Investigating the role of thermosensation in multimodal olfactory perception

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled "**Investigating the role of thermosensation in multimodal olfactory perception**" towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Ganesh Ashish Nair at Indian Institute of Science Education and Research, Pune under the supervision of Dr. Nixon M Abraham, Assistant Professor, Department of Biology, during the academic year 2022-2023.

Dr. Nixon M Abraham

Committee:

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Declaration

I hereby declare that the matter embodied in the report entitled **Investigating the role of thermosensation in multimodal olfactory Perception** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Nixon Abraham and the same has not been submitted elsewhere for any other degree.

Ganesh Ashish Nair

Date: 01/04/2023

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Abstract

Every organism in their environment is exposed to a variety of sensory stimuli. The ability of living things to identify, categorise, and respond towards these external stimuli is most crucial for their survival. These sensory cues include visual, auditory, smell, taste, somatosensory, and temperature. Animals perceive these cues and integrate congruent and eliminate incongruent information from several sensory systems to make appropriate decisions. The mouse olfactory system is unique in a way that it has different subsystems that can detect a variety of stimuli with varying physico-chemical characteristics. The subsystems comprise of the Main olfactory epithelium (MOE), Septal organ (SO), Vomeronasal organ (VNO), and Grueneberg ganglion (GG) which enables the animals to detect and discriminate olfactory cues, mechanical pressure, pheromones like non-volatile cues and temperature cues. Thus far, these subsystems have been studied independently and the interactions among them are not well understood. Additionally, the role of the olfactory system in sensing the temperature also remains elusive. Hence, in this study, we focus on examining the effect of temperature on olfactory perception. We first investigated whether animals could discriminate different temperatures that they experience in their natural environment and the role of GG in sensing these temperatures. To accomplish it, we custom-built a thermo-olfactometer capable of delivering the odorized/non-odorized air of desired temperatures and standardized the critical parameters. When animals were trained to detect and discriminate different temperatures, we observed that the performance of animals reached the asymptotic phase in 1200-1500 trials, which showed a deficit after the GG of the animals underwent axotomy. We showed that animals could successfully discriminate temperatures. In contrast, animals that had sham surgeries showed no decrease in accuracy, thereby indicating the role of GG in temperature discrimination. Moreover, to understand how temperature affects olfactory perception, we trained the animals on a multimodal discrimination task i.e. animals were trained to discriminate different odours coupled to different temperatures. Our results showed faster learning in the multimodal task compared with the temperature discrimination. Taken together, our results reveal that animals can detect and discriminate temperature using their GG and it can modulate the olfactory perception.

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Contributions

Contributor name	Contributor role
Dr Nixon Abraham	Conceptualization Ideas
Ganesh A Nair, Sarang Mahajan	Methodology
Ganesh A Nair, Sarang Mahajan	Software
Ganesh A Nair, Sarang Mahajan	Validation
Ganesh A Nair, Sarang Mahajan	Formal analysis
Ganesh A Nair	Investigation
Dr. Nixon M Abraham	Resources
Ganesh A Nair	Data Curation
Ganesh A Nair	Writing - original draft preparation
Dr Nixon Abraham, Sarang Mahajan	Writing - review, and editing
Ganesh A Nair	Visualization
Dr Nixon Abraham, Sarang Mahajan	Supervision
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Dr. Nixon Abraham (DBT Wellcome Alliance)	Funding acquisition

1. Introduction

One of the most important survival skills of living organisms is their ability to recognize, classify, and respond to specific external stimuli. The animals sample these stimuli, process the information and make appropriate decisions. Many behavioural responses that animals display can be utilized in probing the underlying neural mechanisms. In nature, animals are exposed to a range of sensory signals with varying physicochemical characteristics. These sensory signals include visual, auditory, smell, taste, somatosensory, and temperature cues. Animals sense these stimuli and integrate congruent/incongruent information from different sensory systems to form an efficient percept of the external world.

In general, animals have specialized systems that can sense different sensory stimuli. However, the rodent's olfactory system is unique as it can process a variety of sensory stimuli with distinct physicochemical characteristics. The olfactory system's ability to process stimuli with varying physicochemical properties makes it a good model system for investigating the neural mechanisms involved in multi-sensory decision-making using a single sensory system. The olfactory system of rodents consists of four subsystems capable of sensing a variety of stimuli, including chemical cues (such as volatile scents, volatile and non-volatile pheromones), mechanical pressure, and possibly the thermal stimulus (Grosmaitre et al., 2007; Mamasuew et al., 2008; Tian & Ma, 2008). Although the mechanisms underlying chemical and mechanical information through the olfactory system have been investigated, temperature sensing through the olfactory system and its effects on olfactory perception remains unexplored. Hence, in this study, we aim to investigate the role of the olfactory system in sensing temperatures and the influence of temperature on modulating olfactory perception.

1.1. Rodent olfactory system

The sense of smell in rodents serves numerous functions such as identifying and assessing the quality of food, finding potential mates, avoiding predators, and participating in social interactions (Fuss et al., 2005). The main role of the olfactory system is to interpret chemical signals from the environment in order to perceive the chemical landscape around an animal. Structurally, the olfactory system can be

divided into three components - the nasal cavity, the olfactory bulb, and the olfactory cortex (Barrios, 2014).

1.2. Nasal Cavity

The presence of multiple sensory structures with distinct morphological and molecular characteristics within the nasal cavity reflects the functional diversity of the olfactory system. These structures include the main olfactory epithelium (MOE) (Ronnett & Moon, 2002), septal organ (SO also known as Organ of Masera), the vomeronasal organ (VNO) (Chamero et al., 2012), and Grueneberg ganglion (GG) ("The Grueneberg Ganglion," 2010) (Fig 1), all of which enable the olfactory system of rodents to perceive signals triggered by odorant molecules, mechano, and thermo-sensation.

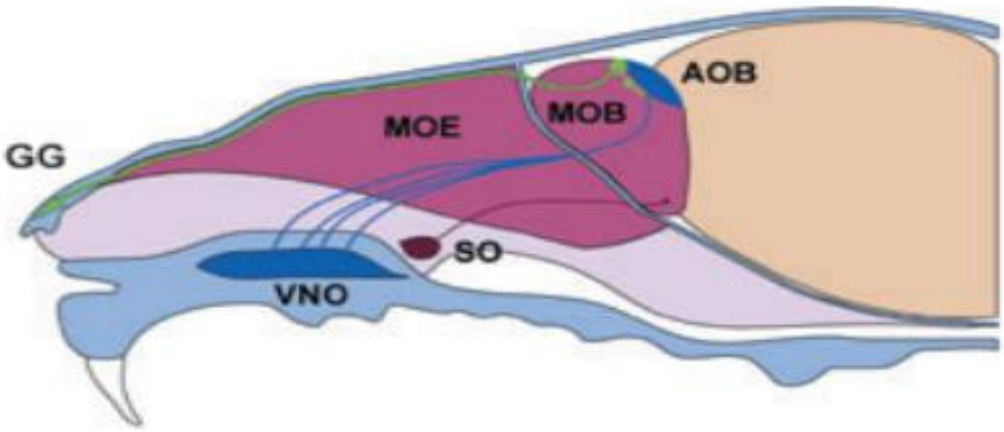

Fig 1: A diagrammatic representation of the olfactory system of mice (Fuss et al., 2005)
MOE - Main olfactory epithelium, SO - Septal Organ, VNO -Vomeronasal Organ, GG - Grueneberg ganglion. The axons of the sensory neurons of these systems project to the olfactory bulb. MOB – Main olfactory bulb, AOB – Accessory olfactory bulb.

The main olfactory epithelium (MOE) occupies the posterior-dorsal region of the nasal cavity and envelops the turbinates and parts of the nasal septum (Fuss et al., 2005). MOE harbors olfactory sensory neurons which project towards and establish connections with the glomeruli of the main olfactory bulb and is responsible for the primary olfactory sensation in mammals. Each OSN in the MOE has a single G protein-coupled receptor except for membrane-spanning four-pass A (MS4A) receptors which are specifically found in necklace sensory neurons (P. L. Greer et al.,

2016). All of these sensory neurons can be activated by different odorants. The binding of odorant molecules to receptors on OSNs triggers a cascade of events that results in the generation of an action potential and subsequent transmission of these signals to the first relay center of the olfactory pathway, the olfactory bulb. At the glomeruli in the olfactory bulb, these OSNs form synapses with the projection neurons of OB, that is mitral and tufted cells. The inhibitory neurons making synapses with mitral and tufted cells facilitate the refinement of olfactory signals. Through these M/T cells these signals are relayed to different parts of the olfactory cortex. The vomeronasal organ (VNO), which is situated in the ventral region of the nasal cavity, harbors vomeronasal sensory neurons (VSNs) with their axons innervating the accessory olfactory bulb (AOB) (Fuss et al., 2005). The VSNs are responsible for detecting pheromones that control specific behaviours, such as mating and social aggression (Dulac & Torello, 2003). The septal organ also harbors OSNs that send their axon projections to glomeruli in ventral OB (Fuss et al., 2005). Additionally, the Grueneberg ganglion (GG) located at the rostral tip of rodent nostril has its axons projecting towards the caudal side of the olfactory bulb. Studies have shown that GG has a role in sensing cold temperatures and alarm pheromone signals (Bumbalo et al., 2017; Mamasuew et al., 2008). As the majority of these subsystems have only been investigated independently, it is currently unclear how the combination of information from these subsystems affects chemical perception. Moreover, in a behavioural context, it also remains unclear whether temperature detection and discrimination can be achieved by GG. Hence, in this study, we aim to investigate these questions by examining how temperature can be sensed by an animal and how it affects the olfactory perception.

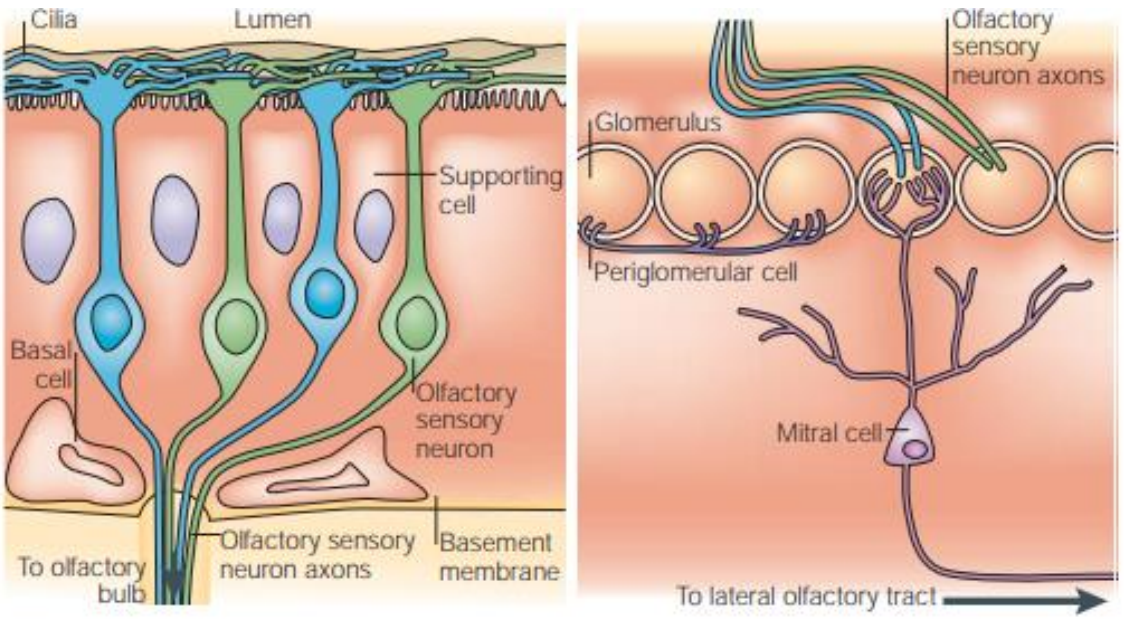

Fig 2: Cross section of main olfactory bulb (Mombaerts, 2004)
Left: Stimulus sensing apparatus. Right: Neurons involved in signal transduction in OB

1.2.1 Grueneberg Ganglion

The Grueneberg ganglion, identified by Hans Grüneberg in 1973, is a group of cells that are located bilaterally in the nasal vestibule. GG neurons, like their counterparts in other nasal compartments, express the olfactory marker protein (OMP) and are also equipped with olfactory receptors mainly from two important families: The V2R family and the trace amine-associated receptor (TAAR) family (Fleischer et al., 2006, 2007; Fuss et al., 2005; Koos & Fraser, 2005). These receptors are activated in response to certain chemicals released by predators or by conspecifics thus stating the role of GG as a detector for alerting semiochemicals (Fleischer, 2021). The GG is lodged in connective tissue in this region of the nose, which is surrounded by the septum, the nasal roof, and a thin layer of epithelial tissue that surrounds the nasal cavity's lumen. Around 800 GG neurons extend out an axon that bundles altogether (Fleischer, 2021) as they project caudally through the dorsal roof of the nasal cavity. These axons remain firmly fasciculate until they reach the caudal edge of the olfactory bulb, at which point they unbundle, evade the modified glomerular complex's glomeruli, and enter the posterior OB. Here they branch out to two lateral sides and form spherical

structures that are interconnected with axons, giving the appearance of the famous beads on a string array, hence the name ‘necklace glomeruli’(Bumbalo et al., 2017).

