Enhancement of relevant information through bottom-up bias is facilitated by tyrosine hydroxylase in the glomeruli layer.

A Thesis

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by

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From May 2023 to Mar 2024

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH, PUNE

Certificate

This is to certify that this dissertation entitled "Enhancement of Relevant Information through Bottom-Up Bias is Facilitated by Tyrosine Hydroxylase in the Glomeruli Layer" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science, Education, and Research, Pune, represents the study /work carried out by Vikas Kumar (20191192) at Stowers Institute for Medical Research, Kansas City, USA, under the supervision of Dr. C. Ron Yu, Professor, The Graduate School of SIMR, SIMR, USA, during the academic year 2023-2024.

Dr. C Ron Yu

I dedicate this thesis to my family for their constant support and to my friends who were an essential source of motivation for me throughout my journey.

Declaration

I hereby declare that the matter embodied in the report entitled "Enhancement of Relevant Information through Bottom-up Bias is Facilitated by Tyrosine Hydroxylase in the Glomeruli Layer" are the results of the work carried out by me at the Stowers Institute for Medical Research, Kansas City, USA, under the supervision of Dr. C. Ron Yu, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Vikas Kumar
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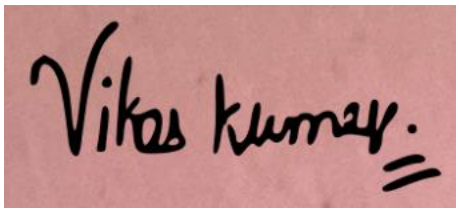
Date: 25th March, 2024



Dr C Ron Yu

5/16/2024

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Abstract:

The olfactory bulb (OB) is responsible for processing odor information and shows experience-based plasticity. OB houses various types of neurons, including tyrosine hydroxylase (TH)-expressing dopaminergic neurons found within the glomerular layer (GL). The expression of TH dopaminergic interneurons in OB has a vital role in odor information processing. Previous studies have focused on the role of TH under odor-enriched conditions, as changes in its expression can occur in an odor-experience-dependent manner, but whether these changes are odor-specific is not known. We systematically address this using odor exposure and odor discrimination paradigms in M72-GFP mice to determine the regulation and selection of TH expression due to the valence of associated odors and the duration of odor exposure. Using acetophenone, which is an odor investigated to activate the M72 glomerulus, we observe that chronic but not acute exposure to the odor acetophenone leads to an approximately **66%** increase in dopamine-expressing TH cells surrounding the M72 glomerulus. Next, we design a reward association paradigm where the total exposure to acetophenone matches acute exposure conditions to test the valence-dependent plasticity of TH cells. Reward association leads to a notable **40%** increase in TH expression surrounding the M72 glomerulus, but not when the same odor is behaviorally irrelevant. This suggests that a valence-dependent increase can occur even with low-odor exposure. OB receives cholinergic feedback from the diagonal band nucleus that dishabituates sensory responses. To test if cholinergic-mediated dishabituation of odors is vital for TH expression, we use a DREADD-based neural activation approach in our passive exposure paradigms. Accordingly, using AAV-hSyn-DIO-DREADD(Gq) viral vectors in Chat-Cre; R26R-tdT; M72-GFP mice, we observed a **60%** increase in TH cell populations in the acute exposure condition with administration of CNO intraperitoneally compared to acute exposure without the CNO injection. This highlights the role of top-down odor dishabituation in modulating TH expression. Overall, our results demonstrate that the odor exposure conditions, along with their relevance to goal-directed behaviors, play a crucial role in regulating dopaminergic cells in OB. These findings are vital for optimizing olfactory bulb circuit adaptation and enhancing animal's ability to recognize relevant odors in their natural environment.

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Contributions

Contributor name	Contributor role
Rahul Garg	Conceptualization Ideas
Rahul Garg	Methodology
Rahul Garg	Software
Vikas Kumar	Validation
Vikas Kumar	Formal Analysis
Vikas Kumar	Investigation
Rahul Garg	Resources
Rahul Garg, Vikas Kumar	Data Curation
Vikas Kumar	Writing – Original Draft preparation
Rahul Garg, C Ron Yu	Writing – Review and editing
Vikas Kumar	Visualization
C Ron Yu	Supervision
Vikas Kumar	Project administration
C Ron Yu	Funding acquisition

Chapter 1

Introduction:

The brain integrates sensory inputs from the surroundings and processes them to generate a suitable response. Neurons are the basic unit of formation in the brain and are highly plastic. They have the ability to reorganize by modifying their connections by weakening or strengthening the synapses with other neurons with experience in a changing environment(Baroncelli et al., 2009; Mandolesi et al., 2017; Mateos-Aparicio and Rodríguez-Moreno, 2019; Han et al., 2022). Studies show that adult neurogenesis occurs in specific sections of the brain with learning(Shors et al., 2002; Döbrössy et al., 2003; Hanson et al., 2017; Kumar et al., 2019). This plasticity is an essential feature required by the individual for various cognitive processes, including memory formation and learning.

1.1. Olfactory System in Sensory Processing

The neurons in the brain collect sensory stimuli from various sensory modalities, one of which is olfaction. The olfactory bulb is a unique brain structure responsible for detecting odors, which makes it important for the survival and behavior of organisms across species, such as foraging, mating, and predator avoidance. Olfactory processing involves complex networks of neural circuits in the brain. Each of the olfactory sensory neurons (OSNs) in the olfactory epithelium expresses one type of olfactory receptor, which binds to different odors with varying efficacy. Then, the olfactory information is sent through the sensory neurons to the OB. The odor signals carried through the OSNs activate different combinations of glomeruli. Glomeruli are very special structures in the olfactory bulb where sensory stimuli from olfactory receptor neurons gather(Friedrich and Korsching, 1998; Kauer and White, 2001; Mori and Sakano, 2024). It acts as an important connection between sensory input and the subsequent processing of odor information. Odor discrimination happens due to the unique combination of olfactory receptor neurons that converge onto a glomerulus. Each of these glomeruli expresses a unique odorant receptor (Treloar et al., 2002). After an odorant molecule binds to its receptor on these neurons, it trigger a cascade of signals that converge onto the glomerulus associated with that receptor(Kurian et al., 2021). This level of organization of glomeruli enables the brain to create a detailed odor map. It facilitates accurate odor recognition and discrimination. From this glomeruli layer, the sensory signals synapse further onto other downstream neurons, the mitral and tufted cells(Mori et al., 1999; Mori and Sakano, 2024). These formations are important for refining the olfactory signals and, finally, enhancing the brain's capability to distinguish and interpret a wide range of odors. The processed odor information is later relayed to higher brain areas and may be followed by a behavioral response. These lay the groundwork for the brain to form perception.

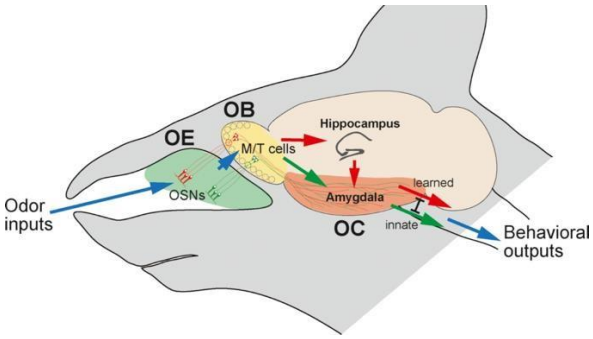


Figure 1.1: Olfactory system in sensory processing(Mori and Sakano, 2024)

1.2. Glomerular Organization and Odor Discrimination

Previous studies have found specific ligands that activate specific OSNs. Acetophenone is recognized for its ability to strongly activate the glomeruli in the olfactory bulb that are associated with the M72 receptor(Bozza et al., 2002; Zhang et al., 2012). The M72 glomerulus is located on the dorsal and dorsolateral sides of either of the olfactory bulbs (Potter *et al.*, 2001). The robust activation of the M72 glomerulus by acetophenone offers a valuable model for testing and finding out the neural mechanisms that govern odor perception and the influence of local olfactory signaling in olfactory processing.

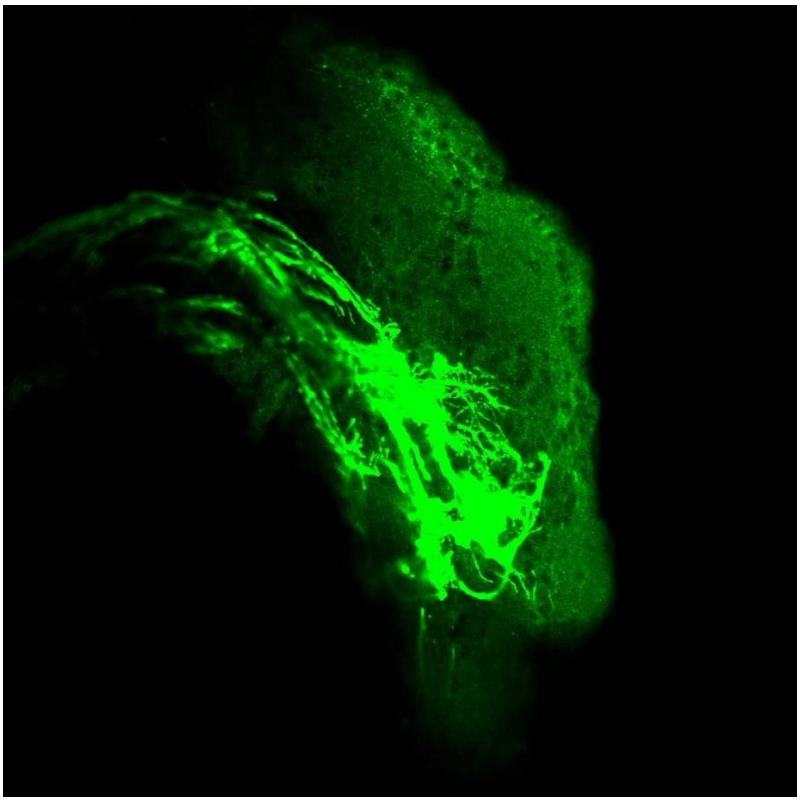


Figure 1.2. showing M72 glomeruli image

1.3. Interneurons in the OB

The glomeruli layer of OB comprises distinct types of interneurons known as juxtaglomerular cells. γ-Aminobutyric acid (GABA), an inhibitory neurotransmitter in the central nervous system, has a significant role in regulating neuronal excitability in the OB. It maintains the balance between excitation and inhibition in the OB, influencing odor processing and discrimination(Tatti et al., 2017). GABAergic neurons express GAD1, and enzymes are known to synthesize GABA, which provides a basal level of GABA synthesis. GAD2 is localized in nerve terminals and is associated with synaptic GABA release(Gaetz et al., 2014; Bolneo et al., 2022). These play a role in the regulation of GABAergic neurotransmission in the OB as well and are found in the glomeruli layer surrounding individual glomeruli. Additionally, GABAergic neurons comprise several subtypes, such as those expressing tyrosine hydroxylase (TH), parvalbumin (PV), and somatostatin (SST). The plasticity of interneuron maps is promoted by Task Learning in the OB(Huang et al., 2016).

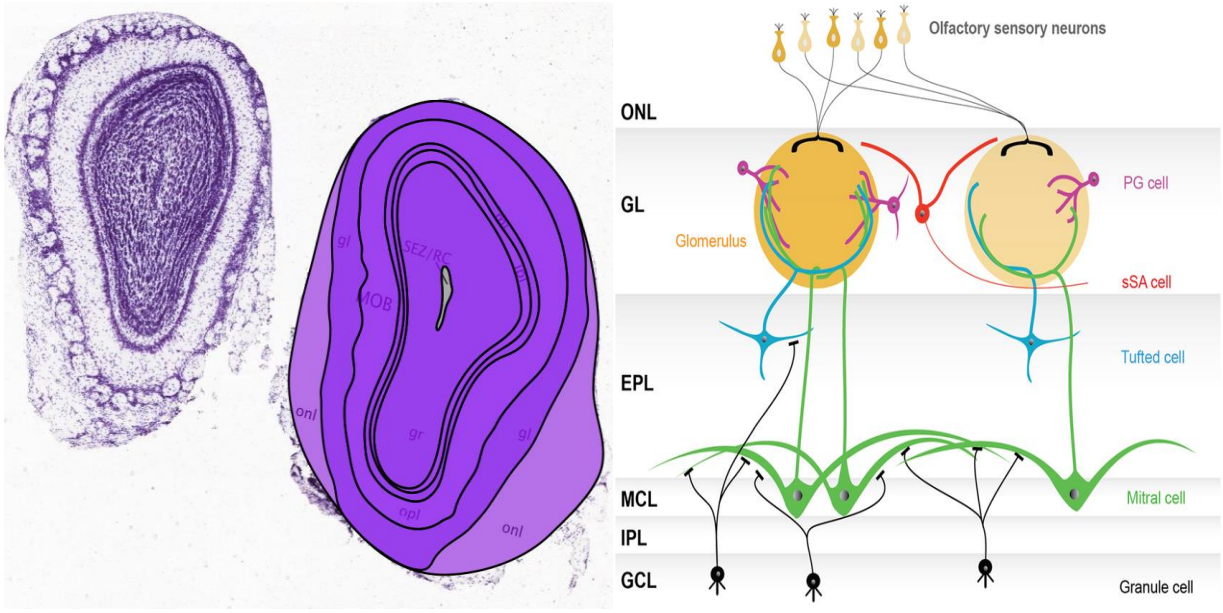


Fig1.3: Representation of the (a) olfactory bulb and, (b) interneurons present in it (Nagayama et al.).

1.4. Dopamine and Glomeruli

To understand the role of glomeruli in odor processing, we need to explore the modulation of the olfactory system and the processing of olfactory stimuli. The neurotransmitter dopamine is historically linked with reward and motivation(Bromberg-Martin et al., 2010), and is also involved in multiple areas of olfactory function, such as odor detection, discrimination, and associative learning (Escanilla et al., 2009; Chaudhury et al., 2010).

