such a response is an automatic reflex to the air pressure rather than the odor and thus has no bearing on sniffing behavior. To address air flow challenges, we will be adding mass flow controllers to my system to keep air and nitrogen levels constant during odor presentation and in between trials.

Along with pressure, another technical issue may have been the trigger and reset values for odor presentation in our experiment's code. Our project, like many others in the field, present odor at the onset of inhalation. In order to determine when an

inhalation occurs, we use the analog signal from the thermistors and convert them into a voltage. Our code says when the voltage reaches above or below a number to reset or start a new trial. However, sniff signals (thermistor signals) are not uniform like a sine wave, but rather vary greatly. To determine the trigger and reset values, I watch the sniff signal and move the cursors on our oscilloscope to capture about 80% of the sniff signal as seen in Figure 11. However, because the sniff signal moves throughout the session, there are times were the time between the odor vial opening and odor onset is longer or shorter depending on the trigger and reset levels set. These may have been too high in some trials and may have contributed to altered sniffing responses.

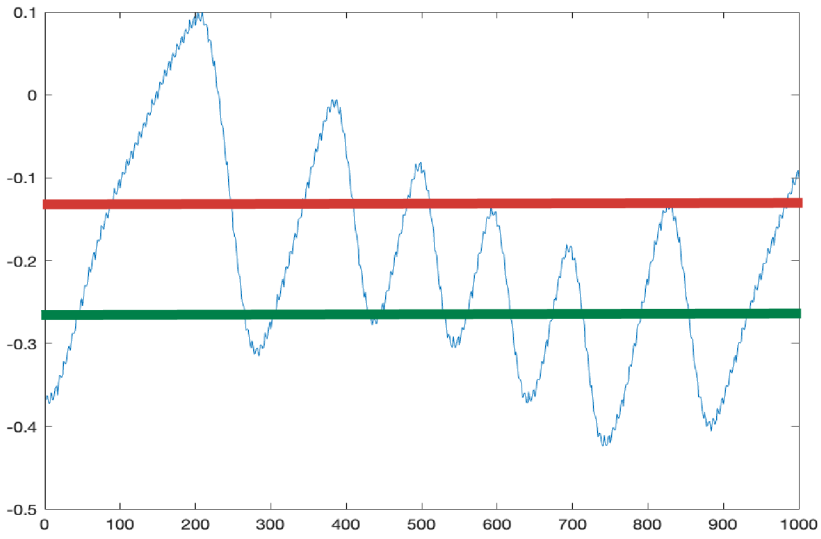

Figure 11: Example of sniff signal trace on the oscilloscope with trigger and reset values.

Red = Reset Value, Green = Trigger Value

Future Directions

In beginning of the project, I had hoped to also have data from an autism model mouse, SHANK3. Unfortunately, due to pandemic shipping delays, we were unable to

obtain the mouse line in time to perform experiments to include in this thesis. In addition to expanding our wildtype data, the first future step is to place SHANK3 -/- mutants through this same paradigm to observe if there is an altered sniffing phenotype present.

In combination with data from SHANK3 -/- mutants, another next step is to balance cohorts for gender and examine if there are differences in sniffing responses between male and female mice. A large European meta-analysis examining olfactory discrimination, identification, and threshold values between neurotypical human females and males determined that females performed better on all olfactory measures (Sorokowski et al., 2019). By balancing gender in both cohorts, we may be able to determine possible neural mechanisms that lead to differences in healthy adults and even determine if there is an altered female phenotype in the SHANK3 -/- mice that may be used a biomarker.

Finally, in addition to performing my paradigm, I also want to examine if differences in sniffing responses when these mice are freely moving versus head fixed. Recent data from our lab suggests there are large differences in the olfactory bulb firing patterns and rates when an animal is head-fixed versus freely moving (Figure 12). By performing freely moving experiments in addition to our current project, we can evaluate both sniffing responses to odors, but also how they perform olfactory navigation. SHANK3 mice, like many other autism model mice, have been shown to display altered behavioral responses in tasks such as novel object testing and social interactions (Delling & Boeckers, 2021; Kabitzke et al., 2018; Peça et al., 2011).

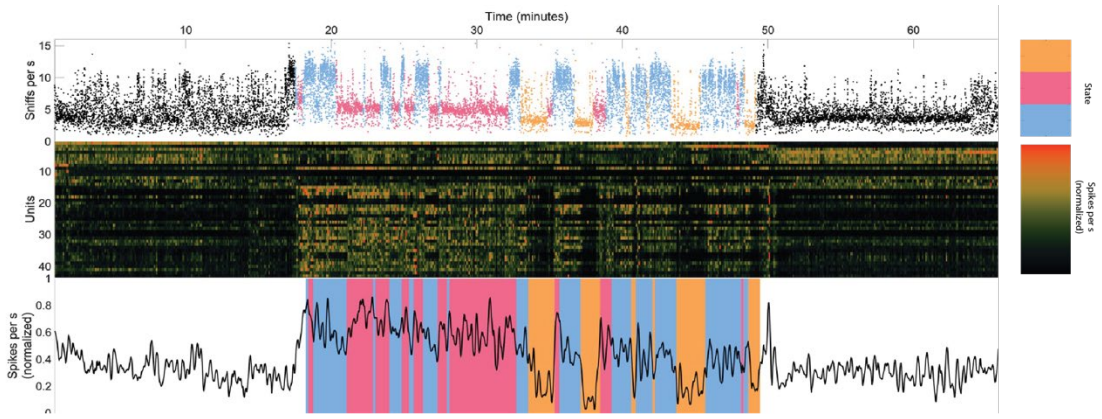

Figure 12: Electrophysiology recordings of the olfactory bulb and sniffing response in a head-fixed and freely moving mouse

Top: Histogram of sniffing speed over time. States denotes similar patterns of neural firing in olfactory bulb determined by hidden-markov model. Colored sections were when the mouse was freely moving, and black was head fixed. Middle: Electrophysiology recordings from olfactory bulb using a 16-channel electrode. Bottom: Normalized graph of top panel to denote sniff speed over course of session.

Conclusions

In this thesis project, I examined wildtype mice's sniffing, orofacial, and olfactomotor responses to attractive, aversive, neutral, and control odorants. I hypothesized that wildtype mice would inhale a larger volume of the attractive odorant than the aversive, neutral, and control odorants. My data did not support this hypothesis and instead showed that mice showed very little difference in inhalation volume between the attractive versus neutral, aversive, and control odorants. Although my project yielded null results, there may have been several experimental design problems that contributed to this along with a small sample size. Both a larger sample size of mice and sessions are needed to make a stronger claim about the effects of attractive and aversive odorants on inhalation volume and olfactomotor responses.

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