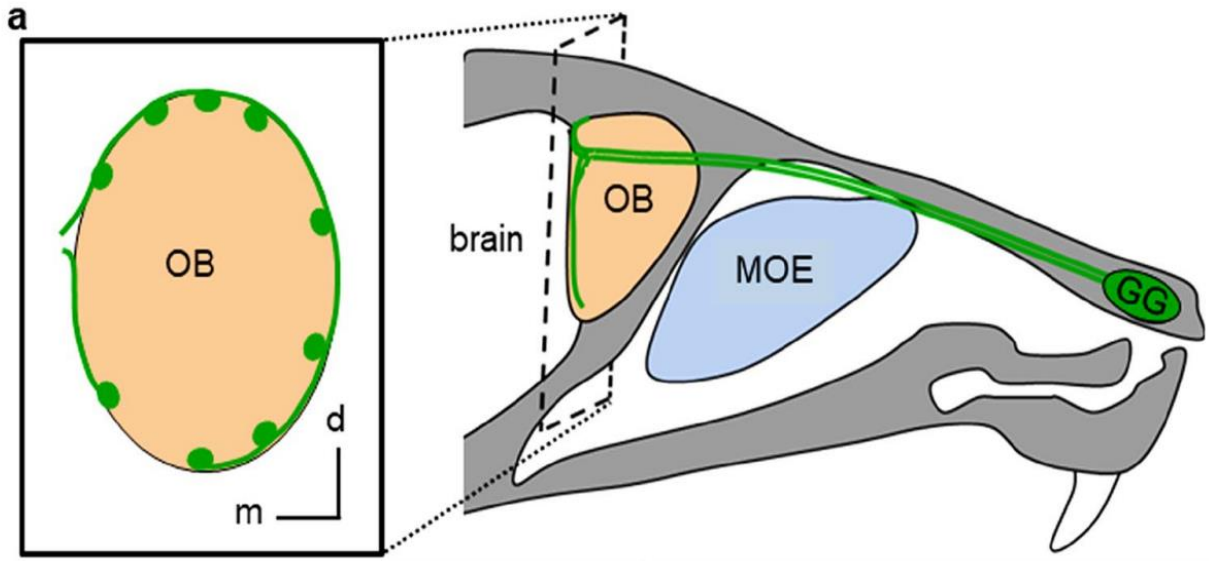

Fig 3: Illustration representing projections of the GG (Fleischer, 2021)
Right side – GG originates at the rostral tip of the rodent nostril and sends its axonal projection to the OB. Left side – A coronal section of the OB represented by the rectangle bounded by the dashed. This section shows the innervation of axonal projections of GG in the caudal side of OB in such a way that the glomeruli are seen to be interconnected (‘necklace glomeruli’).

The architecture of GG along with its expression of olfactory receptors suggests that it has a role in chemoreception as well. The olfactory necklace which envelopes the caudal olfactory bulb at the junction between the main olfactory bulb and the accessory bulb was proposed to function in neonatal suckling (C. Greer et al., 1982). The projection of GG to this region along with its principal expression of olfactory receptors in newborn pups brought out speculations about its role in mother/child interactions (Fuss et al., 2005; Roppolo et al., 2006). Further studies revealed that neonatal mouse' GG is activated by cool ambient temperatures when they are exposed to these stimuli in the absence of the dam, indicating that the GG may serve as a thermosensor with its higher activation shown below 22 degrees Celsius (Mamasuew et al., 2008).

1.2.1.1 Activation of GG neurons by cold temperatures

As the anterior region of the nasal cavity is highly susceptible to temperature variations resulting from changes in ambient temperatures in the atmosphere, the location of the GG neurons in the anterior region of the nose may attribute to their sensitivity toward cool temperatures (Brechbühl et al., 2013; Chao et al., 2015). Specifically, the V2r83-positive cells of GG neurons respond to coolness, while the TAAR-expressing neurons are unresponsive to lower temperatures (Mamasuew et al., 2008). Moreover, not only do cool temperatures activate GG cells, but they also trigger the corresponding glomeruli in the OB. This suggests that electrical signals generated by GG neurons in response to coolness are transmitted through their axons to the OB. Therefore, the GG is recognized as a sensory organ that serves a dual purpose.

In cold-sensitive GG neurons, two different thermosensors, GC-G and TREK-1, appear to be active simultaneously (Fleischer, 2021), as both of these neurons lack the expression of transient receptor potential subtype TRPM8 (Fleischer et al., 2009), which is the primary molecular transducer of cold somatosensation present in trigeminal and dorsal root ganglia. Inactivation of TREK-1 (potassium channels) in response to coolness induces depolarization of the membrane, thereby resulting in GG stimulation (Fleischer, 2021). On the other hand, coolness-induced GG stimulation via GC-G occurs through the cGMP pathway which requires the co-expression of cGMP-sensitive ion channel CNGA3 along with GC-G (Chao et al., 2015). Coolness induced dimerization/ oligomerization of GC-G results in its increased enzymatic activity (Chao et al., 2015). As a result, more cGMP is produced, which in turn evokes the opening of CNGA3 channels (Fig 4), thereby inducing an influx of positively charged ions and thus promoting membrane depolarization (Fleischer, 2021). Hence, two separate thermoreceptors, namely GC-G and TREK-1, function in tandem within GG neurons that are sensitive to cool temperatures

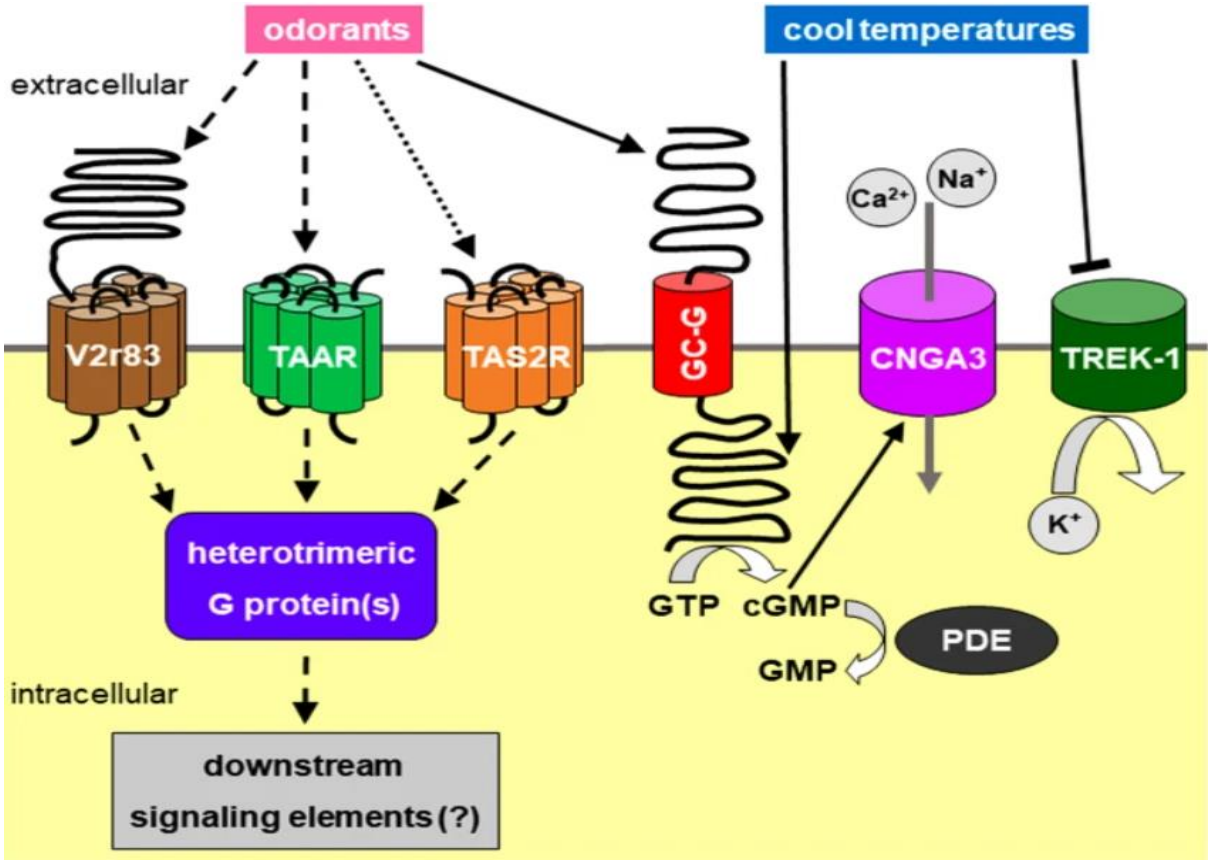

Fig 4: Chemo- and thermo-sensory signaling elements in GG (Fleischer, 2021)
Chemosensory receptors include V2r83, TAAR family receptors, and TAS2R. Coolness-evoked GG responses occur through the thermosensory protein TREK-1. Transmembrane guanylyl cyclase GC-G in GG responds to both odorants and cool temperatures.

Coolness-evoked GG responses in adult animals have not been studied extensively in comparison to the animals in their neonatal stage. Therefore, it remains unknown whether cold temperatures or in general the temperature profiles experienced by the animals in their habitats trigger activation of GG neurons in adults as strongly as they do in pups (Fleischer, 2021). Few immunohistochemical studies have shown that the expression of TREK-1 in conjunction with GC-G and CNGA3 is not restricted to GG neurons of neonates but is also observed in the adult stage (Liu et al., 2009; Stebe et al., 2014). Hence, similar to the neonatal stage, GG might also have a role to play in sensing temperatures in adults. In this study, one of the main focuses is to investigate it.

Moreover, there have been reports suggesting that certain olfactory sensory neurons expressing guanylyl Cyclase-D receptors send their axons to the necklace

glomerular area (Juilfs et al., 1997). Although it has been discovered that sensory neurons stimulated by odor and temperature independently project to this area, there has not been much research into the potential interactions between these two systems. However, due to their projection to the same area, it is likely the signals from both of these neurons interact and thereby affect the olfactory perception and hence the decision-making.

1.3. Our Work

The olfactory system possesses a unique ability to detect a broad range of physical and chemical stimuli, which makes it a strong candidate for investigating how in a single sensory system, information from different modalities interact and influences the decision-making process. Since environmental factors such as airflow patterns and temperature changes can affect the properties of the odor, it is important and relevant to study how these variations impact olfactory perception. Thus far, our lab has examined how altering airflows can modify olfactory perception, and in this research project, we aim to explore the impact of temperature alterations on olfactory perception.

In behavioural neuroscience, to investigate the neuronal mechanisms underlying any behaviour, a robust behavioural paradigm with high-throughput behavioural readouts is needed. Although the GG neurons have been shown to respond to coolness, a behavioural paradigm assessing the temperature sensing behaviour and its effect on the olfactory perception is scarce to date. Therefore, to study the effect of temperature on olfactory perception, we first need to establish a paradigm capable of providing both stimuli to the animal in a precise and controlled manner. This is the first aim of the study and to achieve this, we custom-built a thermo-olfactometer that is capable of delivering odorized or non-odorized air while their temperatures are being regulated efficiently (range of 0-24 degrees Celsius). This was accomplished by installing and integrating thermostats into the custom-built thermo-olfactometer. The temperature can be regulated with a least count of 0.1 degree Celsius and at a time resolution of 800 ms. Additionally, this instrument, which is based on the Go/No-Go paradigm can be used to simultaneously provide multimodal stimuli (odorized air with different temperatures).

Firstly, we started by investigating whether coolness can induce GG responses in adult mice and whether GG has any role to play in detecting and discriminating

different temperatures. Using a Go/No-Go paradigm, we challenged the animals to discriminate two different temperature stimuli; 19 °C vs 22.1°C, in the absence of odorants. These temperatures lie in the range that animals experience in their natural habitats. Animals learning efficiency and discrimination time were used to assess the discrimination performance of animals. Once all the animals reached the asymptotic phase of their learning, a subgroup of animals was subjected to GG axotomy, whereas in other subgroup sham surgery was performed. For sham surgery, a small incision on the nasal cavity near the nostrils was performed. The performance of animals was examined by training them on the same temperature pair that they have learned previously after the surgical interventions and a recovery period. By this, we were able to examine the role of GG in sensing temperatures in adult mice. To further characterize the temperature sensing behaviour, we are in the process of finding the minimum temperature difference that the animals can discriminate. Further, we investigated the effect of temperature on olfactory perception by training the animals to discriminate different odour coupled with different temperatures. Further experiments are required to delineate the mechanisms underlying the integration of temperature and odour information in the olfactory bulb.

2. Materials and methods

2.1. Subjects

A total of 27 C57BL6/J (Male) animals were used in the study. All animals were 8-10 weeks old at the beginning of the behavioural experiments and were maintained on a 12-hour light-dark cycle in temperature and humidity-controlled isolated cages. During the training period, animals were provided with *ad libitum* food and placed on a water-deprived schedule that lasted not more than 12 hours. Throughout the experiment, regular weight checks on the animals were done. Animals that weighed less than 80% of their original weight were removed from the water restriction immediately. All experimental procedures were performed following the guidelines of the Institutional Animal Ethics Committee, IISER Pune, and the Committee for Control and Supervision of Experiments on Animals, Government of India.

2.2. Temperature measurements from potential rodent habitats

The temperature measurements were done from the potential rodent burrows in the IISER Pune campus. The temperature readings were measured with a hot wire anemometer probe. The temperature measurements were done from outside and inside the burrows. The temperature was recorded for 100 seconds with a temporal resolution of 800 ms and was averaged for quantification purposes.

The average temperature inside and outside of the burrows was 20.31 °C and 23.6 °C, respectively. As studies suggest that GG shows higher activation below 22 °C, hence, for the temperature discrimination task, we used the individual temperatures from the readings. The outside temperature was chosen to be 22.1 °C, whereas the inside temperature was 19 °C. Since we did not know the minimum temperature difference that animals can differentiate and as the average temperature difference between the inside and outside was 3.29 °C, we started with training the animals on discriminating the temperatures with a difference of around 3 °C.