In the olfactory bulb, these dopaminergic neurons modulate the activity of olfactory sensory neurons and interneurons, influencing odor processing and perception. Exposure to various odors correlates with an up-regulation of TH expression in OB dopaminergic interneurons situated within the glomerular layer (GL), consequently yielding heightened dopamine levels within the OB(Liu et al., 1999). The studies have suggested a link between passive odor exposure and dopaminergic neuron activity (Bonzano et al., 2014; Quast et al., 2017). They demonstrated that passive exposure to an odor-enriched environment results in increased dopaminergic cell counts in the olfactory bulb. Therefore, dopamine is one of the factors responsible for regulating synaptic plasticity in the olfactory bulb. This is essential for adjusting the olfactory circuits to allow for changes in environmental experiences. Hence, understanding the complex relationship between glomeruli and dopamine is essential to exploring the complexities of olfactory perception.

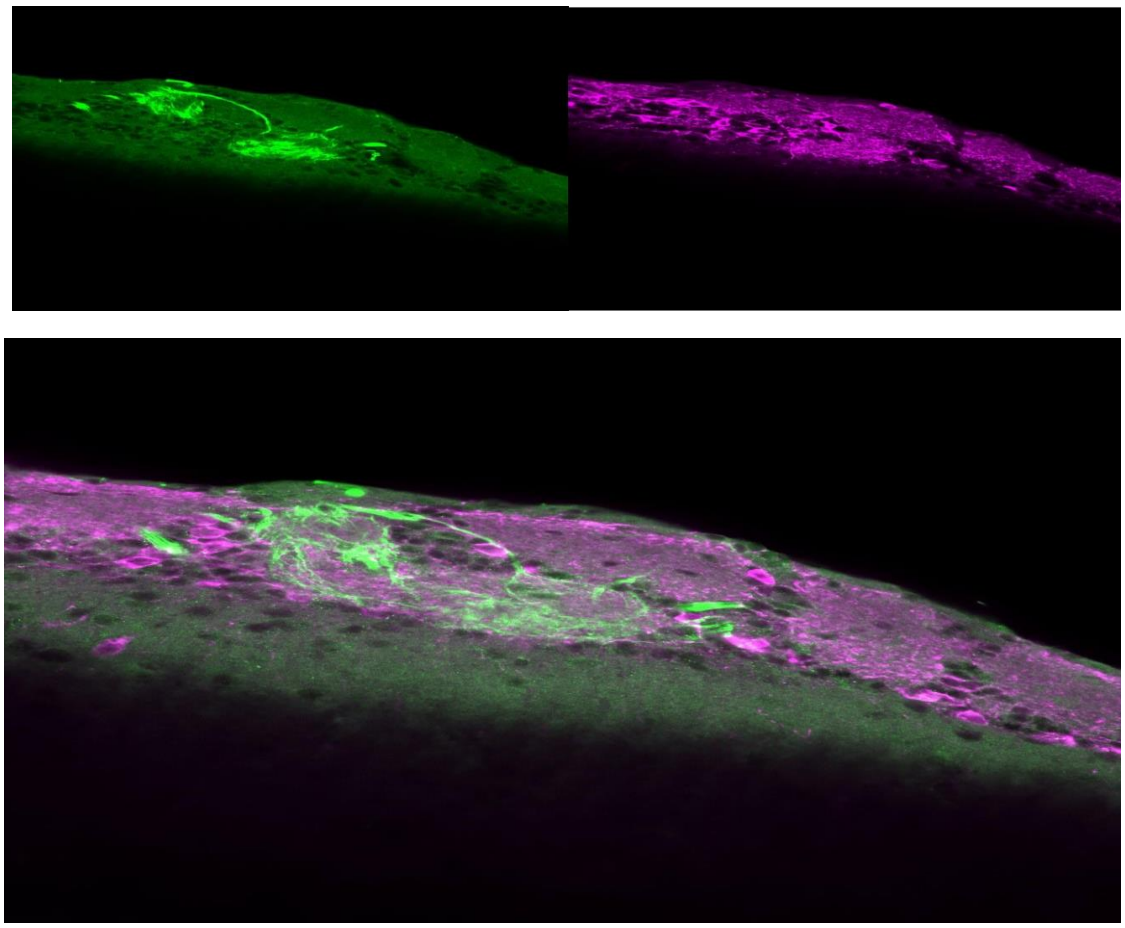


Figure 1.4: A 40x magnification image of M72 glomeruli and TH cells surrounding it. Top left- M72-GFP, top right- TH, bottom- composite.

1.5. Modulation in OB

There remains a notable gap in our understanding regarding this phenomenon. We know very little about its influence on dopaminergic cell function during alterations in odor processing mechanisms, like in scenarios where relevant rewards are associated with an olfactory stimulus. The brain selectively processes and retains relevant olfactory information. Receptors in the OB are selectively activated by odorants, which lead to the activation of distinct neural pathways. TH-expressing dopaminergic neurons might play a role in biasing neural circuits towards particular odorants. It is known to regulate synaptic plasticity in several brain regions(Hanson et al., 2021).

The OB not only processes bottom-up signals from sensory neurons in the nasal epithelium but also receives inputs from various brain regions through top-down signaling pathways(Chen and Padmanabhan, 2022) that modulate the ongoing odor processing. These top-down signals play a very important role in shaping the plasticity of the OB by influencing its response to odors and resulting in olfactory perception. Studies have shown the significance of these top-down inputs in regulating OB function and plasticity(Boyd et al., 2012). The piriform cortex is one of the primary receivers of the OB input. It sends strong feedback to the OB, influencing odor processing and synaptic plasticity(Wu et al., 2020).

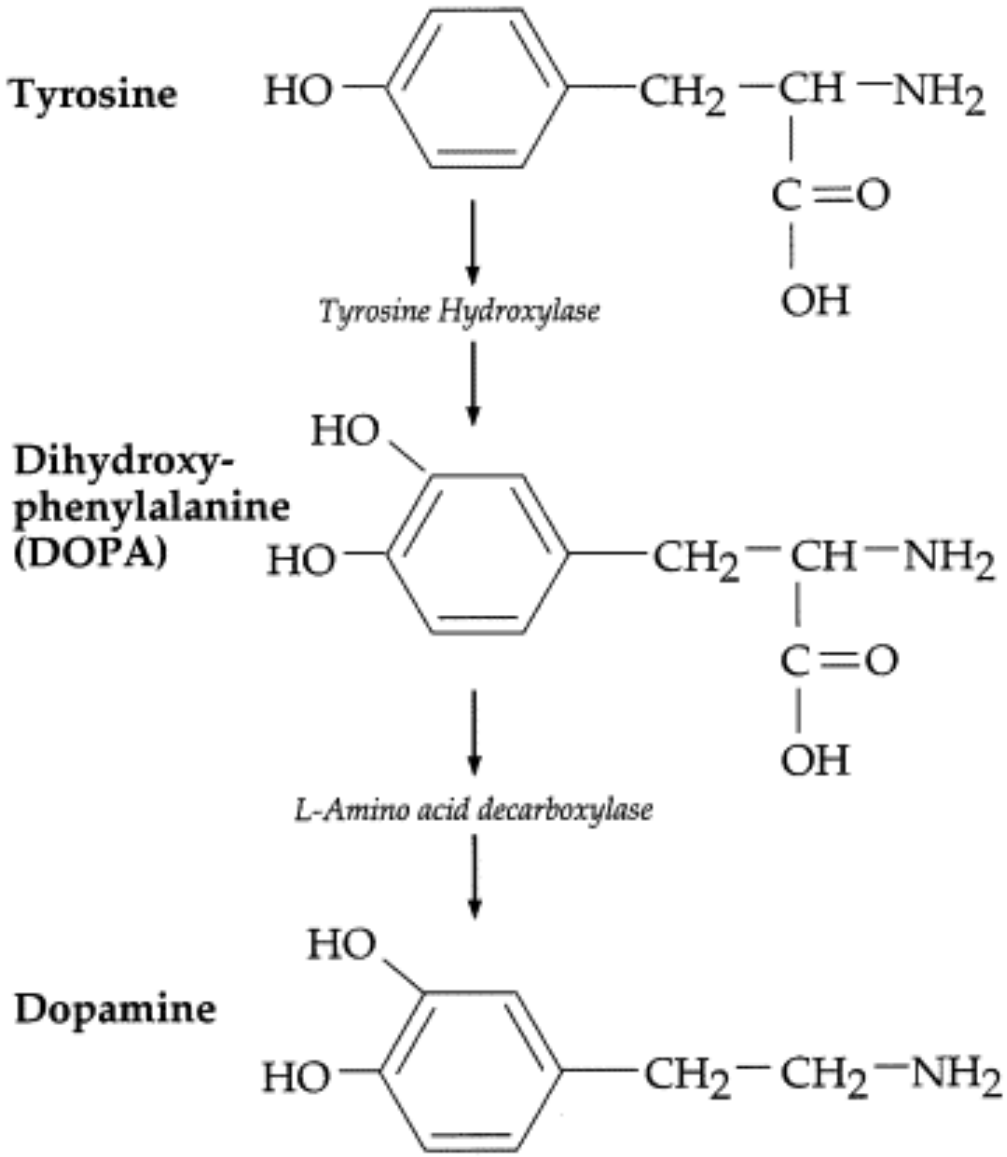


Fig1.5: Showing synthesis of Dopamine.

1.6. HDB Cholinergic Innervation in OB

The horizontal limb of the diagonal band (HDB) is an area located in the basal forebrain. It is known for having a very dense population of cholinergic neurons. These neurons are known to project to various regions of the brain, including the OB(Case et al., 2017). Here, they release the neurotransmitter acetylcholine (ACh). Acetylcholine is a neurotransmitter released by choline acetyltransferase. It helps in transmitting impulses across synapses. It plays a vital role in physiological processes like cognitive function, memory, and attention in the central nervous system(Wilson et al., 2004). The innervation of the glomeruli in the OB by cholinergic projections from the HDB plays a role in modulating olfactory processing. While the peripheral nervous system, it mediates and controls voluntary movements. Acetylcholine released by these projections acts on cholinergic receptors located on different cell types within the glomeruli(Ogg et al., 2018a), including mitral, tufted cells, and interneurons. Prolonged exposure to odors causes habituation in the

neurons(Chaudhury et al., 2010). Acetylcholine is said to play an important role in odor discrimination and processing(Nunez-Parra et al., 2020). Moreover, it is also known to cause dishabituation in the GL(Ogg et al., 2018b). The innervation of glomeruli by cholinergic projections from the HDB represents a key mechanism by which top-down signals from higher brain regions might influence olfactory information processing in the OB(Ma and Luo, 2012; Zheng et al., 2018). Its diverse role in the central nervous system makes acetylcholine a crucial part of our study. We aim to understand the mechanism by which it modulates the circuits in the olfactory bulb, especially in the glomeruli region. Clozapine-N-oxide (CNO) was used as a ligand for DREADD-based neuronal activation, resulting in increased acetylcholine release in the targeted brain region.

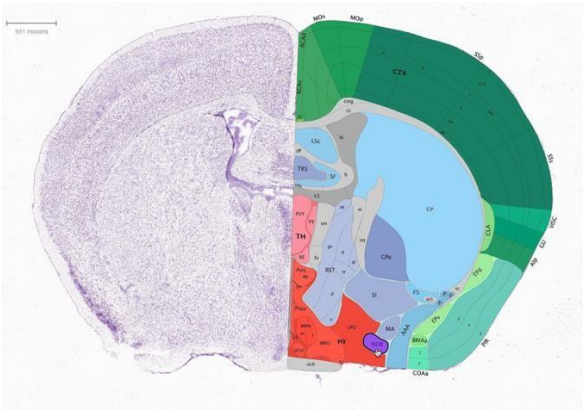


Figure 1.6. A mouse brain atlas showing HDB area(Interactive Atlas Viewer :: Atlas Viewer)

1.7. Motivation

Neural mechanisms that involve dopaminergic signaling and top-down acetylcholine release in olfactory processing remain largely unknown(Zhang et al., 2012; Hanson et al., 2021). The activation of the M72 glomerulus by acetophenone is a good model for exploring the mechanisms that modulate odor perception. Therefore, to investigate plasticity in olfactory processing and selection of relevant olfactory stimuli, we used Chat-Cre;R26R-tdT;M72-GFP transgenic mouse model to determine the regulation and selection of TH expression. We want to highlight the dopaminergic activity of tyrosine hydroxylase interneurons. In our study, we quantified the TH population changes and found a significant difference between experiment groups when an odor is linked to a reward, comparing it to acute exposure conditions where the odor has no associated reward. Thus, we demonstrate that the increase in TH populations is not solely dependent on the duration of odor exposure but also depends on its valence. Our results show a significant increase in the neuronal population when the mice are presented with chronic odors with respect to the controls. Studies show that prolonged exposure to odors causes habituation in the glomeruli(Chaudhury et al., 2010), resulting in a saturated TH population. To investigate further, we use AAV-hSyn-DIO-DREADD(Gq) viral vectors in

Chat-Cre; R26R-tdT; M72-GFP mice, with an administration of CNO injection to increase the activity of the neurons to facilitate acetylcholine release known to promote disinhibition and reinstating odor investigation. Following this, we observe a significant increase in the TH populations for the mice groups exposed acutely to odors when compared to the control. However, no significant difference was noted between the acutely and chronically odor-exposed groups. Through this, we aim to deepen our understanding of the intricate connection between neurotransmitter systems and sensory processing in the olfactory system.

Chapter 2

Materials and Methods:

2.1. Animal Model and Housing

A total of 44 mice were used in this study. All the experiments were conducted using 8-10 week-old Chat-Cre;R26R-tdT;M72-GFP (Stock no. - 006410 (Chat-Cre), 007914 (R26R-tdTomato), 006678 (M72-GFP)) transgenic mice obtained from Jackson Laboratory. This strain was bred through a series of genetic modifications to express both the Chat-Cre recombinase, which drives the expression of Cre in cholinergic neurons and the red fluorescent protein tdTomato (tdT). M72-GFP mice labeled M72 OSNS with a green fluorescent protein GFP. The breeding of this mouse strain followed standard transgenic breeding protocols provided by Jackson Laboratory.

All the Chat-Cre;R26R-tdT;M72-GFP mice were grouped and housed in standard laboratory cages (dimensions: 32x19x12 cm), with environmental enrichment provided in the form of bedding, paper or wooden chew blocks, and plastic toys. For odor association training animals were single-housed. The cages were placed in a temperature-controlled room maintained at normal room temperature with a relative humidity of 50–60%. The mice were maintained under a reverse 12-hour light/dark cycle with ad libitum food access and an inverted bottle with easy access to water. Daily weight measurements were recorded for the training mice, and compensatory water was provided if the mice exhibited reduced water intake during training sessions. Water deprivation was carefully monitored and controlled to ensure the health of the animals throughout the seven-day experimental procedure. All these animals were kept in the Lab Animal Services Facility of Stowers Institute. All the behavior experiments mentioned in the study were done during the dark cycle. The experiment protocols that involved the mice were approved by the Institutional Animal Care and Use Committee at Stowers Institute for Medical Research and were in compliance with the NIH Guide for Care and Use of Animals.

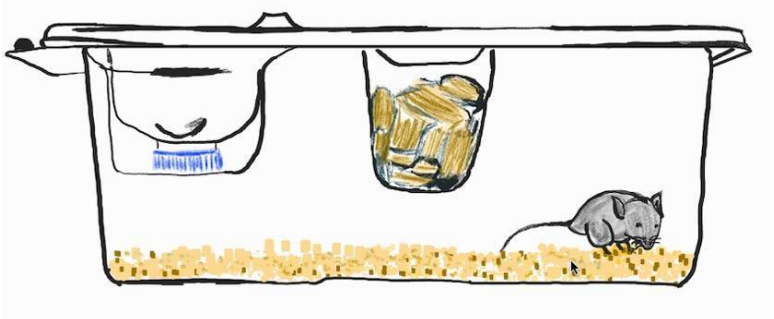


Figure 2.1. Representation of a mouse cage from the LASF facility. Adapted from(Protecting Animals and Workers in a Rodent Facility | NuAire)

2.2. Apparatus:

2.2.1. Lab Setup

Odors were delivered and controlled by an automated olfactometer(Qiu et al., 2014). It has custom lickometer software developed in the National Instrument LabVIEW programming environment. The setup consists of two odor bottles, acetophenone (ACE) and methyl butyrate (MBE), containing the respective odorants, connected to an olfactometer. The air input is separated into two different channels passing through flowmeter with complimentary input of 400mL/min following different paths. One of them proceeds towards the humidifier, while the other ends in the odor vials to carry the air mixed with the odorant, and the combined airflow is presented to the mouse. The humidifier is present to maintain the normal amount of humidity present in the natural environment. It also makes sure that continuous airflow doesn't dry up the nasal epithelium of the animal. The airflow is directed through air tubes to a port opening in an experiment box (EB) with dimensions of 27x7x8 cm (length x width x height), where a mouse is positioned at the beginning of each experiment to detect the odor. An outlet pipe is attached to work as an exhaust and throw out the odor from the EB. Additionally, a nose cone equipped with infrared (IR) beams and a detector is installed within the EB. The nose cone features a port where a mouse can lick, and the breaking of the IR beams can be monitored and counted, particularly in paradigms requiring a reward. Odors were freshly diluted in mineral oil at the desired concentration every other day. Odorants were then diluted and presented to the animals through air tubes at a maintained flow rate of 400 mL/min.

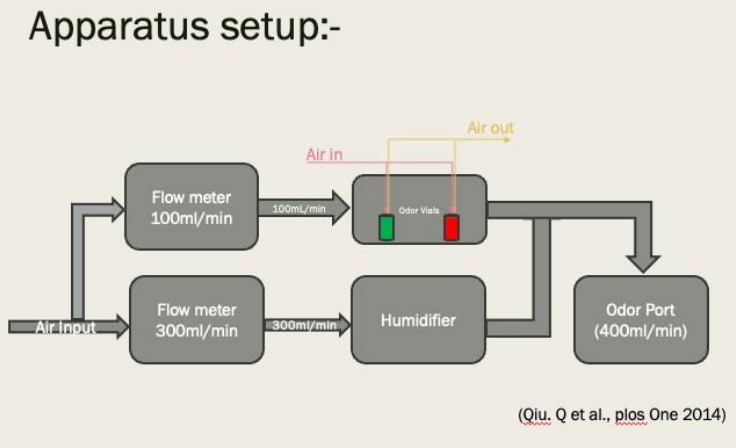


Figure 2.2. Showing the apparatus construction and odor delivery mechanism

Figure 2.3. Image of the Experiment Box for Acute and Training mice groups.

2.2.2. Odor

Acetophenone is a colorless, volatile liquid with a distinct sweet odor well known for its use in animal studies, especially relating to behavioral experiments in olfaction neuroscience. It triggers a specific neural response in animals, making it a valuable tool to study the neural circuits involved.

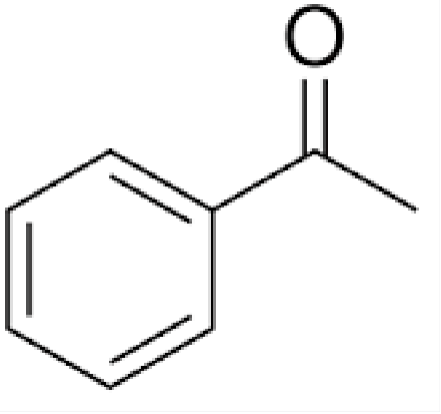


Figure 2.4. Showing the structure of the odor of Acetophenone(Favre and Powell, 2014)

We used two different concentrations of odors throughout the experiment. We dilute 150ul of 100% acetophenone in 4850ul mineral oil to make a 3*10^-2 % concentration of 5ml acetophenone odor. We later added this 500ul of this freshly prepared odor with 4500ul mineral oil using a pipette to make a 3*10^-3% concentration of 5ml acetophenone odor. We follow the same procedures for methyl butyrate. The Experiment box had odor presented with airflow, while the cage exposure mice were exposed to a passive mixture of odorants in the cage environment with controlled airflow, hence, we kept this odor a little more concentrated than the experiment box exposure.

Mice are divided into these three categories based on odor presentation methods:

i. EB environment

ii. Cage environment

iii. No Stimuli groups

2.2.2.1. Experiment Box Environment

A total of 22 mice were allocated to various experimental groups explained below in the EB environment (dimensions: 27x7x8 cm). These groups included the acute exposure (AE) group (n = 4), the no-go group (NG) (n = 3), the go group (GG) (n = 8), and the acute exposure with DREADD (AEV) group (n = 7). All the experiments were conducted within the same EB environment, ensuring consistency in testing conditions. Acetophenone is shown to mainly activate the posterior regions of the olfactory bulb. Hence, in all the mice exposed to acetophenone, non-M72 glomeruli sections were collected from the anterior regions of the bulb.

2.2.2.2. Cage Environment

A total of 9 mice were presented with odors in different experimental groups and tested in their cage environment with dimensions of 32x19x12 cm (length x width x height). This included the following groups: the chronic exposure (CE) group (n = 4), the chronic exposure group with injected virus (CEV) (n = 3), and the short-term chronic exposure (SCE) group (n = 2). Testing in the cage environment allowed for the evaluation of behaviors and responses under conditions more reflective of the animal's natural habitat. Non-M72 glomeruli sections were collected from the anterior regions of the bulb.

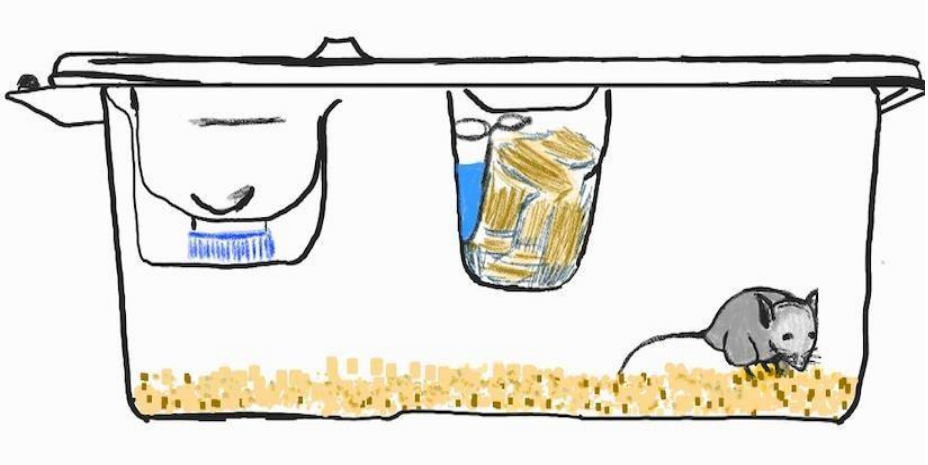


Figure 2.5. Showing the arrangement for chronic exposure group, chronic exposure with DREADD group and short-term chronic exposure for mice. Adapted from (Protecting Animals and Workers in a Rodent Facility | NuAire)

2.2.2.3. No stimulus Groups.

The groups in this section comprised a total of 9 mice and served as the control group for two different experiments. Specifically, there were two subgroups within this section:

No Stimuli Control Group (NS): This subgroup consisted of 5 mice that were 8 weeks old and had no significant or goal-associated odor exposure throughout their lives. They served as the baseline control group for assessing the effects of odor exposure in the absence of any stimuli.

Cholinergic activated no stimuli control group (NSV): This subgroup comprised 4 mice that were also 8 weeks old and had no significant external odor exposure throughout their lives. Additionally, these mice were injected with the AAV carrying excitatory DREADDs, serving as a control group to evaluate the effects of cholinergic manipulation in the absence of odor exposure.

These control groups provided essential reference points for comparing the outcomes of the experimental groups, allowing for the precise assessment of the effects of odor exposure and manipulation of TH cell populations.

2.3. Paradigm and Training

The paradigm for the experiment groups is devised on the basis of their odor presentation methods. Discussed below are the paradigms for the different conditions used in this experiment.

2.3.1. Experiment Box Exposure

Odor exposure methodology in the EB environment varied across experimental groups, including the acute exposure, positive valence go group, negative valence no-go group, and acute exposure with virus groups. All mice were subjected to two different odors, namely acetophenone and methyl butyrate, in the EB, followed by different odor exposure conditions for seven days. The concentrations of both odors used in the EB condition were maintained at 3*10^-3%. This experimental condition targeted two different responses of mice depending on passive exposure or goal-relevant exposure of the odors and therefore, expected two distinct behavioral responses based on the valence:

i. Acute
ii. Training

2.3.1.1. Acute Exposure

We introduced a total of 11 mice in this group, comprising the acute exposure (AE; n = 4) and cholinergic activated cells in acute exposure (AEV; n = 7) subgroups using virus surgery discussed later. For both the acute exposure and acute exposure with DREADDs subgroups, mice were placed in the EB environment and exposed to three continuous sessions of five minutes each of methyl butyrate and acetophenone odors, respectively. Importantly, no valence i.e., rewards or punishments were associated with the odors in this group, and the mice were allowed to inhale the odors passively. This passive exposure paradigm aimed to assess the effects of odor exposure on TH cell populations without the influence of associative learning or behavioral conditioning. For the acute with MBE exposure, we collected glomeruli sections from anteroventral or anterolateral regions. In all the cases, we tried to minimize the effect of odor exposure on the non-M72 glomeruli cases.

2.3.1.2. Training

This group had a total of 11 mice, consisting of the positive value group (GG; n = 8) and the negative value group (NG; n = 3). Prior to training, all mice underwent a pretraining phase aimed at establishing an association between the olfactory stimulus from the odor port and the water reward from the lick tube. During pretraining, mice learned to break an infrared (IR) beam located on the narrow end of EB by poking their snout into the nose cone and triggering the lick port to receive a water reward. This phase facilitated the mice's associating the odor stimulus with the subsequent reward.

Subsequently, training commenced using a go/no-go paradigm for both the positive value (GG) and negative value (NG) groups. All the animals in this group underwent water deprivation for a minimum of 12–14 hours prior to each experiment to ensure motivation during the training sessions.

During the training sessions, mice were required to lick for the rewarded odor, triggering the lick port to receive water as positive valence, while refraining from licking for any other stimuli. The experiment ran for 30-minute sessions daily over a period of 7 days. In the positive value group (GG), mice were rewarded with 5µL of water every time they poked their snout into the nose cone, broke the IR beam, and licked for the odor of Acetophenone in the lick window, making it the rewarded stimulus. No water reward was

provided for poking the nose cone and licking for methyl butyrate. Additionally, triggering the nose cone lick port for the unrewarded

"no-go" odor (methyl butyrate) resulted in the activation of a tone of frequency 4 kHz, serving as a low salience punishment for the mice. To counterbalance the positive value group, a negative value group (NG) was introduced, which was rewarded with water for MBE odor and received the auditory stimulus when poking its snout for the other odor, Acetophenone. For the go/no-go training groups with MBE exposure, we collected glomeruli sections from anteroventral or anterolateral regions.