Burrow Number	Temperature Inside (°C)	Temperature Outside (°C)
1	22.3	24
2	20.3	23.9
3	20.3	23.8
4	21.4	23.8
5	21.0	23.9
6	22.1	24
7	21.7	23.9
8	21.8	23.9
9	20.7	23.8
10	20.9	24.7
11	19.2	24.5
12	19.7	23.4
13	18.7	22.9
14	18.3	23.7
15	17.7	22.4
16	20.7	23.8
17	21.1	23.3
18	19.7	23.4
19	19.9	22.8
20	18.7	22.1
Average	**20.31**	**23.6**
SEM	**0.29**	**0.143**

Table 1: Temperature measurements inside and outside rodent burrows in IISER Pune campus.
SEM –Standard Error of Mean

2.3. Behavioural training

2.3.1. Apparatus

To perform the temperature discrimination tasks, we custom-built a two-channel thermo-olfactometer, which is controlled by custom written in Igor Pro (Wave-metrics).

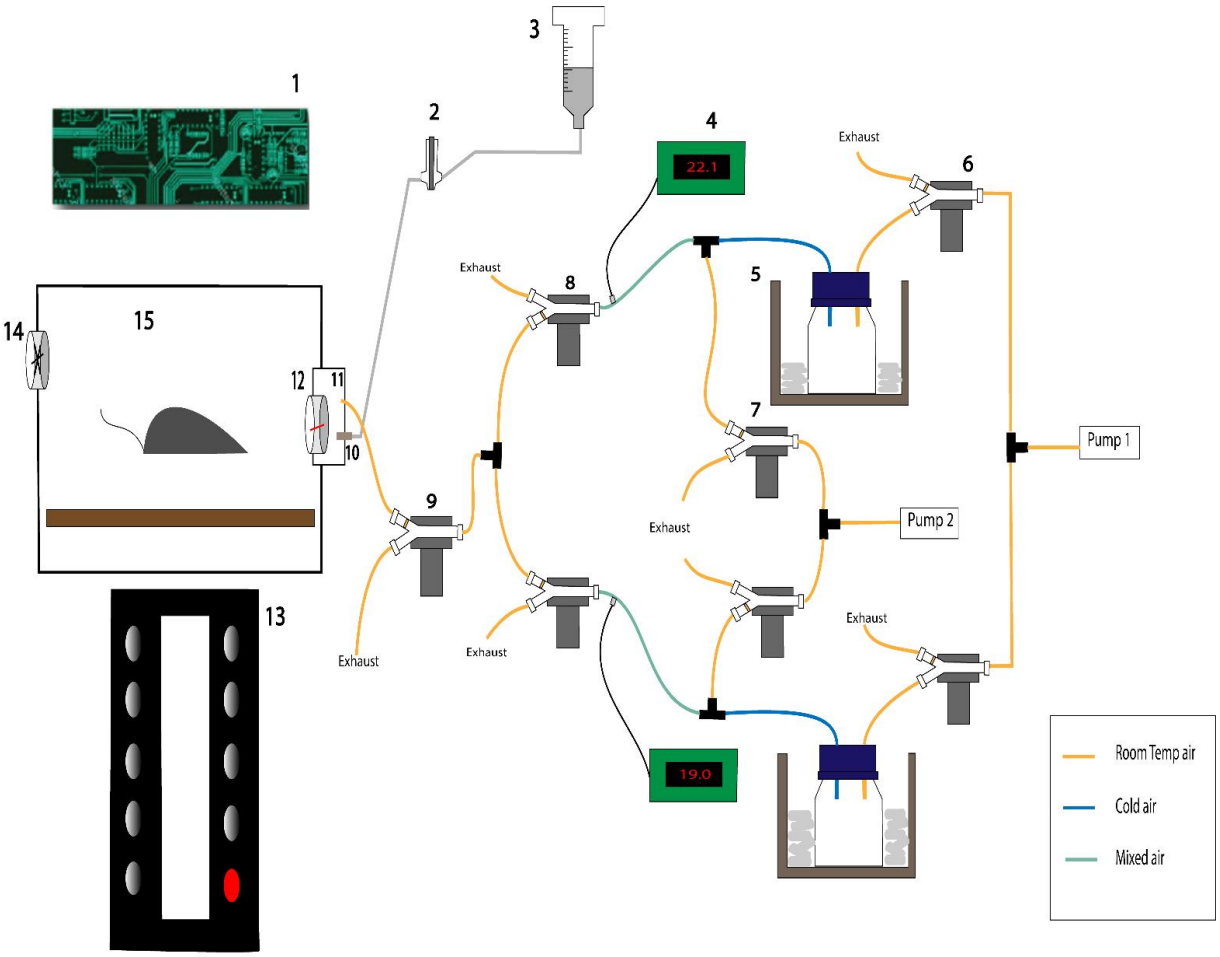

Fig 5: **Diagrammatic representation of the Behavioural training apparatus**

1) Circuit Board. **2)** Water Valve. **3)** Water Source. **4)** Thermostat. **5)** Cooling Chamber. **6,7,8)** 3-way valves. **9)** Final valve (3-way). **10)** Lick port/Water delivery tube. **11)** Stimulus delivery tube. **12)** Sample Port entry guarded by IR beam. **13)** Manual Controlling unit. **14)** Exhaust line. **15)** Animal holding chamber.

Fig 5 shows the apparatus in which the circuits regulating the temperature pair are represented. The influx of the airflow is enabled through two air pumps. The air supplied by pump 1 is diverted to two channels via a 3-way solenoid valve. On each

side, the air then proceeds to a 3-way valve whose default output allows the air to pass onto the cooling chamber which consists of an insulated box. This box holds an odor bottle surrounded by dry ice pellets which can decrease the temperature rapidly. The insulated box minimizes the heat exchange with the environment. This altogether reduces the temperature of the output air from the chamber. The temperature of the output air is monitored by the thermometer probe and is regulated at a specified temperature with the help of the internal thermostat feedback and is then delivered to the animal.

Animals are kept in a chamber wherein one side of this is a sampling port which is guarded by an IR beam. As soon as the animal pokes its head into the sampling port, the IR beam is broken, the trial is initiated and the stimulus is provided to the animal. The thermometer probe helps to monitor and regulate the temperature of the stimulus of each trial. Depending on the stimulus being rewarded or unrewarded the animal has to lick on the water delivery tube/lick tube placed parallel to the sampling port to fulfill the reward criteria and get the water reward.

2.3.2. Task Habituation

The experimental animals were first given a pre-training task to get them acclimated to the setup. For the pre-training task, animals that had above 80% of their body weight following two to three days of water restriction were used. The pre-training task was divided into 9 phases (phases 0 to 8) with an increasing level of complexity. In the initial stage of pre-training, animals receive water as a reward for breaking the IR beam by poking their heads into the sampling port. Once this phase is over, the animals are aware of the location of the water source. In the second phase, animals only receive a reward if they register at least one lick on the lick tube. The length of time that animals must lick to receive the reward is gradually increased in the subsequent stages. The eighth phase's reward criteria correspond to the criteria that are used during the discrimination training. The animals finished the pre-training phase in four to five sessions of 30 minutes each.

2.3.3. The Go\No-Go paradigm

The Go\No-Go paradigm was used to test the temperature discrimination abilities of the animals. During the task, the water-deprived animals poke their head into the sample port causing the disruption of the IR beam thereby initiating the trials. During

this period, a valve (labeled 8 in Fig 5) from a particular channel corresponding to a particular temperature opens, allowing the air of that temperature to flow through the corresponding channel. One of these channels corresponds to the temperature that is coupled to the water reward (S+), whereas the other temperature is neither coupled to a reward nor a punishment (S-).

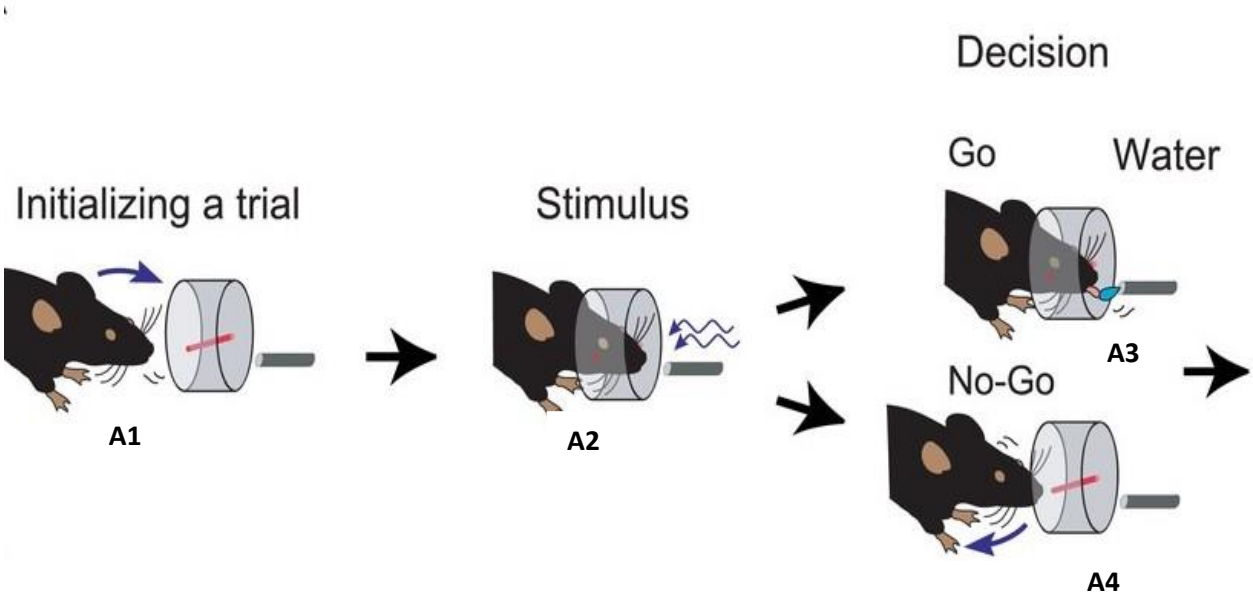

Fig 6: Diagrammatic representation of the Go/No-Go paradigm (Adapted from Abraham et al., 2004)

A1: Sampling Port guarded by an IR beam. **A2**: The animal poking its head into the sampling port results in the breakage of the IR beam in turn resulting in the initiation of the trial and stimulus onset. **A3**: S+ stimulus, the animal stays and licks on the lick port, and if reward criteria are met water is given as a reward. **A4:** S- stimulus, animal retracts its head, and the IR beam is resealed

2.3.4. Reward criterion

The total duration of a trial is 2500 ms and for the initial 500 ms (pre-trial period) the stimulus flows through the exhaust of the final valve (3-way valve). This is done to ensure that the stimulus is homogenous when delivered to the animal. After the first 500 ms, the stimulus is delivered to the sampling chamber for 2000 ms which is followed by an Inter trial interval (ITI) of 5000 ms. After the ITI, the next trial is initiated only when the animal pokes its head into the sample port thereby breaking the IR beam.

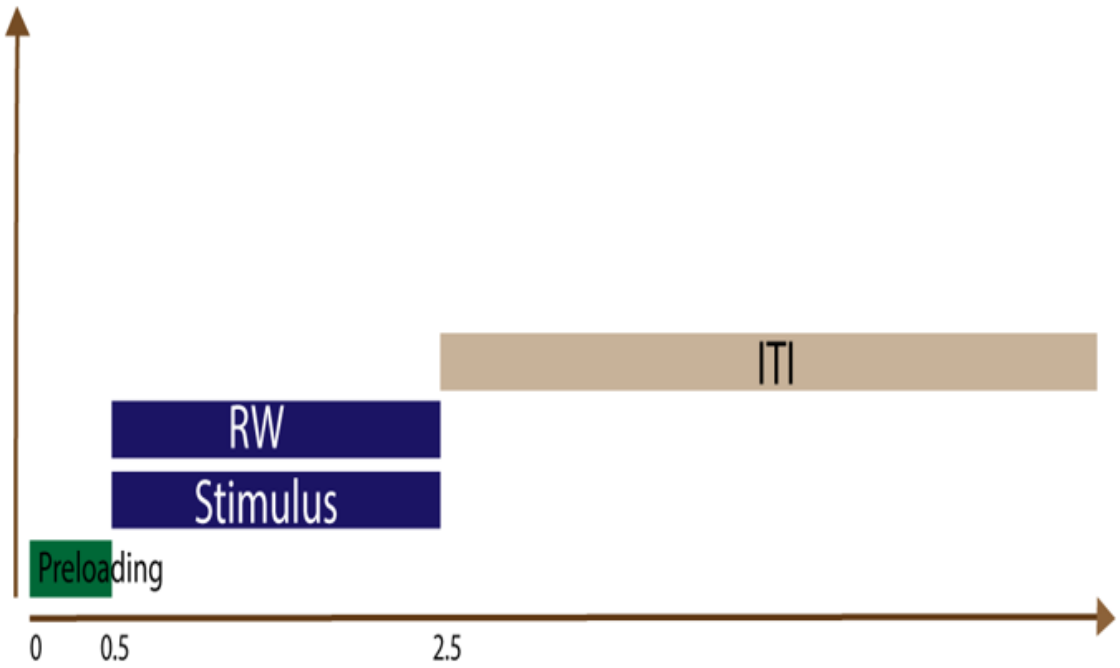

Fig 7 – Behavioural Paradigm

The total duration of each trial is 2500 ms of which the first 500 ms is the preloading time and 2000 ms is the stimulus delivery time. The response window (RW) overlaps with the stimulus delivery period. Each trial is followed by an inter-trial interval (ITI) of 5000 ms

Based on whether the trial is rewarded or non-rewarded, animals need to lick in the response window which overlapped with the stimulus delivery time. The response window of 2000 ms is virtually segregated into four bins of 500 ms each. For an S+ trial to be correct, the animal has to register a single lick in at least 3/4 bins. The correct S+ trials end with a delivery of 3-4 µl of water reward. For an S- trial to be accurate, the animals generally learned not to lick, however, licking in a maximum of 2 bins is allowed.