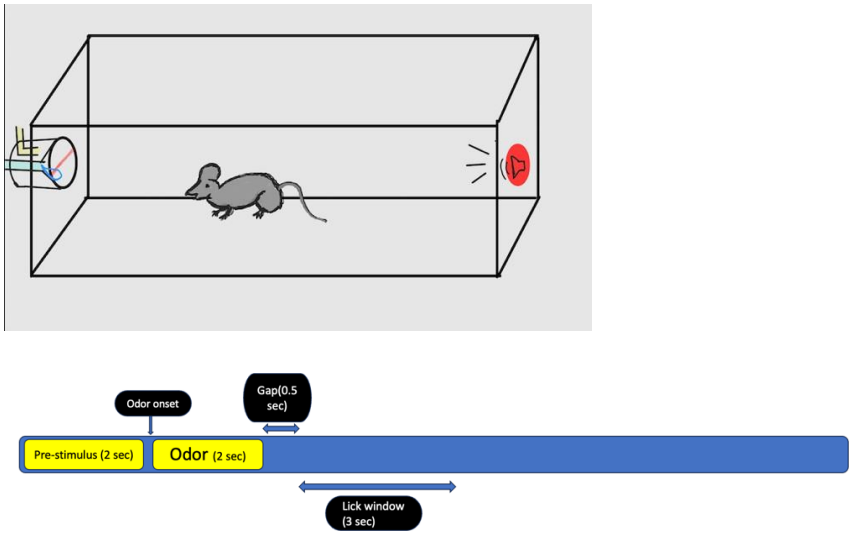


Figure 2.6. above-Schematic representation of the experiment box used for the go/no-go paradigm. bottom- Representation of the paradigm used for training the mice.

2.3.2. Cage Exposure

The remaining 9 mice were subjected to odor exposure within their cage environment under various conditions. All mice in this group were exposed to an odor concentration of 3*10^-2% acetophenone. The volatile odor was contained within a 1.5-ml uncapped centrifuge tube affixed to the feeder at the top section of the cage, ensuring that the mice could not directly access the odor source.

Two main experimental group divisions based on odor exposure durations were employed:

2.3.2.1. Chronic Exposure

Chronic Exposure Group: Mice in this group were exposed to the odor within the cage environment for a continuous duration of seven days.

Chronic Exposure with DREADD Group: Similar to the chronic exposure group, mice in this group were also exposed to the odor within the cage environment for seven days. However, they were previously injected with the AAV to assess the combined effects of chronic odor exposure and cholinergic manipulation on TH cell populations.

2.3.2.2. Short-term Chronic Exposure

Additionally, a subset of mice was assigned to a Short-Term Chronic Exposure (SCE) group (n = 2), where the odor centrifuge tube was present in the cage for only 24 hours. Therefore, mice in this group were exposed to the odor of acetophenone for a lesser duration, in contrast to the 7-day exposure duration experienced by all other experimental groups.

2.4. Water Deprivation

Water deprivation was implemented for animals participating in the training paradigms only within the EB, the go group (GG), and the no-go group (NG). Rest all the other cages were equipped with inverted water bottles with a hole at the bottom to provide free access to water for the animals.

The mice groups were ready for experimentation when the animals reached 8 weeks of age. Prior to the start of training, animals in the GG and NG groups underwent a period of water deprivation lasting 2 days. During this period, free access to water was removed, and each animal was provided with a constant amount of 1.5 ml of water per day to maintain hydration and motivate the animals for training. During the training, the mice were provided water in the EB and compensated by giving extra water totaling 1 ml if they didn’t perform well and couldn’t get sufficient water.

Daily monitoring of animal weight was conducted following each training session. Compensatory water was provided based on the previous day's body weight to ensure that animals did not experience excessive weight loss. Animals were checked daily to ensure that their weight did not decrease by more than 20 percent of their total body weight, ensuring their health and welfare throughout the experiment.

2.5. Virus Injection

AAV2-hSyn-DIO-hM3D(Gq)-mCitrine, 350nL, (titer: 2*10^13), (Addgene viral prep) As the animals used were 8 weeks old and had an average weight of around 20 grams, AAV2-hSyn-DIO-hM3D(Gq)-mCitrine was surgically injected into their brains. Previous studies have highlighted the horizontal limb of the diagonal band of Broca (HDB) as a significant source of acetylcholine in the olfactory bulb(Arneodo et al., 2018). Therefore, the viral vectors were targeted for injection into the HDB area using stereotaxic coordinates with respect to the bregma.

CNO injection (2.5mg/kg body weight) is administered nearly 20 minutes before the odor exposure experiment for acute exposure with DREADD every day in an EB environment. No stimulus with virus and Chronic exposure with virus group mice are injected with CNO every day in a cage environment.

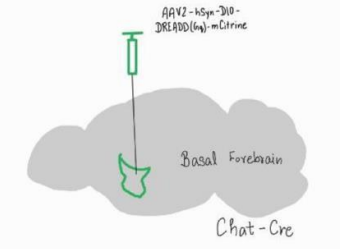


Figure 2.7. A representation of image showing the location of AAV injection (Adopted from (Zhu et al., 2023)).

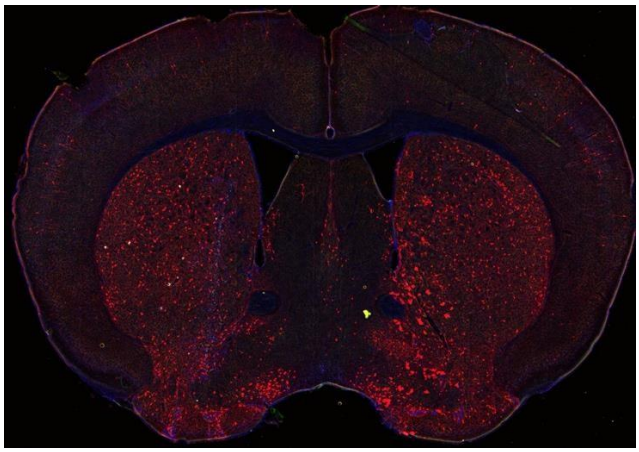


Figure 2.8. Microscopy image of a 10x magnification image of the HDB area where the virus was injected.

2.5.1. Basic Stereotaxic Surgery Methods

A stereotaxic apparatus was used to perform the surgery, which was disinfected using 70% ethanol. The animal was checked for reflexive movements by the toe-pinch reflex. The mice were initially anesthetized using Ketamin/Xylazine (Patterson Veterinary,Vedco) (0.1 ml/10 g body weight) in preparation for the stereotaxic injections. They were given an analgesic buprenorphine dosage (0.1 mL/30 g body weight). The animals were transferred to a stereotaxic frame. Mice were head-fixed to a stereotaxic frame using ear bars, and we made sure that the animals were anesthetized for the whole duration of the surgery. The right and left ear

bars and bite bars of the stereotaxic apparatus were aligned to the same height. Following that, the animal is mounted, the ear bars are inserted into the ears, the upper tooth of the animal is placed in the bite bar, and the nose bar is placed upon its snout to keep the head fixed throughout surgery.

A heating pad set at 37C was placed over the base of the stereotaxic apparatus to maintain the body temperature of the animal during surgery. Eye ointment was applied to keep the eyes lubricated and prevent them from drying out during the procedure. Prior to the incision, the scalp was cleanly shaved, and the surgical site was meticulously cleaned by alternating three rounds of iodine (poviodine swab sticks) followed by alcohol swab sticks to minimize the risk of infection. All surgical instruments were heat sterilized and cleaned with 70% alcohol to maintain sterility.

Using a sterile surgical blade, a midline incision was made on the scalp to expose the skull surface, centrally positioned above the olfactory bulb. A window was then created to expose the skull, followed by careful removal of the pericranium connective tissue using forceps. Alcohol was applied immediately to the exposed skull surface to prevent dehydration, followed by air drying.

Next, the skull was thinned above the horizontal limb of the diagonal band of the Broca (HDB) region using a high-speed dental drill, taking care to avoid damage to the dura mater. Coordinates for the viral injection were determined based on the Angle2 software, with reference to the bregma and the skull surface. The coordinates for the HDB region were set as follows: anterior-posterior (AP) = -0.59 mm, medial-lateral (ML) =±0.74 mm, and dorsal-ventral (DV) = -5.41 mm from the skull surface.

The injections were carried out using an injector Nanoliter 2010 (World Precision Instruments) that was mounted on the stereotaxic frame. It was delivered through a pipette injection made up of glass having a very narrow taper length and a very thin tip diameter. The stereotaxic injection was brought to the target coordinates. By lowering the pipette using a micromanipulator to reach the HDB area, the skull is penetrated. The viral vector was then injected at a controlled rate using a nanoliter pump. The pipette was kept in place for 5 minutes. Later, we started injecting at a slow rate with approx—10 seconds gaps between two subsequent injections. The pipette was left in place for 10 minutes after the injection to avoid viral backflow along the injection tract and to allow for adequate diffusion of the viral vector before being lifted 3-5 mm for subsequent injections. This process was repeated five times on both sides of the hemispheres, injecting a total of 350 nL bilaterally.

Finally, the incision site was sutured using the tissue adhesive Vetbond and allowed to dry. After 5 minutes, the nose clamp, bite bar, and ear bars were removed, and the animal was placed on a heating pad at an optimal temperature to facilitate recovery. Post-operative care included monitoring of general health and behavior for the next 10 days. We waited at least 3

weeks for the AAV to express prior to any odor exposure. This surgical injection was performed on both male and female mice.

A total of 14 animals were injected with AAV. No stimulus control with virus injection (NSV): 4 mice were included in this group. They did not have prior exposure to odor but received 2.5mg/kg body weight of clozapine-N-oxide (CNO) daily without exposure to any odor.

Chronic acetophenone exposure with the DREADD AAV virus group (CEV): 3 mice were included in this group. They received seven days of chronic cage exposure to 1 ml of 3*10-2% acetophenone daily, similar to the CE group. Odors were renewed every alternate day.

Acute exposure (AE) in behavioral setup: 6 mice were included in this group. They were exposed to acute exposure to acetophenone and methyl butyrate alternatively three times, totaling 30 minutes.

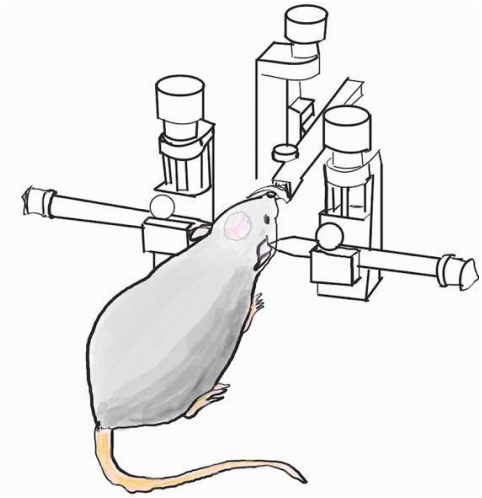


Figure 2.9. A schematic representation of a stereotaxic surgery.

2.6. Perfusion and Dissection

The animals were considered ready for experimentation upon reaching 8 weeks of age. Animals in the EB group, including the training groups, acute exposure (AE), and chronic exposure (CE) groups, were sacrificed after 7 days of odor exposure, except for the 1-day passive odor mice, which were sacrificed immediately after 24 hours of exposure. The no-stimulus (NS) control group was perfused when they reached around 8 weeks of age without any odor exposure.
To begin the perfusion process, the animals were deeply anesthetized using urethane at a concentration of 0.15ml/10gm body weight and checked for the toe-pinch reflex to ensure complete anesthesia. An incision was made to expose the heart, and the animals were then perfused transcardially with 1x phosphate-buffered saline (80g NaCl, 2g KCl, 14.4 g Sodium Biphophate, 2.4 g Potassium Dihydrogen Phosphate, PH = 7.4 ± 0.02). This was followed by 4% paraformaldehyde (PFA) in PBS. Following perfusion, the brains were carefully

dissected and placed in a 4% PFA solution for 24 hours at a temperature of 4°C to further fixate the tissues and preserve them for subsequent sectioning.

2.7. Sectioning

The brain was extracted from the PFA solution and rinsed in PBS for 30 minutes to remove excess fixative. Subsequently, the brain was carefully mounted in low melting agarose and allowed to cool until hardened. Using a vibratome (Leica VT 1000s), we collected the coronal sections of the olfactory bulb by cutting them at a thickness of 60 µm. The sections were collected in 1x PBS within the vibratome sectioning chamber and then directly mounted onto slides for further analysis.

2.8. Analysis

Olfactory bulb M72 and non-M72 glomeruli sections were collected and analyzed.

2.8.1. Immunohistochemistry and Staining

After the animals were perfused, coronal sections of the olfactory bulb were collected on slides. These slides were subjected to antigen retrieval by microwaving for 45 seconds in citrate buffer (pH 6.0). Antigen retrieval helps to improve the accessibility of antigens within tissue sections for antibody binding by reversing the cross-linking of proteins that may occur during tissue fixation. Following this, the slides were allowed to cool for 30 minutes.
Subsequently, the slides underwent a 30-minute wash in PBST (phosphate-buffered saline with 0.1% triton-X) to increase permeabilization and reduce non-specific binding. Primary antibodies against TH (rabbit anti-tyrosine hydroxylase, host rabbit, source: Millipore), GFP (chicken anti-GFP, host: chicken, source: Abcam), and td-tomato (goat anti- tdTomato, host:goat, source: Origene) were prepared at a 1:1000 dilution in a solution containing 90% PBST, 5% DMSO, and 5% donkey serum. The olfactory bulb slides containing the sections were then incubated in the primary antibody mixture for 24 hours at room temperature.

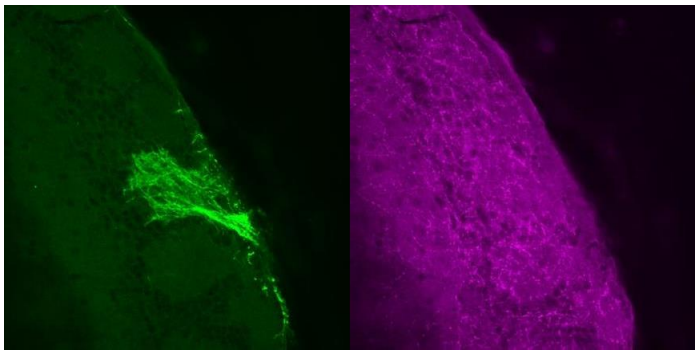
After incubation, the sections underwent three washes with PBS for 30 minutes each, followed by the introduction of secondary antibodies. Secondary antibodies, including Donkey anti-Chicken Alexa 488 (Jackson Immunology), Donkey anti-Rabbit Alexa 640 (source: Invitrogen), and Donkey anti-Goat 546 (Invitrogen), were applied at a 1:1000 dilution in the

same solution used for the primary antibodies. Additionally, DAPI was diluted to a concentration of 1:1000 in the same solution to stain nuclei.