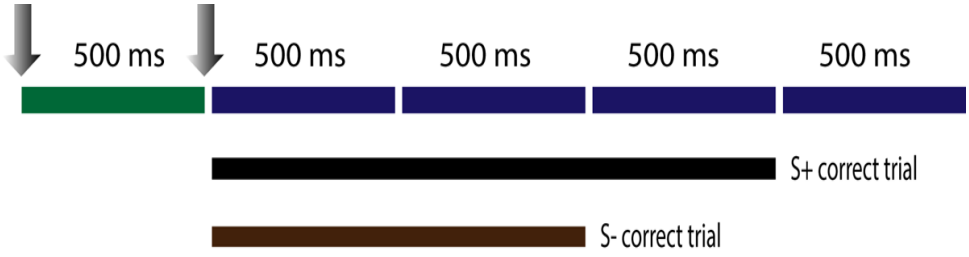

Fig 8: Reward criteria. Reward criteria for S+ and S- trials. Animals have to register a lick in at least three out of four bins for the S+ trial to be correct. For S- trial to be correct, animals are only allowed to lick for a maximum of 2 bins.

To reach optimal performance levels, the animal must complete 4-5 tasks (1200–1500 trials), with each task consisting of 300 trials. The trials are presented to the animals in blocks of 20 trials. In each block, out of 20 trials, half of the trials are S- (non-rewarded), and the other half are S+ (rewarded). The trials are presented in a pseudo-randomized manner such that no more than two trials with the same reward contingency are presented consecutively.

2.3.5. Instrument Standardization

2.3.5.1 Airflow standardization

We used an anemometer to check the consistency of airflow throughout the entire task. We optimized the flow to minimize the variations in the desired temperature and the flow was kept constant at 4 LPM throughout the training experiment. The total output airflow was also confirmed by using the water displacement method.

2.3.5.2 Reward Standardization

The reward was standardized in such a way that 3-4 µl of water was rewarded for each correct S+ trial.

2.3.5.3 Temperature regulation by thermostat:

The temperature was set in the thermostat and the thermostat was coupled to a set of valves. The temperature regulation was ensured with the loop combined action of the temperature probe with the set of valves passing the cool air and the air at room temperature. The thermostat was set in heater mode (H) so that whenever the temperature dropped below the set temperature, the thermostat transmitted signals to the corresponding valves and thereby regulating the temperature back to the set temperature. The protocol for temperature regulation was integrated into the setup. In addition to using Valves 6 and 7, other valves were also used to ensure that the air of different temperature is efficiently delivered to the animal. The functions of these valves are described below.

Function of Valve 8

Valve 8 is a 3-way valve whose default output pumps out the air thereby acting as an exhaust. This was kept as a 3-way valve because the default exhaust would ensure

continuous airflow even during inter-trial intervals (ITI). If this valve opens only during stimulus onset and remains closed during ITI, this would build up the pressure in connections before valve 8, resulting in heating and a quick rise in the temperature.

Function of Valve 9

Valve 9, a 3-way valve which in its default state pumps out the air acting as an exhaust. When a trial is initiated, valve 8 opens and air is directed to valve 9 which acts as an exhaust during the first 500 ms (preloading time). Once this duration ends, valve 9 default output shifts and thereby delivers the stimulus through the sampling port and hence to the animal for the next 2000 ms (stimulus delivery period). Therefore, valve 9 ensures a homogenous stimulus delivery to the animal.

2.3.6 Behavioural readouts

2.3.6.1 Learning Curve

The percentage accuracy of the temperature discrimination behavioural tasks is determined by analysing the number of correct responses by the animals. An animal's ability to learn is evaluated based on its successful responses to the S+ and S- trials. To track the animals' progress, learning curves for the behavioural tasks were plotted. Each point on the curve represents the average accuracy of 100 trials (with 50 S+ and 50 S- trials) which is averaged across all animals. Initially, as the animals could not discriminate the S+ and S- trials, they licked for all trials and exhibited an accuracy of 50% (chance level). However, with more trials, the animals gradually learn, accuracy increases and at a point reach 80% or higher, indicating successful differentiation between the S+ and S- trials. The animals' learning speed varies depending on the complexity of the behavioural tasks employed.

2.3.6.2 d’

Probabilities of correct S+ trials (hit) and incorrect S- trials (false alarms) were computed for d-prime (d') over an average of 100 trials. z score was then calculated from these probabilities. d' was calculated as z(hit)- z (false alarm) per 100 trials. This was then averaged across all animals.

2.3.6.3 Lick Pattern

The lick pattern provides information about the temporal licking behaviour of an animal towards a specific stimulus. For a trial with a stimulus duration of 2500 ms, each trial consists of 125 bins of 20 ms each (2500 ms). The licking behaviour is measured across these 125 bins where binary values are given for lick and non-lick responses [1 or 0, respectively] for each trial. These values are then plotted against the time of the trial. Initially, the lick patterns for naive animals are high and similar for both types of stimuli as they are unable to differentiate the two stimuli. However, as the animals learn, they start to preferentially lick for the S+ trials and avoid licking in the S- trials, resulting in a divergence in the lick patterns.

2.3.6.4 Sample Pattern

The sample pattern is yet another parameter that is used as a readout of the duration that the animal has spent inside the sampling port during a trial. The temporal pattern of the broken IR beam is used as a proxy to estimate the sampling pattern. Similar to the lick pattern, binary values are assigned to the broken and intact IR beam [1 or 0, respectively]. The animal's sampling behaviour is measured across 125 bins for each trial, with each bin being 20ms, and is averaged separately for S+ and S- trials of the entire task. This data is then plotted against the time of the trial. Initially animals lick for all trials irrespective of the reward contingency, resulting in a consistently broken beam and higher sampling rates for both stimuli. However, once the animal learns to discriminate the temperatures effectively, the sampling pattern remains higher for S+ trials, whereas it decreased for S- trials as animals learn to retract their heads for non-rewarded trials. Hence, sample patterns can also be used to assess the discrimination abilities of animals between rewarded and non-rewarded stimuli.

2.3.6.5 Discrimination time (DT)

DT is the time point at which an animal first learns to differentiate between rewarded and non-rewarded stimuli. In our experiment, DTs can be determined using both lick and sample patterns. By comparing the patterns for the rewarded stimulus (S+) and the non-rewarded stimulus (S-), a p-value curve can be generated. The time point where p-value < 0.05 for the last time is considered as the discrimination time. As DT is only relevant during the stimulus delivery period, the duration of preloading time (500 ms) is excluded from the time calculated giving the DT.

2.3.6.6 Area under the curve (AUC)

The area under the curve (AUC) was also used to calculate the discrimination index of the animals. For AUC calculations, the lick probabilities for S+ and S- trials were used. The discrimination index was calculated as AUC = (AUCS+ - AUCS-)/AUCS+.

2.3.6.7 Inter-trial interval (ITI)

ITI refers to the time that animal takes between two consecutive trials. This can be used as a proxy for the motivation of the animal: Over-motivation in animals can result in shorter ITI with the animal licking indiscriminately towards both the rewarded (S+) and non-rewarded (S-) trials, even after learning to distinguish between them. Conversely, if an animal is under-motivated, it may retract its head more frequently, resulting in false positives for the non-rewarded trials. Since there is no way to measure these false positives directly, we assess them by examining the animal's responses to the rewarded trials (S+). Under-motivated animals may retract their head in response to both the rewarded and non-rewarded trials.

2.3.7 Data Analysis

The behavioural task data were analysed in a custom-written program in IGOR-Pro or Python. Graph Pad Prism 8 was used to plot graphs and perform the statistical analysis.

2.4 Axotomy of Grueneberg Ganglion

The animals were administered with a combination of Ketamine and Xylazine, injected intraperitoneally, in a ratio of 12:5 as anesthesia, equated according to their body weights as 2 µL per gram of body weight. Surgery was performed once the animals were deeply anesthetized, indicated by an absence of reflexes upon toe pinching and no movement of the whiskers.

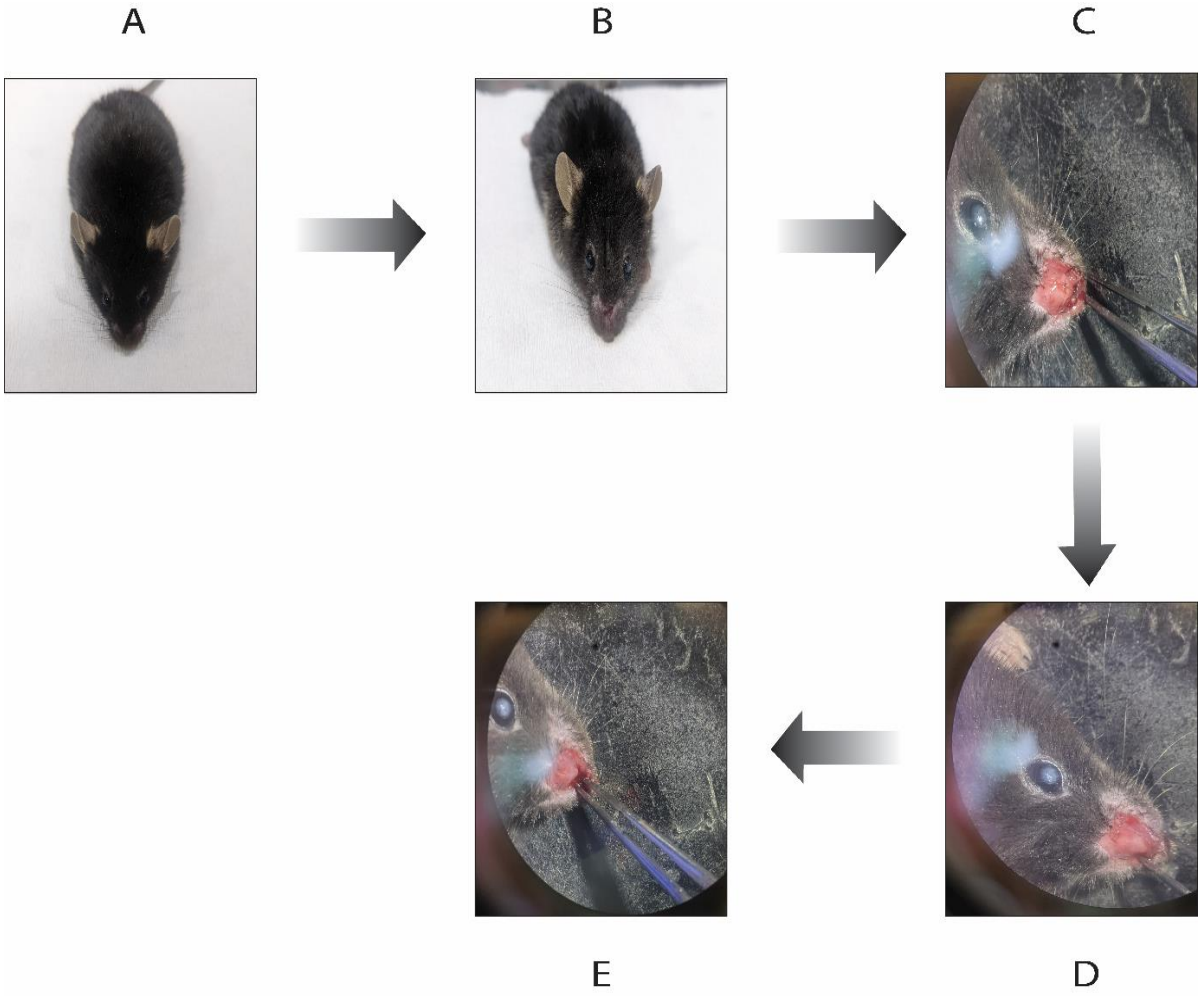

Fig 9: Procedural stages of GG axotomy.

A - The animal was left unconscious after administering a combination of ketamine and xylazine. **B** – A small incision is done on the skin covering the nasal cavity. **C** – The incision is extended till the rostral tip of the nose and patch is created. **D** – The patch is cleaned and collaterals of GG are located. **E** – GG is axotomized.

A small incision is made on the skin covering the nasal cavity. This is extended to the rostral tip of the nose. This incision is then made into a patch so that the nasal compartments are visible. The collaterals of GG entering beneath the nasal septum is visible followed by its axotomy.

3. Results

3.1 Temperatures inside and outside of rodent burrows are significantly different

In order to gain insight into the temperature patterns that animals are exposed to within their natural habitats, we utilized a hot wire anemometer to measure temperatures both inside and outside of suspected rodent burrows. The temperature readings were taken from early June to late August 2022. The average temperature inside and outside the burrows was obtained to be significantly different with 20.31 °C measured from inside and 23.63 °C measured from outside (Fig 10). Since while olfactory navigation and food foraging, animals usually move from inside the burrows to outside, the varying temperature profiles inside vs. outside can affect the odor properties. Hence, animals need to possess a sensory system that can sense this temperature difference, integrate this information with the olfactory centers, and enables efficient olfactory decision-making. Using this observation of differences (3 °C) in the temperature we attempted to address this question. Since GG is a potential candidate for temperature sensing and it shows higher activation below 22 °C degrees, we investigated the role of GG in temperature discrimination by training the animals to discriminate non-odorized air of 19 °C vs. 22.1°C (these temperatures are readings from a single trail from inside and outside, respectively, with the temperature difference being 3 °C.

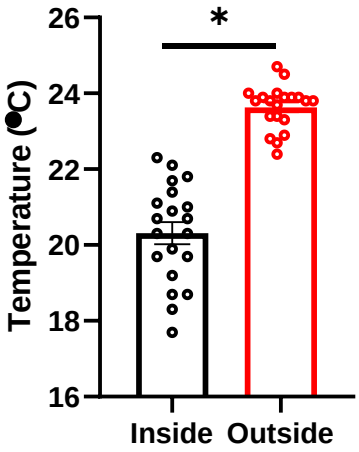

Fig 10: **Temperature measurements inside and outside rodent burrows in IISER Pune campus.** The temperature measured from inside the burrows was found to be significantly lower than outside (n = 20 in each category, mean value inside = 20.31°C, mean value outside = 23.63°C, unpaired two-tailed t-test, p = < 0.0001).