The sections were then kept in the secondary antibody mixture for 24 hours. Following another three washes with PBS for 30 minutes each, the sections were mounted onto glass slides using mounting media. A coverslip was applied, and the slides were stored in a refrigerator at room temperature. A mounting medium was used to preserve fluorescence over time.
Coronal sections from the mice with AAV injections were also subjected to immunostaining using the same procedure, with primary antibodies against TH, GFP, and td-Tomato.

2.8.2. Microscopy

Imaging of the olfactory bulb sections was carried out using a Nikon spinning disc microscope equipped with a 40x water immersion objective lens. The specific microscope model used was the Nikon AT-AT.
Due to the complex and irregular three-dimensional structure of the glomeruli and the presence of numerous fibers marked with GFP, images were captured in z-stacks. Care was taken to comprehensively cover the spatial arrangement of glomeruli and cellular components within the tissue. Each glomerulus spanned across multiple slides, typically ranging from 2 to 4 slides. Z-stack images were acquired at multiple focal planes to capture the entire depth of the tissue, allowing for detailed analysis of the spatial distribution of labeled cells and structures. The confirmation of the DREADD injection was done by imaging the cholinergic cells in the HDB region using an LSM 800 microscope.



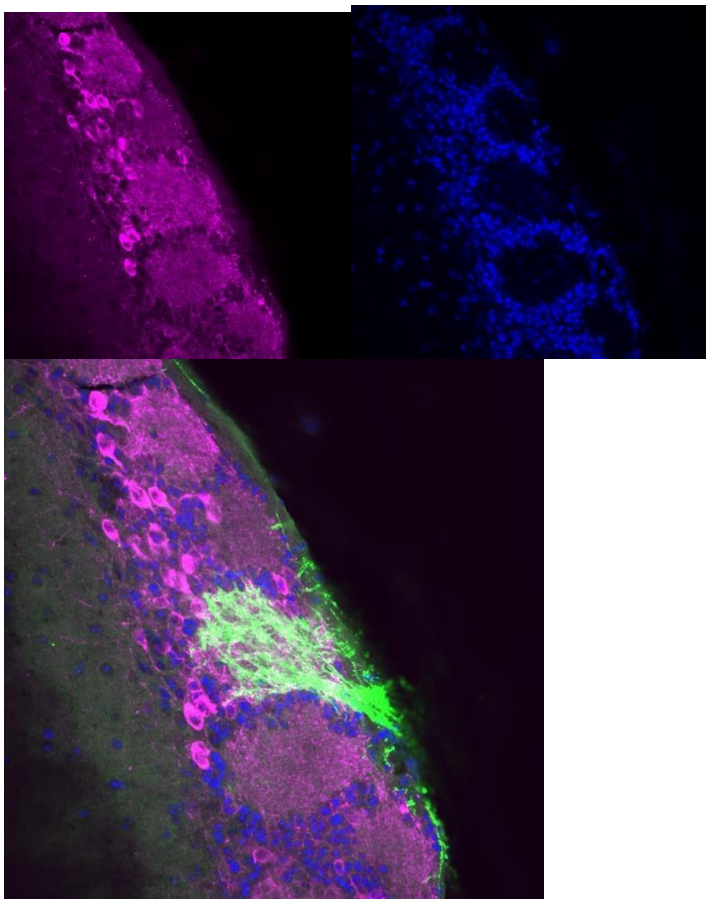


Figure 2.10. This image shows a 40x magnification image. First- GFP, second- tdTomato, third- TH, fourth-DAPI, fifth- composite

2.8.3. Quantification

Fiji:

It is an open-source platform based on ImageJ (Fiji)—source:NIH. Images were acquired in the '.nd2' format and subsequently converted to the '.tif' format for further analysis using Fiji software. Fiji, an open-source image processing package based on ImageJ, was utilized for quantitative analysis and visualization of the immunostained sections. It was an essential tool used in our study for M72 glomeruli and non-M72 image analysis.

Origin Pro:

All the plots were made from Origin Pro from Origin Lab(https://www.originlab.com/Origin)

Quantification of TH cells around M72 and Non-M72 glomeruli Sections :

Quantification of the number of TH cells surrounding the M72 glomeruli and non-M72 glomeruli was performed using microscopy images stained with anti-TH, anti-GFP, and anti-tdtomato antibodies. These antibodies were chosen to enable clear differentiation between TH-positive cells and glomeruli labeled with GFP. Vibratome sections, each with a thickness of 60 µm, were spread across 2-4 slides, resulting in different distributions of TH cell populations around the glomeruli. From multiple sections containing glomeruli, the two largest volume sections were selected for analysis of glomerular volume and TH cell counts. Serial sections were systematically examined for the TH cells that were in immediate proximity to the glomeruli of interest and were included in the analysis. These glomeruli exhibit distinct fiber patterns surrounding them and the non-spherical nature of glomeruli made it complicated to accurately calculate the precise volume. Hence, for the volume and density, a close estimation of the volume was made by integrating the areas across full z-stacks to get an approximate volume.

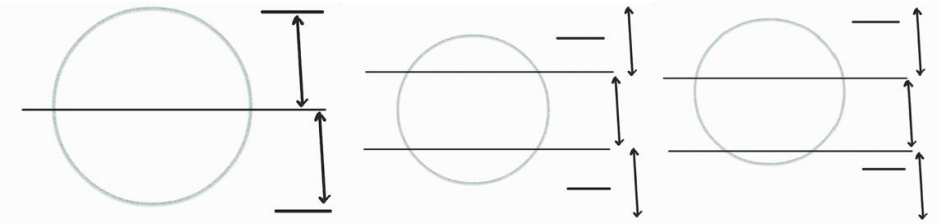


Figure 2.11. A schematic representation showing the irregular collection of glomeruli on slides.

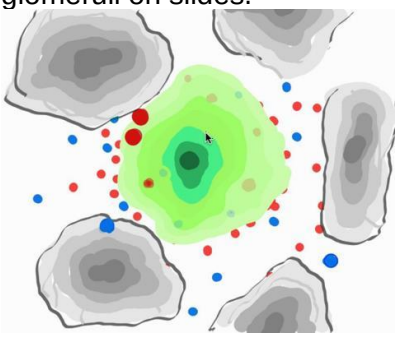


Figure 2.12. Representation of M72 glomeruli and TH interneurons surrounding it (Braubach et al., 2018).

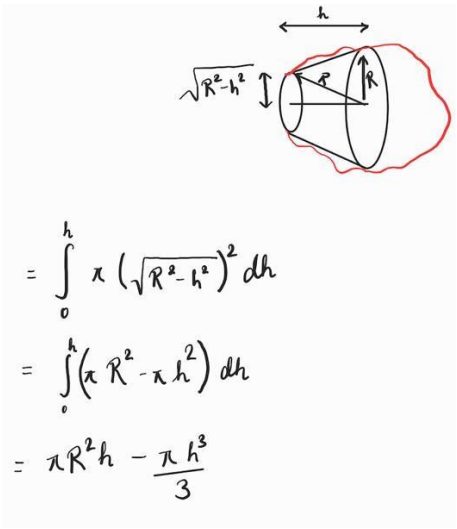


Figure 2.13. A representation showing the approximation of volume calculation of an irregular glomeruli.

2.8.4. Storage of Data

MS-Excel Data Storage:

The storage of M72 and non-M72 glomeruli volume, density, and TH counts was the focus of the experiment. Glomeruli having complex structures with irregular shapes presented challenges in quantification. The number of TH cells observed varied across different experimental conditions, reflecting the influence of odor exposure and other factors. The majority of TH cells were very closely located near the beginning of the glomeruli section, and very few were located far toward the center of the section on the periphery. The midpoint area was calculated along with the height of the sections. This was then approximated to the closest hemisphere. A glomerulus was estimated to have two hemispheric volumes depending on the location of the midpoints (largest area).

Spreadsheets were constructed in Microsoft Excel to record the number of TH cells surrounding each glomerulus, along with the corresponding areas of the starting, middle, and ending z-stacks of the sections, denoting the height of the section from the center. We calculated volume, TH counts, and finally the density. This facilitated further analysis and comparison across experimental conditions.

Mouse No.	TH_count	start area	start stack	mid area	mid stack	end area	end stack	Stack_height C1	Stack_height C2	Height C1	Height C2	volume C1	Volume C2	volume total	Comb_vol	TH_comb	Density
809334-1_1	18	3694.05	1	6675.791	34	6407.79	46	33	12	19.8	7.2	124056	47675.03	171731.0538	177205.9	23	0.00013
809334-1-2	5	264.08	58	1322.003	65	1322.003	65	7	0	4.2	0	5474.867	0	5474.86716			

2.14. Showing the data collection and storage for the glomeruli.

2.8.5. Statistical Analysis

Statistical comparisons were conducted by a two-tailed Student's t-test. Significance was established at $P < 0.05$. Cell counts, glomerular volumes, and cell densities around glomeruli are presented as mean ± SD and are derived from at least 8 different glomeruli, combining two concurrent M72 glomeruli sections (near M72 glomeruli for M72 group and at random anterior location far away from M72 glomeruli for Non M72 group) for each animal. All statistical analyses were performed on Origin Pro (Origin Lab).

Chapter 3 Results

3.1. Baseline Assessment of TH Interneurons in Mice

The glomeruli layer in the OB is shown to have a presence of tyrosine hydroxylase (TH) interneurons in the olfactory bulb (Bonzano et al., 2014, 2016). Studies suggest their involvement in olfactory processes such as learning and odor discrimination. In adult mice, these interneurons are randomly distributed throughout the glomeruli layer. We sought to examine the baseline distribution of TH interneurons in the olfactory bulb of mice under no stimulus conditions. To achieve this, we compare the TH Interneurons surrounding the M72 Glomeruli known for their specificity to acetophenone odor and any other randomly selected non-M72, both in the same five mice.

We did not observe any M72 glomeruli with higher TH expression when compared to non-M72 glomeruli (Figure 3.1, left). Overall, our data analysis revealed no significant difference in the number of TH cell counts between the two groups (p-value = 0.09, two-tailed student's t-test). The mean count of TH cells surrounding M72 glomeruli was 21.72 ± 7.2. This was comparable to the mean count of TH surrounding a random non-M72 glomeruli (27.92 ± 12).

We wanted to confirm if the volumes of the glomeruli might significantly vary leading to higher TH interneuron count depending on the size and volume of the glomeruli. On testing, the volumes of M72 and non-M72 had no significant differences (Figure 3.1, center), with a p-value of 0.63 (two-tailed student's t-test). The mean volumes for M72 glomeruli were 183987.33595 ± 72449.75168, whereas for non-M72 glomeruli was 201731.8017 ± 135123.99549.

Specifically, we wanted to confirm if the density distribution for TH interneurons was statistically insignificant (Figure 3.1, right). On statistical testing, we observed no changes in the densities of any of the animals in the group with a p-value of 0.1 (two-tailed Student's t-test). The mean density of TH for M72 glomeruli was 1.27E-4 ± 7.03E-5, while for the randomly picked non-M72 groups was 1.77E-4 ± 9.7E-5 and, hence proving no statistical significance.

This observation for M72 and non-M72 glomeruli supports our hypothesis that in the absence of odor exposure and lays a groundwork for further experimentation. The randomly spread distribution of TH interneurons shows unbiased odor perception in the olfactory bulb. All these findings show that under basal conditions and with no external olfactory stimuli, we found no detectable differential distribution of TH interneurons.

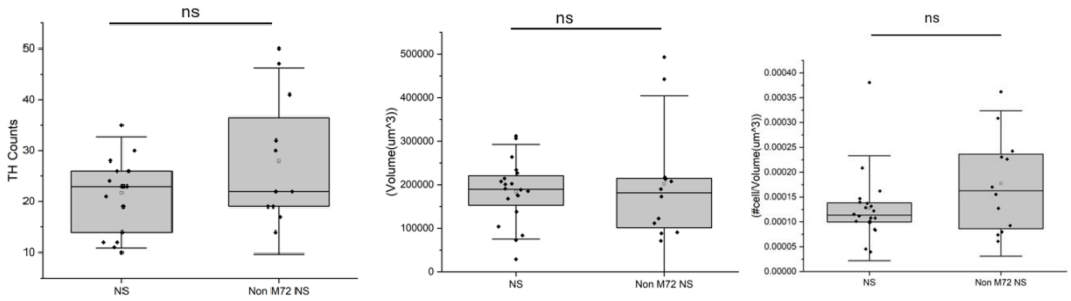


Figure 3.1. Plots showing the comparison between M72 glomeruli of no stimulus (NS) and Non M72 glomeruli of no stimulus mice (Non M72 NS). Left- shows the TH (p-value = 0.09), center- shows the volume for M72 and non-M72 glomeruli (p-value 0.63), right- shows the density of the TH population around the glomeruli (p-value 0.1)

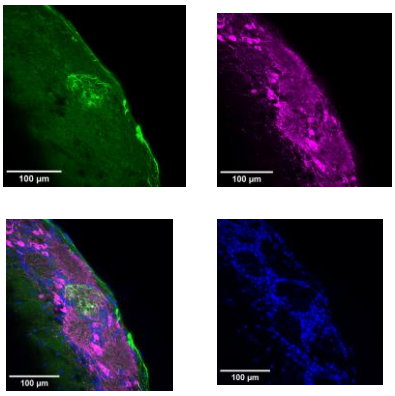


Fig3.2: 40x spinning Disk confocal imagining for No stimuli groups.