3.2 The custom-built thermo-olfactometer efficiently regulates the temperatures

We custom-built a thermo-olfactometer capable of delivering odorized/non-odorized air at different temperatures. The temperatures of the air were regulated using the thermostats that were integrated with the instrument.

For temperature regulation, the temperature was first set in the thermostat coupled to a set of valves. The temperature regulation was ensured with the loop

combined action of the temperature probe with the set of valves passing the cool air and the air at room temperature. The default setting of the system is the passing of cool air through the valves. Since the thermostat was set in heater mode (H), whenever the temperature dropped below the set temperature, the thermostat transmitted signals to the corresponding valves and thereby regulating the temperature back to the set temperature. Specifically, the pumping of cool air was restricted, while room temperature air was pumped in to increase the temperature. The protocols/algorithm for temperature regulation was integrated into the setup and is as follows (Note: for ease of explanation, temperature regulation is explained with the help of half of the instrument that is responsible for regulating one of the temperature pairs)

If

a. Measured temperature < set temperature
   e.g. set temperature on the thermostat is 19 °C
   If the temperature measured by the probe is <= 18.8 °C

The thermostat relays signals to valves 6 and 7 causing the default outputs (cool air) of valves 6 and 7 to close. This diverts the flow of valves to the outside of the main stimulus delivery tube, while simultaneously allowing room air to be pumped in. The room air was allowed to be regulated inside the tube with the help of Valve 7. Since cool air was diverted and room temperature air was pumped in, this mechanism led to an increase in the temperature of the total air, thereby, increasing the overall temperature till the set criteria were met (Fig 11a).

If

b. Measured temperature >= set temperature
   e.g. set temperature on the thermostat is 19 °C
   If the temperature measured by the probe is > 19 °C

The default outputs of valves 6 and 7 open with valve 6 passing air to the cooling chamber and valve 7 acting as an exhaust (Fig 11b). This pattern ensures that the temperature of the air is cooled till the time it reaches the set temperature. Figure 12 shows the integration of the thermostat into the setup and the regulation of temperature during a trial. During the temperature discrimination task, the program was coded in such a way that all of the temperature regulation occurred during the intertrial interval (ITI), which is the time in between the trials, to negate any interference and potential effects on the behaviour of the animal.

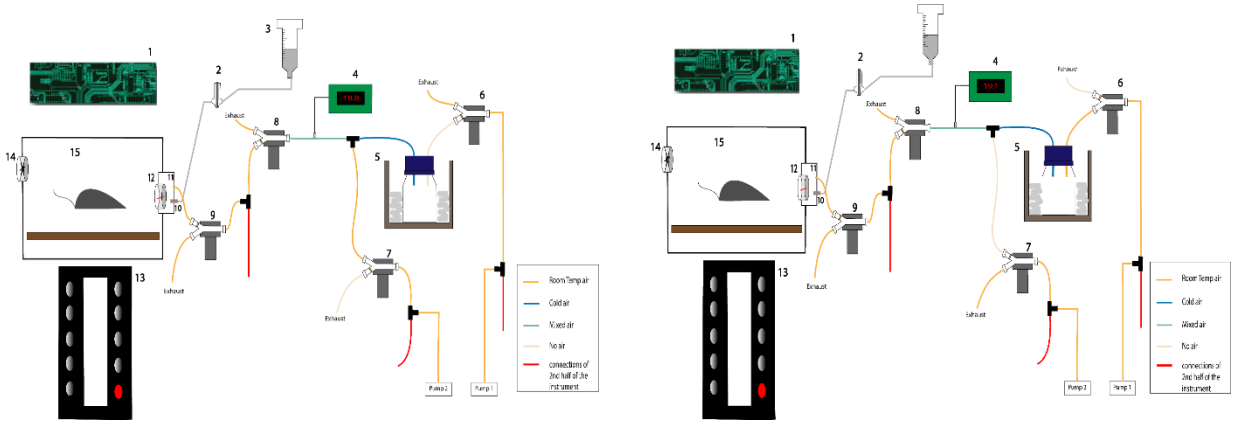

Fig 11: **Schematic representation of temperature regulation.** a) When temperature <= set temperature. b) When temperature > set temperature

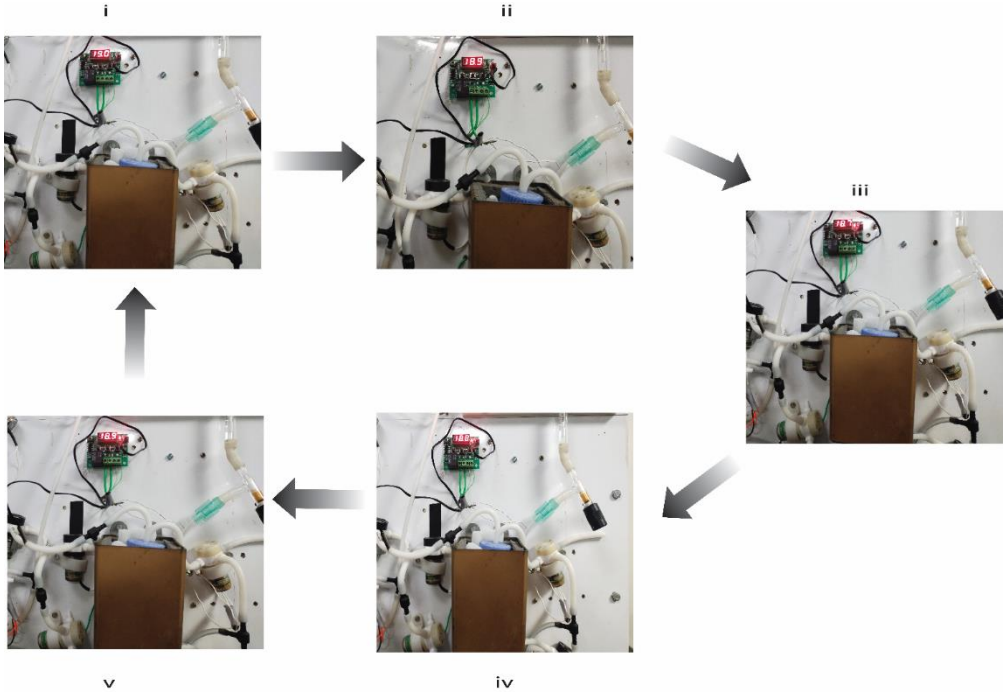

Fig 12: Temperature regulation by the thermostat integrated into the setup. Here the set temperature is 19 °C with the regulation least count being 0.3 °C. **i** – The temperature drops from room

temperature and reaches 19.0 °C. **ii** – The temperature continues to drop. **iii** - At 18.7 °C the thermostat sends signals to the valves (indicated by the red LED in the thermostat) to which it is integrated. **iv,v** – The combined loop action of the valves causes the temperature to rise until the temperature reaches the set temperature. Once it reaches the set temperature the thermostat stops relaying signals to the valves (red LED switched off), back to i.

3.3 Animals could successfully detect and discriminate between temperatures

To investigate whether animals are capable of detecting and discriminating temperatures that they encounter in their natural habitat, the animals were trained on a temperature discrimination task with a temperature difference (Δt) of 3.1 degrees Celsius. Initially, the animals performed at 50% accuracy (chance level) as they couldn't differentiate rewarded (S+) and non-rewarded (S-) trials. However, over time, the animals learned to associate the S+ and S- cues with the respective reward contingency and performed accordingly, showing an increase in the average accuracy as the tasks progress. The animals reached a performance level of above 90% accuracy within 1200-1500 trials (Fig 13a). Moreover, d', which is another way of showing the performance of animals that takes into account the correct hit and false alarms, also showed an increase as the learning progressed (Fig 13b). The preference of animals towards any temperature was mitigated by counterbalancing the stimuli i.e. for half animals in a group one temperature is S+ and for the other half, the other temperature is S+.

As the discrimination task depends on the animal's ability to discriminate the stimuli and its motivation to perform the task (as water is used as a reward), the animal's motivation level can have a profound impact on the percentage correct. Additionally, the motivation levels of animals were monitored using the inter-trial interval (ITI), which is essentially the time taken by animals in between two consecutive trials. A large ITI indicates low motivation in animals, while a smaller ITI indicates over-motivation. Previous results from our lab show that the optimal ITI lies in the range of 10-15 seconds. During learning, ITI was observed to be in the similar range for all the tasks, suggesting that animals performed with optimal motivation. Taken together, our findings suggest that animals are capable of efficiently discriminating temperature differences and reaching the asymptotic phase of learning in 1200-1500 trials. Further studies are required to understand the neural mechanisms underlying temperature detection and discrimination in mice.

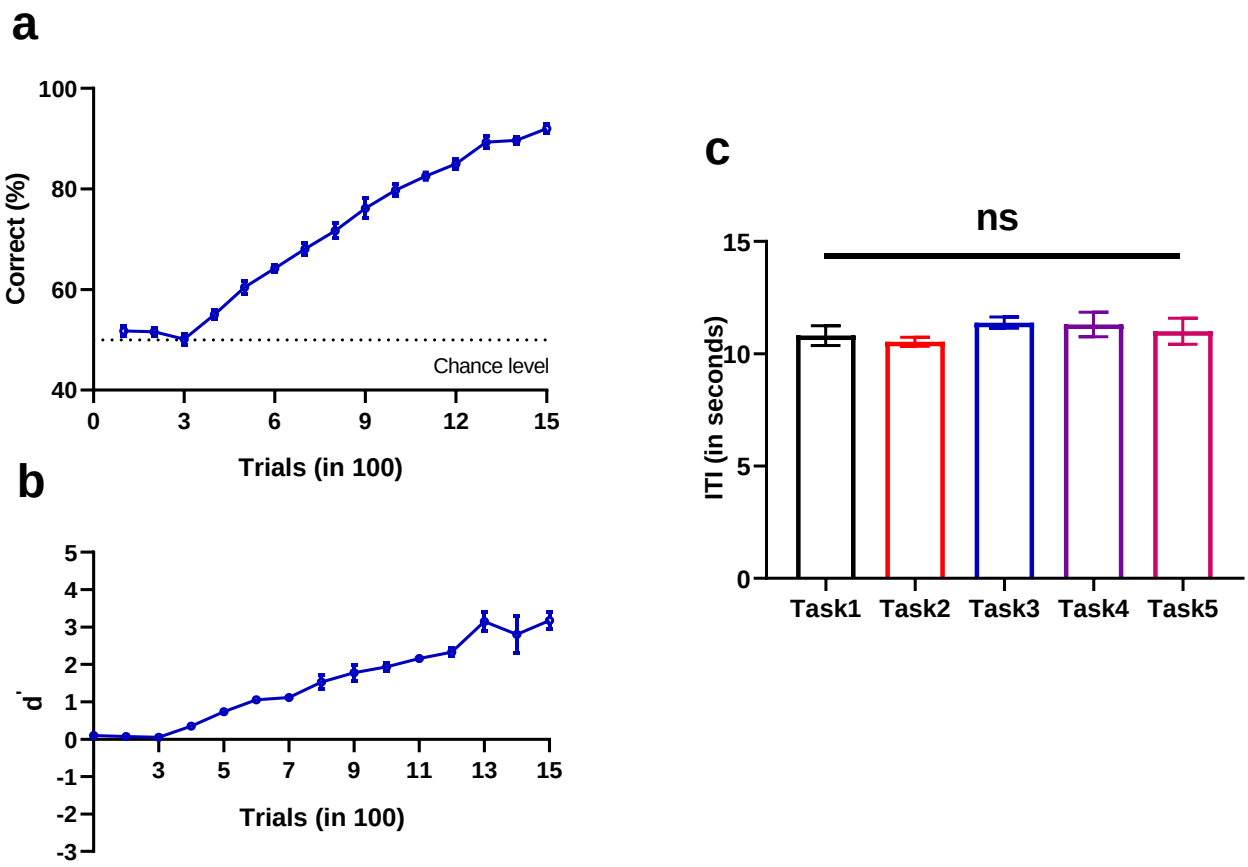

Fig 13: Performance of animals during a temperature discrimination task: 19 °C vs. 22.1 °C (n = 13).

a) The percentage of correct responses (correct percentage) exhibited by mice in a temperature discrimination task is represented as the learning curve. Each data point in the learning curve is the average accuracy of 100 trials averaged across the 13 animals (Mean ± SEM) **b)** d' was calculated as z (correct S+) – z (incorrect S-) per 100 trials. Each point on the graph is d' of each animal per 100 trials averaged across all animals (Mean ± SEM). **c)** The inter-trial interval (ITI) between two consecutive trials is used as a measure to evaluate the animal's motivation level in different tasks (Mean ± SEM). The ITIs shown by animals across different tasks were similar (One-way repeated measures ANOVA, $F = 0.8469$, $p = 0.44$).

3.3.1 Learning-dependent refinement in licking and sampling behaviour

While animals are being challenged to differentiate different temperatures, they had to lick for the S+ stimulus and refrain its licking for an S- stimulus for successful discrimination. In addition, since the lick and sampling port are coupled and the sampling port is guarded by the IR beam, the profile of the beam break can be used as a readout for the sampling pattern. Hence, licking and sampling patterns can be used to assess the learning of the animals as well.

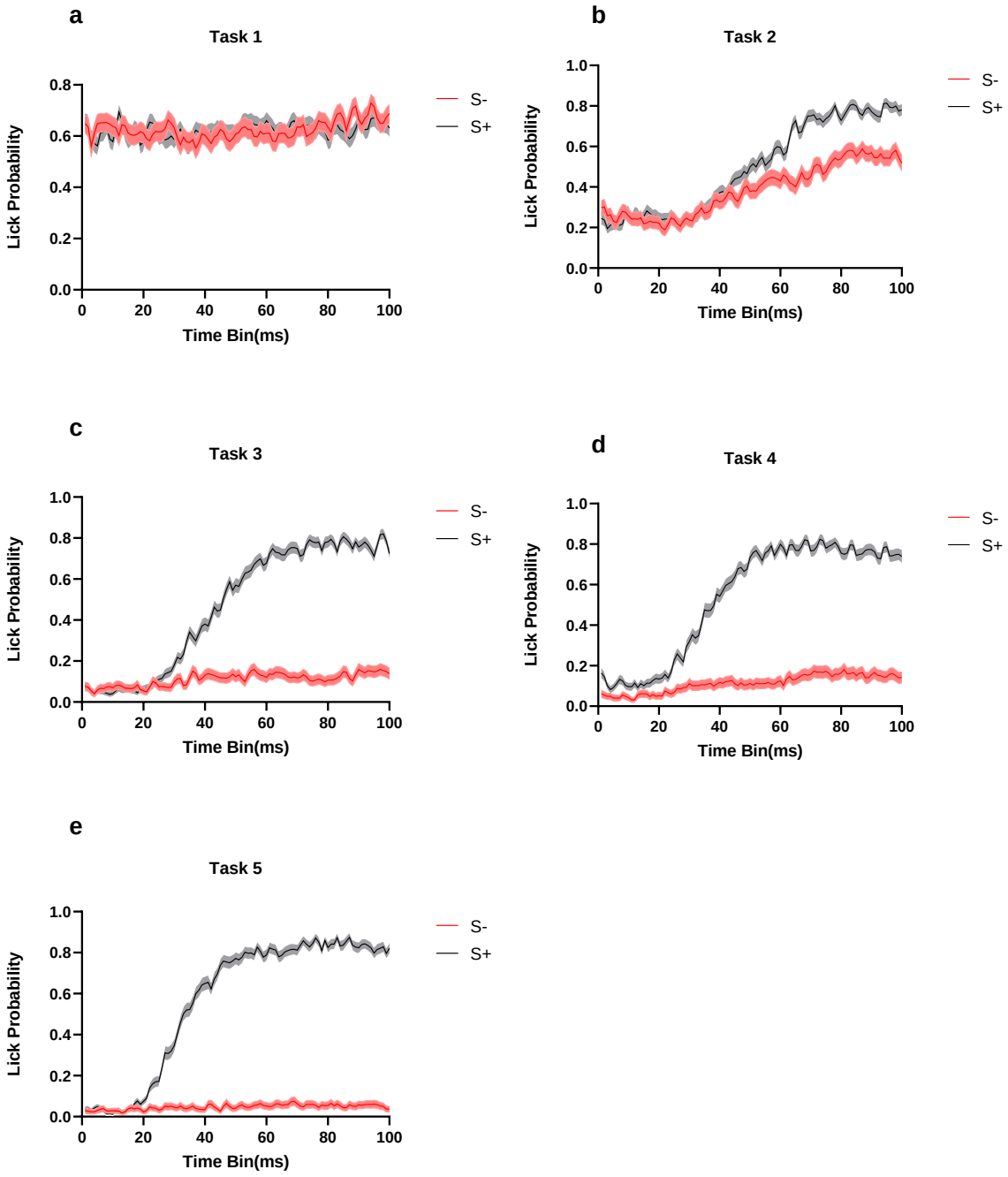

Fig 14: Lick probability of animals for S+ and S- stimuli across 5 different tasks during temperature discrimination

a) In task 1, the animals are unable to differentiate the two stimuli therefore lick for both stimuli are overlapping. **b)** Animals show some learning and hence they refrain from licking for a few S- trials. **c,d)** Animals gradually learn to discriminate the two stimuli which is evident in the gradual divergence between the S+ and S- lick curves. **e)** Once animals reach an accuracy of over 80%, they only lick for S+ trials and refrain from licking for almost all S- trials.

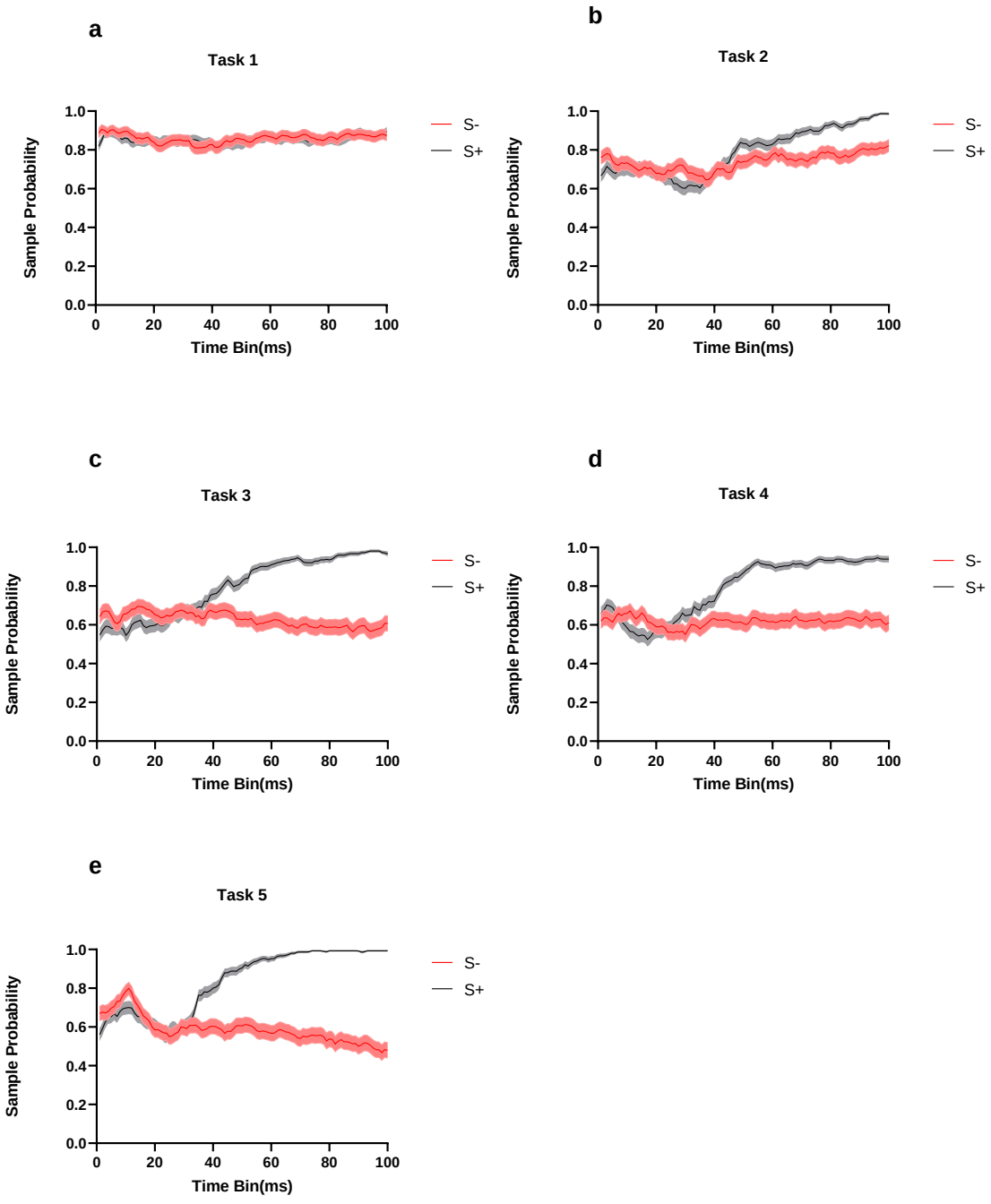

Fig 15: Sampling behaviour of animals for S+ and S- stimuli across 5 different tasks during temperature discrimination

a) In task 1, the animals are unable to differentiate the two stimuli therefore sample patterns for both stimuli are overlapping. **b)** Animals show some learning and hence they refrain from licking for a few S- trials and retract their head for S- trials. **c,d)** Animals gradually learn to discriminate the two stimuli

which is evident in the gradual divergence between the S+ and S- sampling pattern curves. **e)** Once animals reach an accuracy of over 80%, they only lick for S+ trials, thereby showing higher sampling, whereas they refrain from licking for almost all S- trials, and hence sampling decreases for S- trials.

During the course of learning, when animals are in Task 1, the licking and sampling behaviour of the animals for both the S+ and S- stimulus are highly overlapping. However, as the learning progresses animals learn to not lick for S- trials and retract its head (resealing of the IR beam), and the refinement in the licking and sampling behaviour takes place. This refinement can be observed as the increase in divergence between the S+ and S- licking and sampling patterns as a function of the learning i.e. as the animal learns to discriminate the stimuli, the divergence in the lick and sample pattern increases (Fig 14 and Fig 15).

3.3.2 Licking and sampling patterns are readouts to quantify the discrimination time of the animals

In addition to the learning accuracies, we also quantified the reaction time of the animals that they take to discriminate the S+ and S- stimuli. It is the last time point where the difference in lick or sampling between the S+ and S- stimuli is significant. This reaction time is termed as the discrimination time (DT) and can be calculated from both the lick and sample pattern (Fig 16). The DTs calculated from lick and sample patterns were found to be non-significant. However, for an S- trial, although the animal learns to not lick to register a correct trial, there is no rule set for the sampling. Therefore, even though the animal does not lick it can still sample the stimulus which can lead to variabilities while calculating the DT from the sampling pattern. Hence, the lick pattern is a more robust readout of the DT and is used in further analysis.

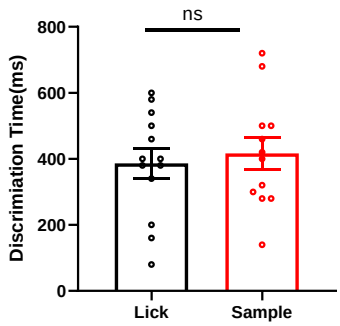

Fig 16: Comparison of DTs calculated from the lick and sampling pattern (average ± SEM**).** No difference in the DTs were observed when they were quantified using either lick or sample pattern (two-tailed paired t-test, p = 0.6485).

3.3.3 Licking-derived discrimination time and discrimination index can be used as additional readouts for performance

As the learning progress, the divergence of the licking responses for S+ and S- stimuli increases (Fig 14). When DT was quantified and compared across different tasks, a decrease in DT was observed as the learning progressed. In Task 1 the average DT was 2000 ms, Task 2 – 1620 ms, Task 3 – 838.5 ms, Task 4 – 509.2 ms, and Task 5 – 386.2 ms (Fig 17a). This decrease in DTs with learning provided complementary evidence for an increase in the divergence between the S+ and S- licking responses. Moreover, owing to this divergence, we quantified yet another parameter known as the discrimination index, which can also be used as the readout for learning. We quantified the discrimination index by taking the differences in the area under the curve of S+ and S- stimuli into account. As the learning progressed, the discrimination index increased (Fig 17b). These results show that animals can discriminate different temperatures accurately within 386 ms. Additionally, these findings also indicate that the DTs and the discrimination index calculated based on the licking pattern can also be used as a readout for learning.

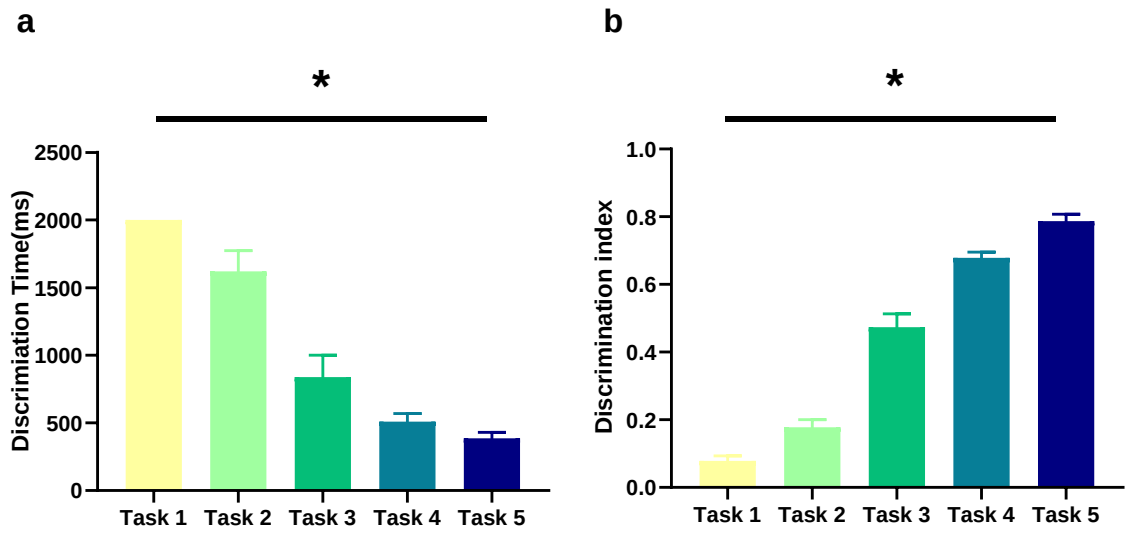

Fig 17: Discrimination time and discrimination index for wild-type animals calculated from the lick pattern for different tasks. a) Discrimination time (DT) for five tasks. The DT decreases as the learning progresses (one-way repeated measures ANOVA, $F = 177.9$, $p < 0.0001$). **b)** The discrimination index is calculated using the area under the curves for the licking responses of animals for S+ and S- stimuli. The discrimination index is observed to increase as a function of the learning (one-way repeated measures ANOVA, $F = 56.95$, $p < 0.0001$).

3.4 Grueneberg Ganglion (GG) mediates the temperature discrimination in rodents

Our results so far demonstrate that animals can detect and discriminate the temperatures efficiently. However, the sensory system mediating this discrimination remains elusive. Since GG is previously known to sense cool temperatures, we investigated whether temperature discrimination can be mediated by GG. To achieve this, a subset of animals that were previously trained on temperature discrimination were subjected to axotomy of GG. These animals were then given a recovery period of 2 days. Following the recovery, the water deprivation cycle was started. Animals were then challenged to discriminate the same temperature pair (19°C vs 22.1°C, which they learned to discriminate before the GG axotomy). The animals were trained for 300 trials and their performance was compared with their performance before the treatment and with that of the animals that underwent sham treatment.