3.2. To test the odor influence on the glomeruli

To investigate the effect of passively exposed conditions on the mice to understand the influence of exposures to odors, we conducted an experiment and exposed the mice chronically to odors of acetophenone for continuous seven days. To understand the plasticity that occurred due to the chronic odor exposure, we compared them with the non-M72 glomeruli in the same mice. We quantify to analyze the number of TH populations, volumes, and density.

The analysis revealed a significant increase in the number of TH cells between the two groups, chronic exposure M72 vs. chronic exposure non-M72 (p-value = 0.03, two-tailed student's t-test). Specifically, the mean count of TH cells surrounding M72 glomeruli (35.6 ± 6.6) was significantly higher (Figure 3.2, left) than the non-M72 surrounding a random non-M72 glomeruli (26.25 ± 14.6). Also, we could not find any difference between the population of TH interneurons of this group and the M72 or non-M72 TH population of the no-stimulus groups. This conclusion states that the odor strongly activates the M72 glomeruli.

On the contrary, the volumes of M72 and non-M72 glomeruli for chronic exposure groups also showed no significant differences (Figure 3.2, center), with p-value= 0.13 (two-tailed student's t-test), which reveals that there is no increment in the volume of the glomeruli due to the odor exposure in seven days. The mean volumes for M72 glomeruli were 182292.53471 ± 86455.3319, whereas, for non-M72 glomeruli were 257673.11205 ± 151840.38445. The consistency in the volume of this group demonstrated that even with the heightened exposures to odors, the TH population increases, which might indicate

towards density increase.

We calculated the density distribution for the abovementioned group and found them statistically significant too (Figure 3.2, right), with p-value = 0.006 (two-tailed student's t-test). The mean densities of chronic exposure M72 glomeruli were 2.36882E-4±1.06717E-4, whereas the non-M72 groups for chronic exposure were 1.16104E-4±5.36803E-5 and hence showed statistical significance. The density of TH interneurons increased due to the involvement of the M72 glomerulus in odor processing.

The lack of increase in TH populations in the non-M72 glomeruli highlights that experimental manipulations like acetophenone exposure selectively impact the targeted M72 glomeruli. These findings conclude that the duration of habituation may influence neurogenesis processes within the olfactory bulb. These findings show the importance of habituation duration in modulating TH cell populations and density within the olfactory bulb.

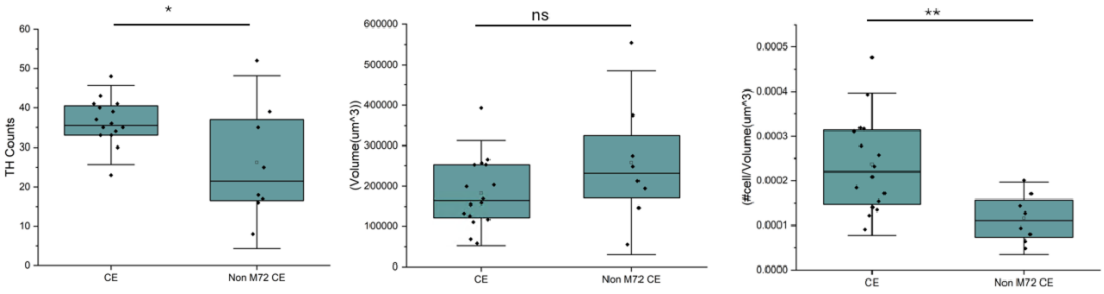


Figure 3.3. Plots showing the comparison between M72 glomeruli of the Chronic Exposure group (CE) and Non M72 glomeruli of the Chronic exposure group. Left- shows the selective increase in TH counts (p-value = 0.03), center- shows the volume for M72 and Non-M72 glomeruli (p-value= 0.13), right- shows the density of the TH population around the glomeruli (p-value = 0.006). Error bars indicate standard deviation.

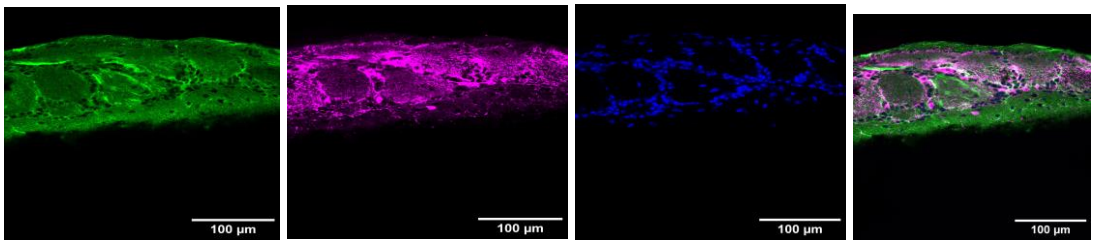


Fig 3.4: 40x confocal microscopy for Chronic exposure groups.

3.3. Effects of odor exposure duration on TH INTERNEURONS

After previously demonstrating the baseline and extreme conditions for no odor exposure and chronic odor exposure, respectively, we tested the influence of different durations of passive odor exposure on TH cells in the olfactory bulb. To understand the influence of chronic exposures on mice, we build a simple olfactory habituation paradigm to note the mice's odor investigation behaviors in the natural environment. We look into acute passive exposure (AE) vs. chronic passive exposure (CE) to get an understanding of the neuronal plasticity that occurs in these cases. The primary objective of this experiment was to assess the impact of temporary and permanent habituation of glomeruli on TH cell counts and density.

The change in the neural circuits would indicate the activation of the glomeruli. To investigate the effects of passive odor exposure on tyrosine hydroxylase (TH) cell counts and density in the olfactory bulb, we compared the TH cell populations for acute exposure and chronic exposure in the EB and cage environment paradigm, respectively, in mice. Acute exposure was exposing the mice to acetophenone and methyl butyrate for 30 minutes per session, three times daily, for seven days. For chronic exposure, the mice were continuously exposed to acetophenone over seven days.

As hypothesized, the results demonstrated a significant difference between the two exposure conditions. Specifically, mice subjected to acute passive exposure exhibited lower TH cell counts when compared to those exposed chronically (Figure 3.3. left). Accordingly, we found a significant difference in the number of TH cells between the two groups (p-value = 0.0012, two-tailed student's t-test). The mean counts of TH cells surrounding M72 glomeruli for CE (35.6875±6.66052) were significantly heightened than the M72 surrounding AE glomeruli (23.6666±11.61075). The statistical tests show an approximately 26% increment in the TH populations of the chronic exposure group as compared to the acute exposure group. Similar to previous groups, we tested out if there lies any increment in the volumes between these groups. The volumes of chronic

exposure M72 and acute exposure M72 had no significant differences with p-value= 0.38 (two-tailed student's t-test), which reveals that there is no increment in the volume of the glomeruli due to the odor exposure in seven days (Figure 3.3, center). The mean volumes for CE M72 glomeruli were 182292.53471±86455.3319, whereas the mean volumes for non-M72 glomeruli were 205650.89109±54950.99291. Not having any increase in volume points at an increase in density for both the groups, being higher for chronic exposure groups due to higher TH counts.

Next, we determined the density distribution and noted that it was also statistically significant, with p-Val= 1.41156E-4 (two-tailed student's t-test). The mean densities of CE M72 glomeruli were 2.36882E-4±1.06717E-4, whereas, for the AE M72 groups, they were 1.37205E-4±6.85084E-5 and hence showed statistical significance (Figure 3.3, right). This result concludes that permanent habituation of glomeruli leads to an increase in TH cell counts and density.

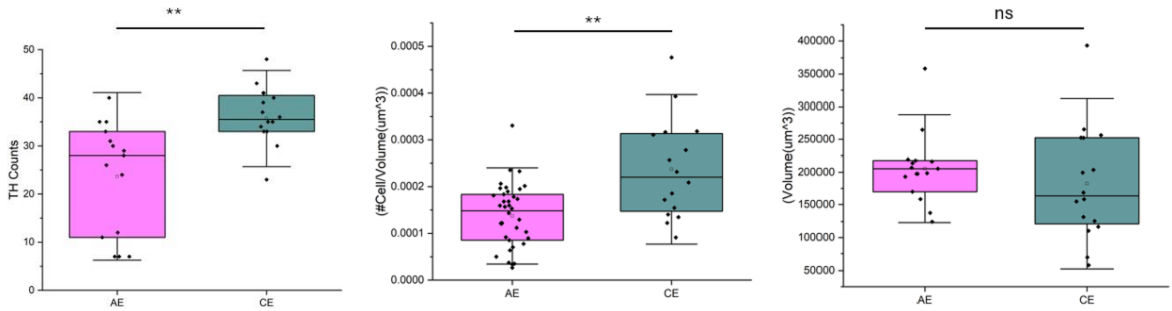


Figure 3.5. Plots showing the comparison between M72 glomeruli of the acute exposure group (AE) and chronic exposure groups (CE). Left- shows the increase in TH counts (p-value = 0.0012), center- shows a non-significant increase in the volume for M72 glomeruli (p-value= 0. 38), right- shows the density of the TH population around the glomeruli (p-Val= 1.41156E-4).

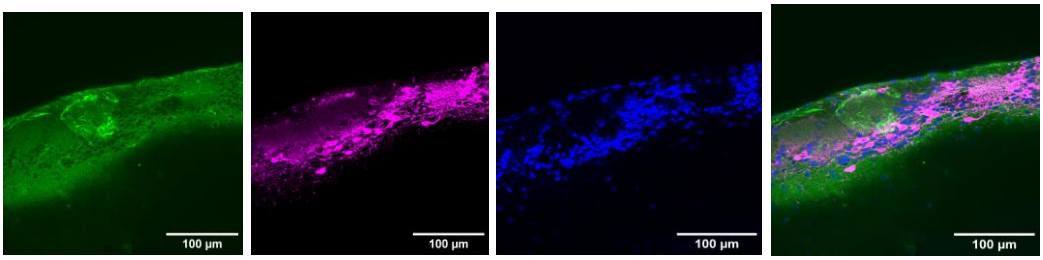


Fig 3.6: 40x imaging spinning disk imaging for Acute exposure groups.

3.4. Impact of goal relevance and training on TH Populations in Mice

The above conditions tested the control, acute, and chronic exposure in the mice, where the mice were freely exposed to odor, where the odors were not goal-relevant and had no importance attached to them. Further, we wanted to investigate the neuronal changes that are evoked in the GL when the odor has a reward attached to it. To investigate the influence of odor relevance and training on tyrosine hydroxylase (TH) interneuron

populations in the olfactory bulb, mice were subjected to a go/no-go paradigm where the odor stimulus acetophenone was positively associated with the water reward. Conversely, the odor methyl butyrate was negatively associated with a tone as a punishment, which promoted water rewards upon accurate licking of the lick port in the lick window. To counterbalance the experimental design, we designated acetophenone as the no-go stimulus, withholding water rewards upon licking for acetophenone and setting off the punishment tone for the negative valence no-go NG group.

Following analysis of TH cell counts in the olfactory bulb, it was observed that mice trained with acetophenone exhibited higher TH cell counts compared to the acute exposure (AE) group (Figure 3.4, left). The analysis allowed us to directly show a significant difference in the number of TH cells between the two groups. A two-tailed student's t-test helped us reveal the p-value= 0.03, and the mean count of TH cells surrounding M72 glomeruli for positive valence go (GG) group (29.59259 ± 5.95663), which was significantly greater than the M72 surrounding acute exposure glomeruli (23.66667 ± 11.61075), which had approximately similar duration and setup for odor exposure. This indicated the relevance of odor exposure to the TH population in the M72 glomeruli region. We characterized that this might be because it would play a role in reward-olfactory association and learning.

We were curious if the volumes of the glomeruli increased due to the odor being relevant. We looked into the data and found no significant differences between the two groups GG and AE (Figure 3.4, center) with p-value = 0.44 (two-tailed student's t-test). Surprisingly, this indicates that there is no increment in the volume of the glomeruli due to the odor exposure. The data for the mean volumes for GG M72 glomeruli were 191211.87312 ± 61156.09556, whereas for non-M72 glomeruli were observed to be 205650.89109 ± 54950.99291.

Also, a statistically significant increase was seen in the densities of the two (Figure 3.4, right), with a p-value of 0.04 (two-tailed student's t-test). The mean densities of GG M72 glomeruli were 1.75477E-4 ± 7.16488E-5, whereas the acute exposure M72 groups, they were 1.27511E-4 ± 7.24255E-5 and hence showed a statistically significant increase.

The observed increase in TH cell counts in the trained group may suggest a positive correlation between odor relevance and dopaminergic neuron activity. We assume acetophenone being the positive valence odor in the go/no-go paradigm possibly triggers dopaminergic responses associated with reward processing and might reinforce learning. The TH populations might be modulated by the salience of the olfactory stimuli, which might facilitate odor perception and discrimination in reward-based learning tasks.

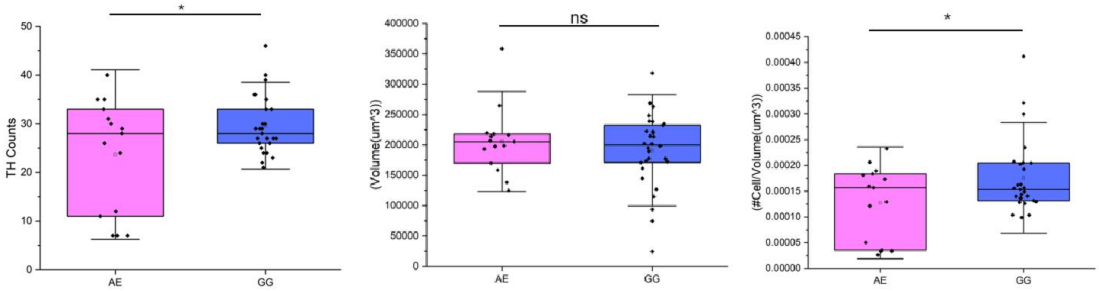


Figure 3.7:. Plots showing the comparison between M72 glomeruli of acute exposure group (AE), and training positive value go group (GG). Left- shows the TH counts (p-value= 0.03), center- shows the volume for M72 glomeruli (p-value = 0.44), and right- shows the density of the TH population around the glomeruli (p-value 0.04).

The results previously shown were based on valence. Next, we also did statistical testing for the negative valence no-go group keeping a low measure for salience. The analysis revealed no significant difference in the number of TH cells between the two groups (Figure 3.5, left), no-go vs. acute exposure (p-value = 0.6, two-tailed-Student's t-test) due to a significant reduction in the salience of the olfactory stimulus. To confirm that the no-go olfactory stimulus is not important for the mice and hence doesn't require further processing, we checked the mean count of TH cells (Figure 3.5, left) surrounding M72 glomeruli for No-Go (26.16667±13.81907) was not significantly different than the M72 surrounding Acute Exposure glomeruli (23.66667±11.61075). However, these results need further testing due to the limited mouse participation in this experiment, hence, can be concluded this with further experimentation. Following the previous result, the volumes of NG M72 and AE M72 had no significant change with p-value= 0.41 (two tailed student's t-test), which reveals that there is no enhancement in the volume of the glomeruli due to the odor exposure in the testing period of seven days (Figure 3.5, center). The mean volumes for NG M72 glomeruli were 183589.26028 ± 53131.42779, whereas for non-M72 glomeruli, they were noted to be 183589.26028 ± 53131.42779. The density distribution was statistically not significant (Figure 3.5, right), with p-value= 0.89 (two-tailed-Student's t-test). Specifically, the mean densities of NG M72 glomeruli were 1.41298E-4 ± 5.70671E-5, whereas, for the AE M72 groups, they were 1.37205E-4 ± 6.85084E-5 and hence, showed no statistical changes.

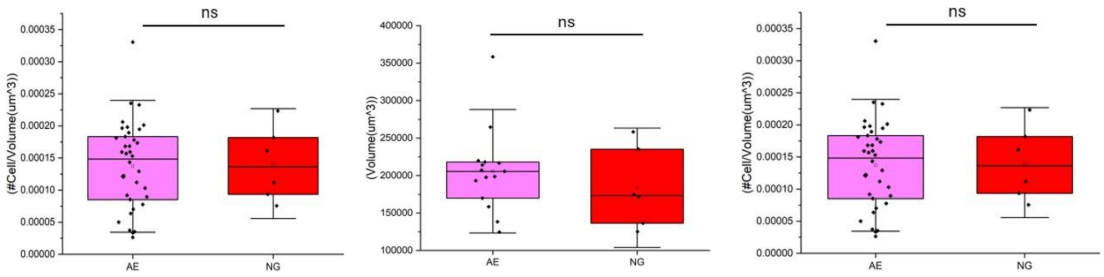


Figure 3.8:. Plots showing the comparison between M72 glomeruli of the acute exposure group (AE) and training negative value no-go group (NG). Left- shows the TH counts (p-value = 0.6), center- shows the volume for M72 glomeruli (with p-value= 0.41), and right- shows the density of the TH population around the glomeruli (p-Val= 0.89).

Also, the Go-group shows a significant difference with the NS group but not with the CE group. This result suggests that factors other than simple exposure and relevance may influence the TH populations in the OB. Duration of odor exposure, individual variability in response to odor stimuli, or the adaptive nature of the allocation of neuronal resources, including TH populations, could all be variables that couldn't be accounted for in this experimental design. The salience of odors differs between chronic and trained conditions. This might also happen as passive exposure involves the presentation of odors without any explicit behavioral task, whereas the training go/no-go paradigm requires active engagement and discrimination. We assume that these two processes might have different modes of activation, leading to different TH populations. We assume that chronic exposure might lead to sustained dopaminergic activation, resulting in a greater TH count over time when compared to the training-induced dopaminergic responses that are task-specific and temporary. We need to do further experimentation to explain the specific mechanisms underlying these divergent effects and their implications for olfactory processing and behavior.

We collected non-M72 glomeruli and M72 glomeruli sections for all the mice in all the groups to keep the data consistent and concrete. We saw that the TH interneurons are randomly distributed throughout the bulb in the wild-type mouse.

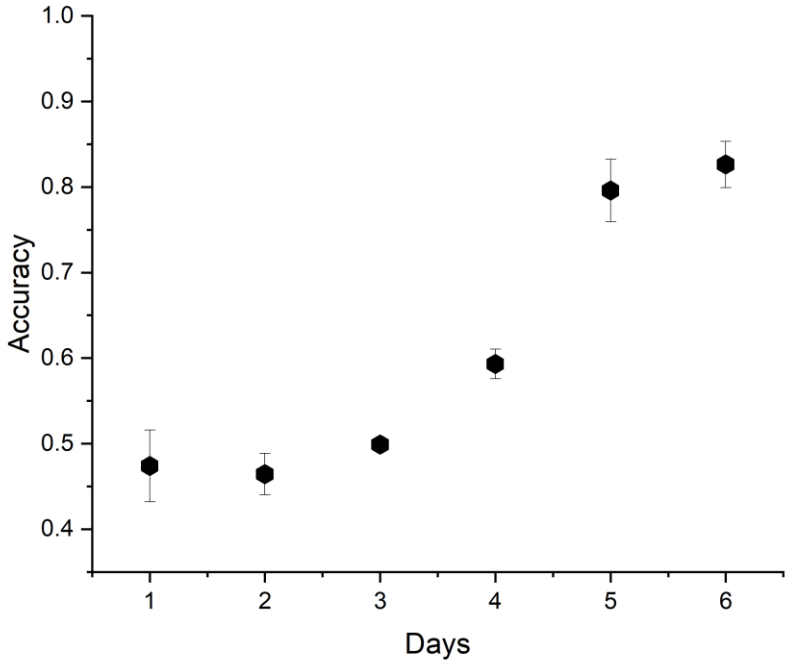


Fig 3.9: Showing the training accuracy curve for the training groups. The error bars show the Standard deviation.

3.5. Disinhibition of Glomeruli influences TH interneurons.

The conditions where mice are exposed to prolonged odor exposure would result in the habituation of the M72 glomeruli, leading to a saturated TH cell population. Hence, to investigate the role of odor exposure in modulating TH cell populations in the olfactory bulb, we employed a strategy to dishabituate glomeruli using neural activation by injecting designer receptors exclusively activated by designer drugs (DREADDq) in the basal forebrain of mice. Through this, we aim to assess whether disinhibition of glomeruli leads to an increase in TH cell populations in the GL regions, providing us with a baseline for the groups exposed to odors in this condition. To quantify this, we analyzed the cholinergic activated no stimulus groups with the control no stimulus group to compare the enhancement in the TH population in the Glomerular layer surrounding the M72 glomeruli. The analysis highlighted that there was a significant effect of the introduction of viral injections in the number of TH cells between the two groups (Figure 3.6, left), M72 glomeruli of NSV and M72 NS (p-value <0.0001, two-tailed student's t-test). The quantification of the TH data showed the mean count of TH cells surrounding M72 glomeruli for M72 NSV (21.72222 ± 7.26641) was significantly greater than the M72 surrounding NS glomeruli (32.5 ± 3.11983).

This answers the question that the presence of more acetylcholine in the GL regions increases TH cell counts globally and is not restricted to the M72 glomeruli region only after the CNO injection. However, the volumes of NSV M72 and M72 NS had no significant differences with p-value = 0.3 (two-tailed student's t-test), which reveals that there is no increment in the volume of the glomeruli due to the odor exposure in 7 days (Figure 3.6, center). The mean volumes for NSV M72 glomeruli were 207216.33429 ± 81534.40285, whereas, for M72 NS glomeruli was 183987.33595 ± 72449.75168.

As found and implied in the data above, the density distribution showed statistical significance with p-Val= 0.03, (two-tailed-Student's t-test). The mean densities of M72 NSV glomeruli demonstrate a value of 1.76809E-4 ± 6.07535E-5, whereas for the M72 NS groups, they were 1.27659E-4 ± 7.03463E-5 and hence showed statistical significance (Figure 3.6, right).

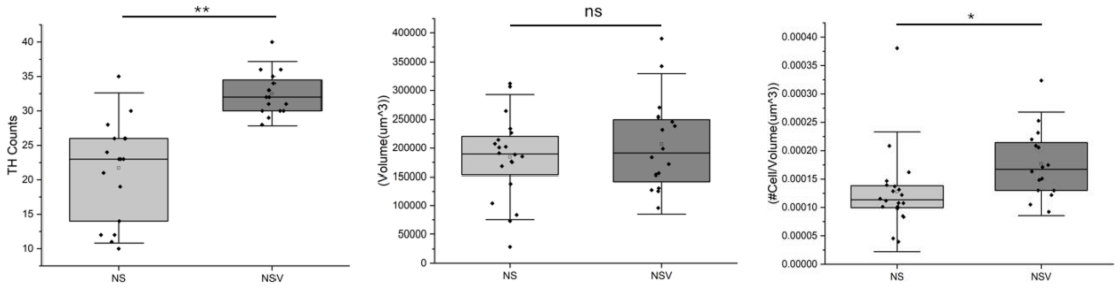


Figure 3.10: Plots showing the comparison between M72 glomeruli of No Stimulus groups (NS) and no stimulus with virus injection group (NSV). Left- shows the TH counts (p-value <0.0001), center- shows the volume for M72 glomeruli (p-value = 0.3), and right- shows the density of the TH population around the glomeruli (p-Val= 0.03).

This control condition allowed us to evaluate the effects of baseline disinhibition in the absence of odor stimulation in an increased cholinergic condition. Also, there was a global increase in the TH counts as compared to the control no-stimulus group throughout the olfactory bulb.

3.6. Comparison of TH Counts between Acute and Chronic Odor Exposure after the virus injection

The above results showed a global enhancement in the TH cell populations in the glomeruli layer of the OB. Next, we sought to determine the plasticity of dopaminergic neurons under different odor exposure conditions after the virus injection. Therefore, to discover the impact of odor exposure duration on tyrosine hydroxylase (TH) cell populations, we aimed to explore whether acute and chronic exposure to acetophenone elicited differential effects.

Strikingly, the analysis provides no significant difference in the number of TH cells between the two groups (Figure 3.7, left), enhanced cholinergic input in M72 glomeruli of chronic exposure (CEV) group and no stimulus (NSV) group (p-value = 0.35, two tailed student's t-test). Also, the mean count of TH cells surrounding M72 glomeruli for M72 CEV (35.6875 ± 6.66052) was not significantly different than the M72 surrounding AEV glomeruli (33.34615 ± 8.44721). Also, the volumes of CEV M72 and M72 AEV had no significant differences with p-value= 0.73 (two tailed student's t-test), which reveals that there is no increment in the volume of the glomeruli due to the odor exposure (Figure 3.7, center). The mean volumes for CEV M72 glomeruli were 182292.53471 ± 86455.3319 whereas, for M72 AEV glomeruli, they were 174091.77017 ± 67045.32439.

Surprisingly, no change was seen in the density distribution too, with p-value = 0.5 (two-tailed-Student's t-test). The mean densities of M72 CEV glomeruli were 1.97888E-4 ± 6.12051E-5, whereas, for the M72 ACV groups, they were 2.16654E-4 ± 7.99472E-5 and hence showed no statistical significance (figure 3.7, right).

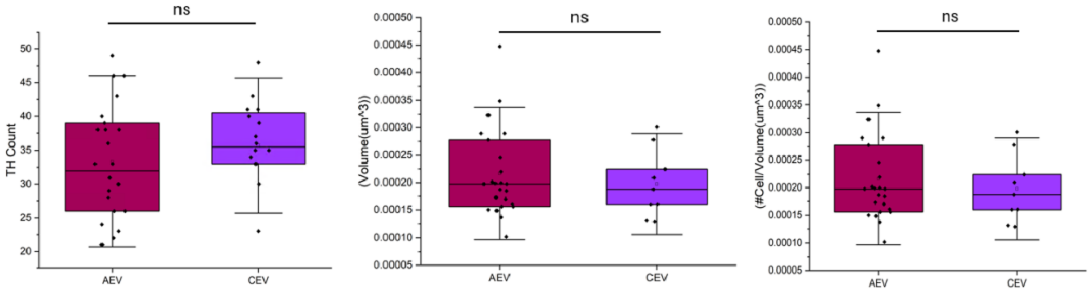


Figure 3.11: Plots showing the comparison between M72 glomeruli of acute exposure group with injected virus (AEV) and chronic exposure group with virus (CEV). Left- shows the TH counts (p-value = 0.35), center- shows the volume for M72 glomeruli (p-value= 0.73), and right- shows the density of the TH population around the glomeruli (p-value = 0.5).

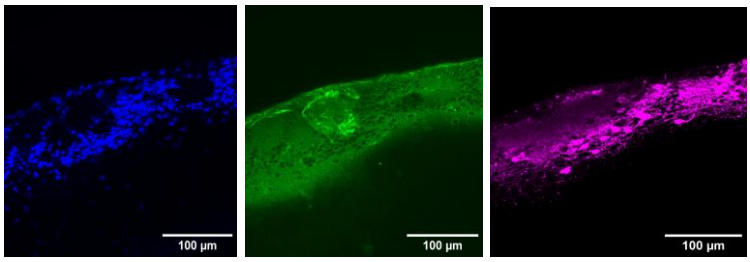


Fig 3.12: 40x imaging for cholinergic activated acute exposure groups.

Notably, analysis of mouse groups revealed that chronic exposure to acetylcholine resulted in similar TH cell counts compared to acute odor exposure conditions. We assume the glomeruli are active and saturated with TH, considering the odor exposure

was only for seven days. The groups might show significant differences when exposed to these conditions for longer period.