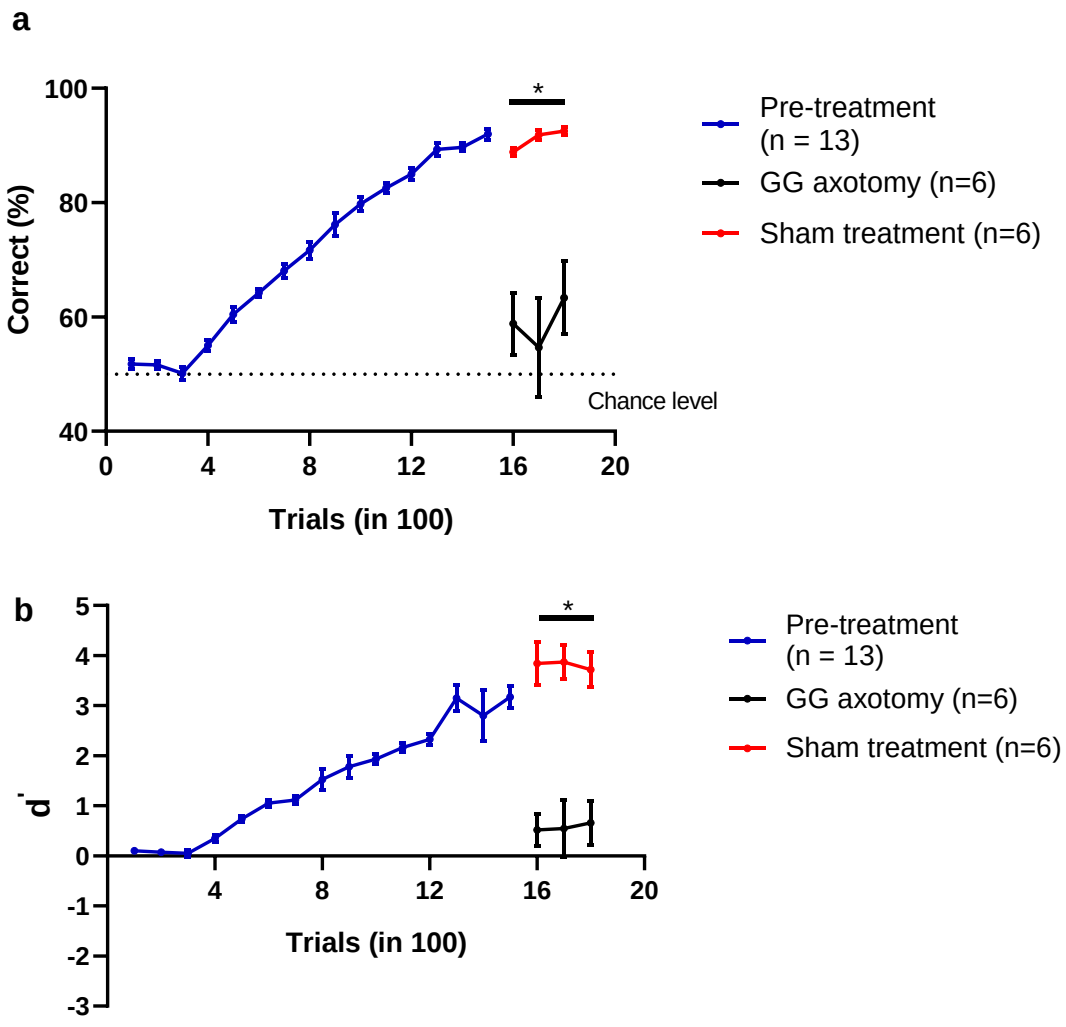

Fig 18: GG mediates the temperature discrimination in mice

a) Comparison of accuracies of mice after the GG axotomy (n=6) and sham treatment (n=6). The accuracy of animals that underwent GG axotomy was significantly lower than that before the treatment and the animals with sham treatment (two-way repeated measure ANOVA, F = 23.63, p = 0.0007). **b)** Comparison of d' of animals post GG axotomy. The d' of animals that underwent GG axotomy was significantly lower than that before the treatment and the animals with sham treatment (two-way repeated measure ANOVA, F = 42.03, p < 0.0001).

Following GG axotomy, the animals showed a significant deficit in their ability to discriminate the temperatures. Both the accuracies and the d' post-GG axotomy were significantly lower than those observed before axotomy and sham treatment (Fig 18a and Fig 18b). In addition to the accuracies and d', we also compared the discrimination time and index before and after the GG axotomy and sham treatments. The DTs post the axotomy increased significantly to that of the DT before the axotomy. Moreover, the DTs for the GG axotomy subgroup were significantly higher than that of the animals with sham treatment (Fig 19a). Further, the discrimination index post-axotomy was significantly lower than that of the discrimination index before the treatment and animals with sham treatment (Fig 19b).

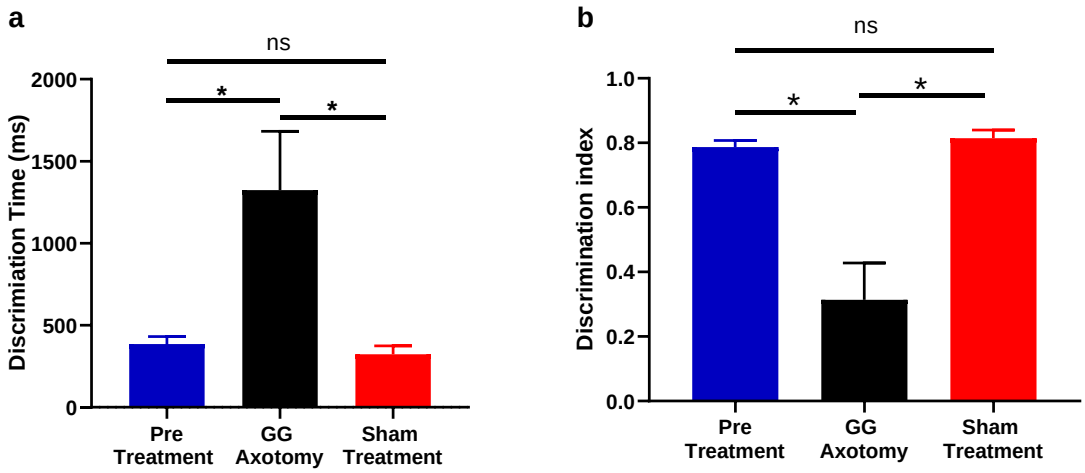

Fig 19: The GG axotomized animals show higher discrimination time and lower discrimination index

a) Comparison of DTs of mice after the GG axotomy and sham treatment. The DTs of animals that underwent GG axotomy were significantly higher than that before the treatment and the animals with sham treatment (one-way ANOVA with Tukey's multiple comparisons, F = 10.82, p = 0.0005). **b)** Comparison of discrimination index of animals after the GG axotomy. The discrimination index of animals that underwent GG axotomy was significantly lower than that before the treatment and the

animals with sham treatment (one-way ANOVA with Tukey's multiple comparisons, F = 24.85, p < 0.0001).

Further, we compared the ITIs in order to investigate whether the motivation level of animals had any influence on the observed differences in the learning and reaction time observed. ITIs were calculated and compared for both groups which are axotomized group (pre vs. post-treatment, Fig 20a) and the sham group (pre vs. post-treatment, Fig 20b). For both groups (pre and post-treatment) ITIs remained consistent and in the ideal range implying that the animals had an optimum level of motivation with no influence on the readouts. Hence, our results provide evidence for a possible role of GG in temperature detection and discrimination in rodents.

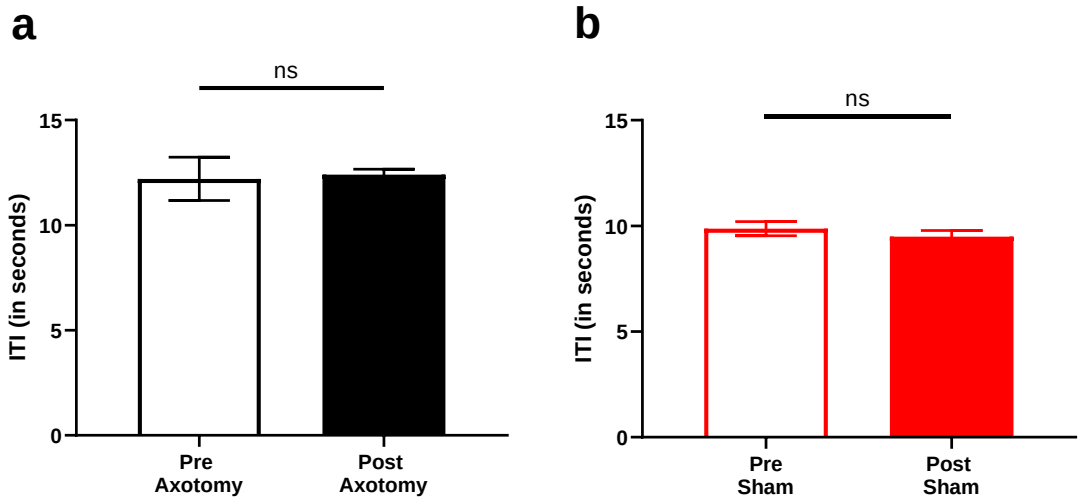

Fig 20: The ITI of animals before and after the treatments were similar

a) Comparison of ITIs of mice before and after the GG axotomy. The ITIs of animals was similar before and after the GG axotomy (two-tailed paired t-test, p = 0.8499). **b)** Comparison of ITIs of mice before and after the sham treatment. The ITIs of animals was similar before and after the sham treatment (two-tailed paired t-test, p = 0.4966).

3.5. Animals' ability to discriminate the multimodal stimuli is higher than the unimodal stimuli

Thus far, our results confirm that the GG is essential for discriminating the temperatures. As GG is a subsystem within the olfactory system, it remains unclear whether temperature can influence olfactory perception. Therefore, to investigate the

effects of temperature on olfactory perception and decision-making, we challenged the animals to discriminate two different odours coupled with the airflows of different temperatures. In this multimodal discrimination task, the odorants (Benzaldehyde and Valeraldehyde) that do not show any differences in their photoionisation detector (PID) profiles at both temperatures (19 vs. 22.1 °C) were chosen. Animals were trained to discriminate benzaldehyde at 22.1 °C vs. valeraldehyde at 19 °C.

The average accuracy of the animals in this multimodal task (temperature + odor) reached more than 80% within 600-900 trials. On comparing its learning pace with that of the unimodal “temperature” discrimination task, the discrimination learning pace was significantly higher for the multimodal task (Fig 21a). The d’ across the progression of training was also significantly higher for the multimodal task (Fig 21b). In addition, we also quantified and compared the discrimination time and lick-associated discrimination index for both the unimodal and multimodal groups. While discrimination time across different tasks was observed to be higher when animals were challenged to discriminate among the unimodal stimuli, the discrimination index calculated based on the lick pattern was significantly higher for the multimodal training (Fig 22a and 22b). The differences observed in the learning are not because of any differences in the motivation levels as the overall ITIs for both groups of animals across the training phase were in the optimal range and similar (Fig 22c). Taken together, these results demonstrate that animals learn to discriminate multimodal stimuli faster than unimodal stimuli (only temperature). However, further experiments are required to probe the neuronal circuitry underlying the multimodal (temperature + odour) information processing in the olfactory centers of the brain.

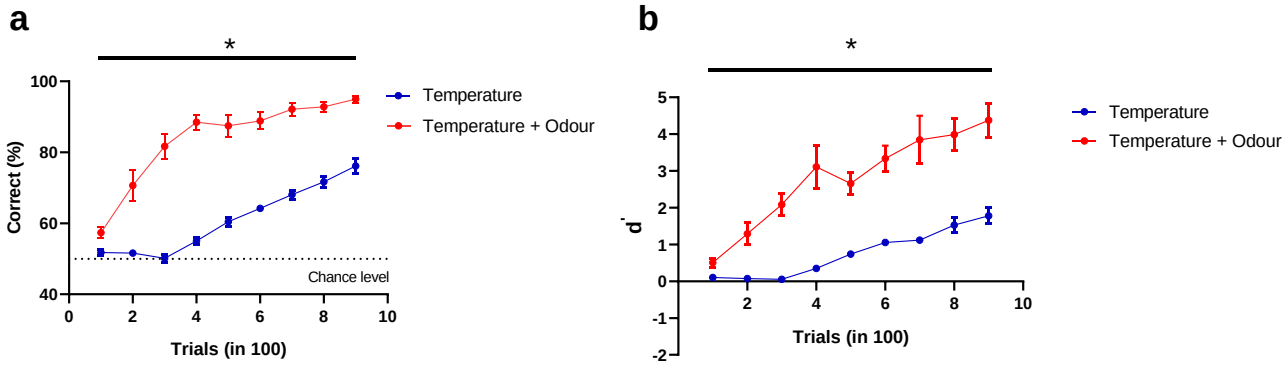

Fig 21: The learning pace of animals is higher for multimodal stimuli

a) Comparison of learning of mice while they are being trained to discriminate unimodal (temperature) and multimodal (temperature + odour) stimuli. The learning of animals was significantly higher when animals were trained to discriminate the multimodal stimuli (two-way ANOVA, $F = 243.3$, $p < 0.0001$). **b)** Comparison of d' of mice while they are being trained to discriminate unimodal (temperature) and multimodal (temperature + odour) stimuli. The d' of animals was significantly higher when animals were trained to discriminate the multimodal stimuli (two-way ANOVA, $F = 42.03$, $p < 0.0001$).