3.7. Investigation of TH Cell Density in Response to short-term chronic odor Exposure

Following the observation of maximal TH cell counts in response to chronic odor exposure, our investigation extended to understanding the underlying mechanisms driving changes in TH cell density. Therefore, we wanted to elucidate whether short-term chronic odor exposure (SCE), such as continuous exposure over 24 hours, would lead to any changes in TH cell density. We saw some changes upon data analysis, revealing that TH cell counts increased significantly (Figure 3.8, left) following one day of continued odor exposure compared to the NS group(p-Val=0.03). The volume changes were insignificant for any of the groups, but the density of SCE with no stimulus group showed significance (p-value = 0.04), and no stimulus group and chronic exposure group had p-value = 0.0007 (Figure 3.8, center). Also, on the comparison, the chronic exposure group has obviously higher TH densities than SCE (p-Val= 0.01) (Figure 3.8, right).
Statistical tests highlight the significance of chronic exposure in influencing the changes in the olfactory bulb. It is hypothesized that chronic exposure can rewire the neural circuits, leading to an increase in TH cell density. We believe that the increased activity of neurons involved in the processing of odor and discrimination may contribute to the recruitment of additional TH-expressing cells in the OB regions. These results show how TH cell density can change over time with repeated exposure to odors. It tells very much about the ability of the olfactory system to adapt and improve its capacity to process and differentiate odors.

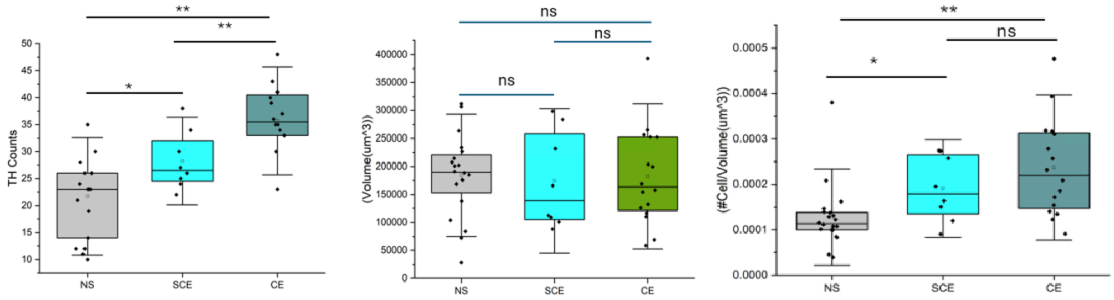


Figure 3.13:. Plots showing the comparison between M72 glomeruli of the No stimulus (NS) group, short-term chronic exposure group (SCE), and chronic exposure group (CE). Left- shows the TH counts ((NS and SCE, p-Val=0.03), (CE and SCE, p-Val= 0.01)), center- shows the volume for M72 glomeruli, there is no statistical difference in volumes of any group, right- shows the density of the TH population around the glomeruli ((SCE and NE, p-Val= 0.01), (NS and CE, (p-Val= 0.01)).

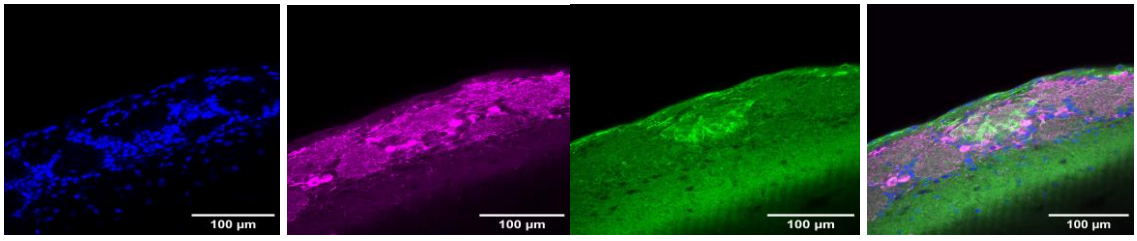


Fig 3.14: 40x imaging for short term chronic exposure groups.

3.8. Comparison of Non-M72 Glomeruli and Density

The comparison of the TH population surrounding the non-M72 glomeruli shows no

significant differences across all mice groups (Figure 3.9, left). This states that the TH cells are randomly arranged in the glomeruli layer of the olfactory bulb. Additionally, by looking at the volumes (Figure 3.9, center) and volumes (Figure 3.9, right) of all groups

used in the experiment, we get an idea of how the spatial distribution and packing of TH cells change within the olfactory bulb.

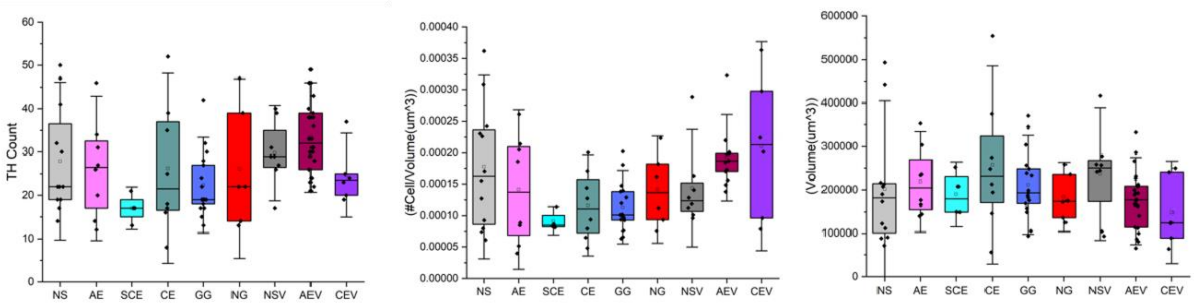


Figure 3.15: Plots showing the comparison between non-M72 glomeruli of all the groups in the experiment. First- no stimulus (NS) group, second- acute exposure (AC) group, third- short-term chronic exposure (SCE) group, fourth- chronic exposure (CE)group, fifth- positive valence go (GG) group, fifth- negative valence no-go (NG) group, sixth- no stimulus virus injected (NSV) group, seventh- acute exposure virus injected group (AEV), eighth- chronic exposure virus injected (CEV). Left- shows the TH counts for all the groups used in the experiment, center- shows the volume across all the groups for M72 glomeruli, right- shows the density of the TH population around the glomeruli for all the groups. There is no statistical difference in TH population, volumes, and densities between any groups for non-M72 groups.

CHAPTER 4

Discussion:

Animals experience a plethora of odors in their environment. Over time, several odors that are experienced over the lifetime of a mouse can be essential for their survival and might require further processing such as those associated with food or predators while others may not be relevant to the current goals and hence can be ignored. This process involves interactions between the feedforward olfactory circuit and feedback projections that modulate the ongoing olfactory processing. This study answers many questions about the processing of relevant and non-relevant olfactory stimuli and how experience-dependent plasticity occurs at very early stages of odor processing. The olfactory bulb glomeruli layer is the first stage of olfactory processing. The odorants bind to the OSNs in the olfactory epithelium and send the signals forward to the glomeruli in the glomeruli layer. This consists of incoming sensory neurons surrounded by interneurons and dendrites of the second-order neurons Mitral and tufted cells. The glomeruli layer gets OSN projection from individual types of receptors. These receptors are unique and specialized to bind to only one type of odorant. The glomerulus under study here consists of OSNs expressing the M72 receptor, which binds to its ligand, acetophenone. There are several interneurons present within the glomeruli layer implicated in gaining control of the incoming odor information, one of which is tyrosine hydroxylase (TH) expressing short axon cells. To study plasticity in the glomeruli layer, we quantified the number of TH-positive interneurons present around specific glomerulus and compared it under different behavioral conditions as discussed below.

First, we quantified the baseline expression of TH-positive cells surrounding the M72 glomerulus and non-M72 glomeruli in adult mice without providing external odorant exposure. The observation from this control experiment suggests that the TH interneurons are randomly

spread throughout the glomeruli layer. Next, we probed how exposure to odors without an associated valence affects this layer. Particularly we tested whether passive exposure to acetophenone affects the TH-positive cells surrounding the M72 glomeruli. We studied this effect by dividing our animals into acute exposure (30min/day for 7 days) and chronic exposure (24hrs/day for 7 days) groups, to test whether the different duration of odor exposure will affect the habituation of sensory responses differently. In chronic exposure but not acute exposure groups we observed an increase in the TH populations specifically around the M72 glomeruli. How is the increase in TH counts achieved? Habituation of glomeruli occurs with prolonged exposure to the same odor, which may recruit interneurons responsible for gaining control. The increase could be dependent on neurogenesis or expression of TH by cells that were previously TH-negative as suggested by Bonzano and colleagues.

We further asked whether this plasticity prevalent in the GL is recruited when an olfactory stimulus is associated with a reward. After training mice on an odor association paradigm that exposed them to odors at levels much lower than the acute odor group, we observed an increase in the TH-positive cells specifically around the M72 glomeruli when acetophenone was a CS+ but not a CS- odor. This suggests that as acetophenone becomes relevant, increased plasticity in the glomerular layer may facilitate a bottom-up bias. We used water as a positive valence for training the water-deprived mice while a 4kHz punishment was used as a negative valence. It is possible that the saliency of our negative valence stimulus was low which at higher saliency may also become relevant for the mouse and promote bottom-up bias for example a foot shock.

Finally, given these observations, we hypothesized that the bottom-up bias that enhances relevant odors may be facilitated by top-down olfactory processing pathways, where the signals from the higher centers of the brain may bias the processing for the rewarded olfactory stimulus.
We sought to test the influence of increased activation of cholinergic neurons in the basal forebrain which have been implicated in olfactory processing and goal-directed behaviors. Here, we used DREADD-based approach for activation of the cholinergic cells while being able to monitor the M72 glomeruli using Chat-Cre; R26R-tdT; M72-GFP mice. Post injection of CNO, prior to which there is presumably minimal cholinergic activation, we observed an increase in TH-positive cells across the entire glomerular layer with and without any external odor stimulation. Strikingly, we observed no significant increase in the TH counts between the cholinergic-activated acute exposure and chronic exposure group which suggests that prolonged cholinergic activation saturates the expression of TH-positive interneurons. This increased activation was non-specific as TH-positive neurons surrounding both M72 and non-M72 glomeruli were increased for acetophenone exposures. Further studies will need to dissect how a global acetylcholine release biases the early sensory layer for a particular odor. The olfactory bulb receives top-down input from other dedicated olfactory cortices which might together with the basal forebrain shape the incoming input and over time.

Anomalies:

The M72 glomeruli are generally present at dorsolateral and dorsal regions in the glomerular layer. They are normally separated by some distance from each other within the glomeruli

layer of the olfactory bulb. While navigating through and analyzing all the images, we found some interesting and rare microscopy images. The images were collected using a Nikon AT-AT microscope.

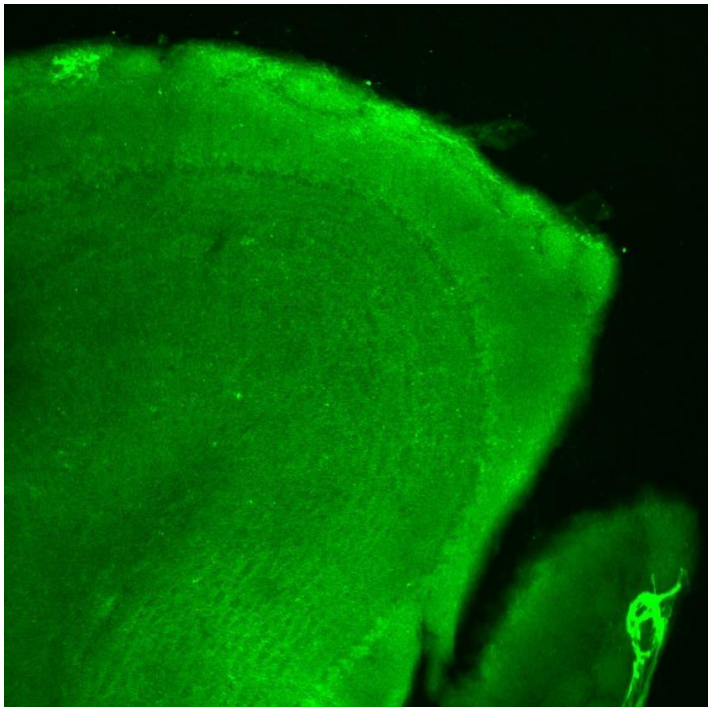


Figure 4.1. The image shows two M72 glomeruli on the same slide.

This microscopy image was collected using 10x magnification. It has two M72 glomeruli sections present in the same section.

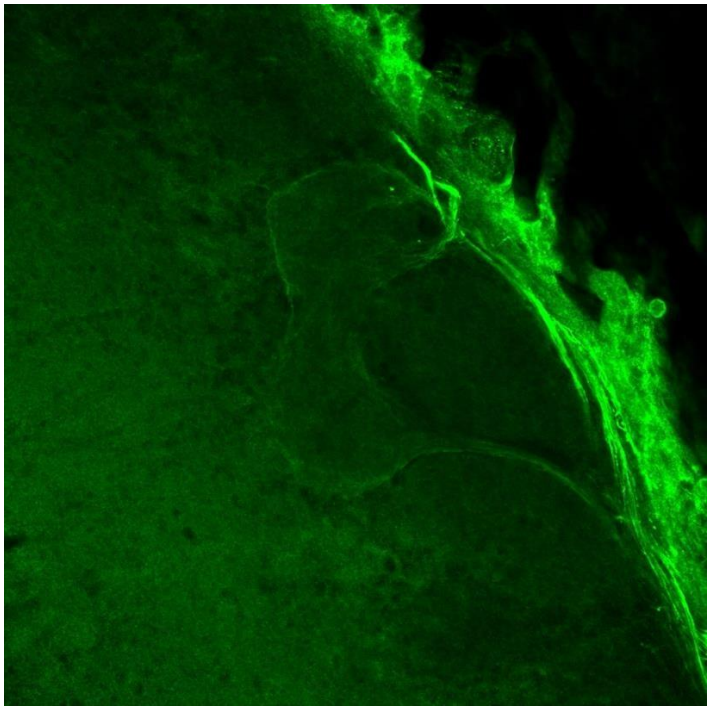


Figure 4.2. Image showing two M72 glomerulus just adjacent to each other.

The image was collected with 40x magnification using water immersion. This image shows two M72 glomerulus not only in the same section but also adjacent to each other (Figure 4.1).

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