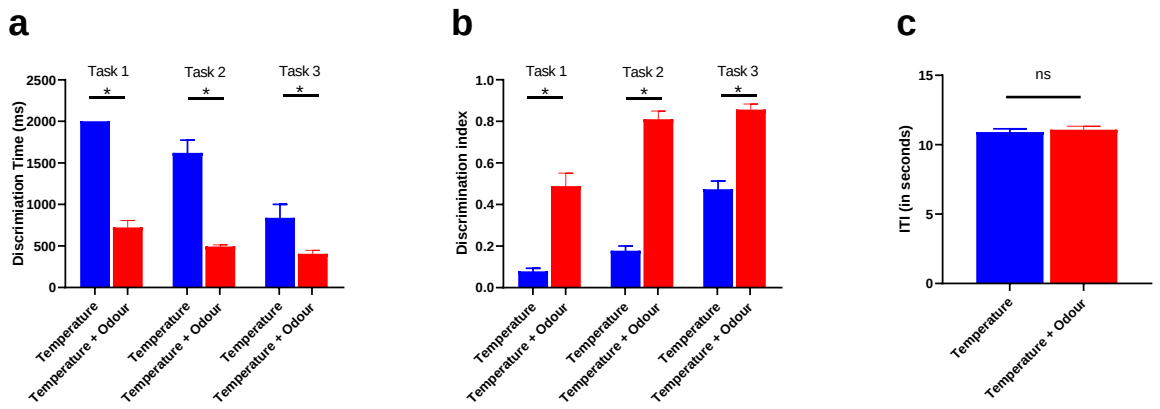

Fig 22: The animals trained on multimodal stimuli show lower discrimination times

a) Comparison of discrimination time (DT) of mice while they are being trained to discriminate unimodal (temperature) and multimodal (temperature + odour) stimuli. The DT of animals across different tasks was significantly higher when animals were trained to discriminate the unimodal stimuli (one-tailed unpaired t-test for each task, $*p < 0.05$). **b)** Comparison of discrimination index of mice while they are being trained to discriminate unimodal (temperature) and multimodal (temperature + odour) stimuli. The lick-associated discrimination index of animals across different tasks was significantly higher when animals were trained to discriminate the multimodal stimuli (one-tailed unpaired t-test for each task, $*p < 0.05$). **c)** Comparison of ITIs of mice while they are being trained to discriminate unimodal (temperature) and multimodal (temperature + odour) stimuli. The overall ITI of animals during discrimination training was similar between the two groups (one-tailed unpaired t-test for each task, ns: not-significant, $p > 0.05$).

4. Discussion

In the natural environment, animals encounter a variety of sensory signals that are sensed and processed by different sensory systems. While most sensory stimuli are unimodal and can only activate a specific sensory apparatus, the rodent's olfactory system is unique in that it can sense stimuli with different physicochemical properties. Olfactory sensory neurons (OSNs) in the main olfactory epithelium (MOE), septal organ (SO), and vomeronasal organ (VNO) can sense volatile and non-volatile

odorants as well as mechanical pressures, while the trigeminal nerve fibers within the nasal cavity, known as the nasal trigeminal subsystem or Grueneberg Ganglion (GG), can detect temperature cues in the environment (Grosmaitre et al., 2007; Mamasuew et al., 2008; Tian & Ma, 2008). While there has been extensive research on the mechanisms involved in chemical and mechanical sensing, the sensory system responsible for temperature sensing and its underlying mechanisms has not been fully understood. In our study, we developed a thermo-olfactometer to investigate the sensory system responsible for temperature sensing. We trained animals to perform temperature discrimination using this apparatus. In this study, the temperatures used for discrimination were selected based on readings obtained from inside and outside of potential rodent habitat burrows (19 °C vs 22.1 °C). We wanted to ensure that the temperatures used in the study were etiologically relevant to the animals and resembled their natural habitat conditions. Therefore, we measured the temperature fluctuations from inside and outside of the rodent burrows and selected the range of temperatures that the animals are more likely to experience in their natural environment. These temperatures were then used in our custom-built thermo-olfactometer to test the animals' ability to discriminate between them. We observed that animals were able to do so effectively, achieving discrimination within 1200-1500 trials with a discrimination time of approximately 380 milliseconds. This suggests that the animals have a well-developed sensory system for temperature detection.

Further, in order to study the olfactory subsystem involved in temperature detection and discrimination, we conducted a procedure known as GG axotomy on the GG and tested the rodents' ability to perform a temperature discrimination task that they had previously learned. We observed a notable decrease in accuracy and d’, as well as a decrease in the discrimination index, and an increase in discrimination time, in the rodents that underwent axotomy of the GG. These findings show that the Grueneberg ganglion is involved in mediating temperature discrimination in rodents. Our findings provide further evidence to support the notion that GG plays a critical role in temperature perception, thereby expanding the information provided by previous studies showing the role of GG in sensing cool temperatures in neonatal mice (Mamasuew et al., 2008).

The olfactory system is complex as it consists of different subsystems, and the cross-talk between information from different subsystems within it can affect olfactory perception. One of the subsystems is the GG which consists of receptors that can

sense a type of odorant (Chao et al., 2015; Fleischer, 2021). Given the newfound involvement of GG in temperature sensation, it becomes crucial to explore the potential cross-talk between odor and temperature information processing and how this interaction might modulate olfactory perception. A potential effect of the cross-talk is multisensory enhancement. Multisensory enhancement is a phenomenon wherein an increase in the ability of animals to discriminate coupled sensory stimuli is observed compared to a single sensory system. The enhancement effect is particularly pronounced when the sensory cues are weak or at subthreshold levels. In such cases, the brain integrates weak sensory signals from different modalities to create a stronger perceptual experience (Lunn et al., 2019). To test whether a similar phenomenon can occur in the olfactory system, we conducted a multimodal discrimination task where one temperature was coupled with one odorant. Our result shows that coupling temperature with odorant leads to faster learning compared to a unimodal temperature discrimination task. This result supports the idea that the integration of temperature information can influence and enhance olfactory perception. Further experiments including odour discrimination tasks at the same temperature are necessary to confirm this finding. These experiments will also provide a more comprehensive understanding of the effect of temperature on olfactory perception.

4.1 Future directions

As our study has demonstrated a possible role of GG in adult mice in detecting cool temperatures associated with their natural habitat, our next objective is to look at the neural mechanisms underlying this behaviour. To accomplish this, we intend to quantify neural activity using c-Fos (of both axotomized and sham groups) in the necklace glomeruli region. To confirm the role of GG in thermosensation in rodents we also aim to combine multimodal experiments with GG axotomy where GG axotomized mice will be trained to discriminate multimodal stimuli (different odours coupled with different temperatures). Further to confirm the effect of temperature on olfactory perception, an odour alone control is necessary for which we aim to train animals to discriminate two different odours (the same odours used for the multimodal experiment but at the same temperature). To dissect the neural mechanisms underlying these behaviours, we will quantify their neural activity using c-Fos in different olfactory bulb regions. This will enable us to identify the regions and their relative activation in a

variety of discrimination tasks (unimodal and multimodal). Further, to provide a causal link between behaviour and neuronal activity, in-vivo imaging will be used to record from these different regions while animals are actively involved in these behavioural tasks.

5. References

Abraham, N. M., Spors, H., Carleton, A., Margrie, T. W., Kuner, T., & Schaefer, A. T. (2004). Maintaining Accuracy at the Expense of Speed. *Neuron*, *44*(5), 865–876. https://doi.org/10.1016/j.neuron.2004.11.017

Barrios, A. (2014). Behavioral Plasticity: A Nose for Every Season. *Current Biology*, *24*(21), R1057–R1059. https://doi.org/10.1016/j.cub.2014.09.062

Brechbühl, J., Moine, F., & Broillet, M.-C. (2013). Mouse Grueneberg ganglion neurons share molecular and functional features with C. elegans amphid neurons. *Frontiers in Behavioral Neuroscience*, *7*. https://doi.org/10.3389/fnbeh.2013.00193

Bumbalo, R., Lieber, M., Schroeder, L., Polat, Y., Breer, H., & Fleischer, J. (2017). Grueneberg Glomeruli in the Olfactory Bulb are Activated by Odorants and Cool Temperature. *Cellular and Molecular Neurobiology*, *37*(4), 729–742. https://doi.org/10.1007/s10571-016-0408-6

Chamero, P., Leinders-Zufall, T., & Zufall, F. (2012). From genes to social communication: Molecular sensing by the vomeronasal organ. *Trends in Neurosciences*, *35*(10), 597–606. https://doi.org/10.1016/j.tins.2012.04.011

Chao, Y., Chen, C., Lin, Y., Breer, H., Fleischer, J., & Yang, R. (2015). Receptor guanylyl cyclase- G is a novel thermosensory protein activated by cool temperatures. *The EMBO Journal*, *34*(3), 294–306. https://doi.org/10.15252/embj.201489652

Dulac, C., & Torello, A. T. (2003). Molecular detection of pheromone signals in mammals: From genes to behaviour. *Nature Reviews Neuroscience*, *4*(7), 551–562. https://doi.org/10.1038/nrn1140

Fleischer, J. (2021). The Grueneberg ganglion: Signal transduction and coding in an olfactory and thermosensory organ involved in the detection of alarm pheromones and predator-secreted kairomones. *Cell and Tissue Research*, *383*(1), 535–548. https://doi.org/10.1007/s00441-020-03380-w

Fleischer, J., Mamasuew, K., & Breer, H. (2009). Expression of cGMP signaling elements in the Grueneberg ganglion. *Histochemistry and Cell Biology*, *131*(1), 75–88. https://doi.org/10.1007/s00418-008-0514-8

Fleischer, J., Schwarzenbacher, K., Besser, S., Hass, N., & Breer, H. (2006). Olfactory receptors and signalling elements in the Grueneberg ganglion. *Journal of Neurochemistry*, *98*(2), 543–554. https://doi.org/10.1111/j.1471-4159.2006.03894.x

Fleischer, J., Schwarzenbacher, K., & Breer, H. (2007). Expression of Trace Amine-Associated Receptors in the Grueneberg Ganglion. *Chemical Senses*, *32*(6), 623–631. https://doi.org/10.1093/chemse/bjm032

Fuss, S. H., Omura, M., & Mombaerts, P. (2005). The Grueneberg ganglion of the mouse projects axons to glomeruli in the olfactory bulb. *European Journal of Neuroscience*, *22*(10), 2649–2654. https://doi.org/10.1111/j.1460-9568.2005.04468.x

Greer, C., Stewart, W., Teicher, M., & Shepherd, G. (1982). Functional development of the olfactory bulb and a unique glomerular complex in the neonatal rat. *The Journal of Neuroscience*, *2*(12), 1744–1759. https://doi.org/10.1523/JNEUROSCI.02-12-01744.1982

Greer, P. L., Bear, D. M., Lassance, J.-M., Bloom, M. L., Tsukahara, T., Pashkovski, S. L., Masuda, F. K., Nowlan, A. C., Kirchner, R., Hoekstra, H. E., & Datta, S. R. (2016). A Family of non-GPCR Chemosensors Defines an Alternative Logic for Mammalian Olfaction. *Cell*, *165*(7), 1734–1748. https://doi.org/10.1016/j.cell.2016.05.001

Grosmaitre, X., Santarelli, L. C., Tan, J., Luo, M., & Ma, M. (2007). Dual functions of mammalian olfactory sensory neurons as odor detectors and mechanical sensors. *Nature Neuroscience*, *10*(3), 348–354. https://doi.org/10.1038/nn1856

Koos, D. S., & Fraser, S. E. (2005). The Grueneberg ganglion projects to the olfactory bulb. *NeuroReport*, *16*(17), 1929–1932. https://doi.org/10.1097/01.wnr.0000186597.72081.10

Liu, C. Y., Fraser, S. E., & Koos, D. S. (2009). Grueneberg ganglion olfactory subsystem employs a cGMP signaling pathway. *The Journal of Comparative Neurology*, *516*(1), 36–48. https://doi.org/10.1002/cne.22096

Lunn, J., Sjoblom, A., Ward, J., Soto-Faraco, S., & Forster, S. (2019). Multisensory enhancement of attention depends on whether you are already paying attention. *Cognition*, *187*, 38–49. https://doi.org/10.1016/j.cognition.2019.02.008

Mamasuew, K., Breer, H., & Fleischer, J. (2008). Grueneberg ganglion neurons respond to cool ambient temperatures. *European Journal of Neuroscience*, *28*(9), 1775–1785. https://doi.org/10.1111/j.1460-9568.2008.06465.x

Mombaerts, P. (2004). Genes and ligands for odorant, vomeronasal and taste receptors. *Nature Reviews Neuroscience*, *5*(4), 263–278. https://doi.org/10.1038/nrn1365

Ronnett, G. V., & Moon, C. (2002). G Proteins and Olfactory Signal Transduction. *Annual Review of Physiology*, *64*(1), 189–222. https://doi.org/10.1146/annurev.physiol.64.082701.102219

Roppolo, D., Ribaud, V., Jungo, V. P., Lüscher, C., & Rodriguez, I. (2006). Projection of the Grüneberg ganglion to the mouse olfactory bulb. *European Journal of Neuroscience*, *23*(11), 2887–2894. https://doi.org/10.1111/j.1460-9568.2006.04818.x

Stebe, S., Schellig, K., Lesage, F., Breer, H., & Fleischer, J. (2014). The Thermosensitive Potassium Channel TREK-1 Contributes to Coolness-Evoked Responses of Grueneberg Ganglion Neurons. *Cellular and Molecular Neurobiology*, *34*(1), 113–122. https://doi.org/10.1007/s10571-013-9992-x

The Grueneberg ganglion: A novel sensory system in the nose. (2010). *Histology and Histopathology*, *25*, 909–915. https://doi.org/10.14670/HH-25.909

Tian, H., & Ma, M. (2008). Activity plays a role in eliminating olfactory sensory neurons expressing multiple odorant receptors in the mouse septal organ. *Molecular and Cellular Neuroscience*, *38*(4), 484–488. https://doi.org/10.1016/j.mcn.2008.04.006