Olfactory subsystems in rodents: effects of temperature on odour perception

A Thesis

Submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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April, 2020

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Certificate

This is to certify that this dissertation entitled **Olfactory subsystem in rodents:effects of temperature on odour perception**, towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Felix Jose K at Indian Institute of Science Education and Research under the supervision of Dr. Nixon M Abraham, Assistant Professor, Department of Biology, during the academic year 2019-2020.

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Committee: Biology

Guide: Dr. Nixon M Abraham

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Declaration

I hereby declare that the matter embodied in the report entitled **Olfactory subsystem in rodents:effects of temperature on odour 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 M Abraham and the same has not been submitted elsewhere for any other degree

Felix Jose K
02/04/2020

Dr. Nixon M Abraham

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Abstract

In nature, an individual is continuously inundated with multiple sensory stimuli. Adequate reception of information from these stimuli is quintessential for decision-making. The process of decision-making requires activation of sensory peripheral organs that further transmit sensory inputs to higher centers of the brain. One mode of information processing is the activation of parallel neural pathways. Alternatively, information from multiple sensory cues can be integrated, leading to multi-sensory percept formation. Different sensory systems are specialized to detect different kinds of sensory stimuli based on their physicochemical nature. Therefore, information processing through multiple sensory systems is required for multisensory percept formation. However, the rodent olfactory system is known to process information about different types of stimuli. The olfactory system comprises of four subsystems that can detect stimuli of various kinds, e.g., temperature, chemical cues, and mechanical pressure. As a part of my thesis, I investigated decision-making in a temperature and odour dependent task. We used the Go/No-Go operant conditioning paradigm to train the animals to discriminate among different temperatures, different odourants, and different pairs of temperature-odourants coupled stimuli. We observed that the animals were able to perform temperature and odour discrimination tasks accurately. The pace of learning during the multi-sensory scenario was quicker than that of temperature discrimination, however, similar to that of odour discrimination. Our results suggest that odour cues may have higher importance over temperature cues, while animals rely on different olfactory subsystems to make the decisions. Investigating activation profiles in areas receiving information from Main Olfactory Epithelium and Gruenberg Ganglion will facilitate us in determining varying mechanisms for different training paradigms that we used.

Acknowledgments

I would like to thank my thesis supervisor, Dr. Nixon M. Abraham for his valuable mentorship and constant guidance during my thesis work. I would also like to thank my TAC member, Dr. Raghav Rajan for following my project and giving me appropriate suggestions. I am thankful to Sarang Mahajan for his immensely valuable mentorship during my work. And also special thanks to Sidharth Adityan, Meher, Priya, Lisni and Atharva for helping me at all points of the thesis work. I am grateful to, Shruti, Eleanor, Anindya, and Meenakshi for their constant support and encouragement. I am also thankful to Kaushik, Suhel, Rajdeep, Sanyukta, Kishalay, Avi, Arpan and Rishab for making my stay in the lab joyful. I am appreciative to NFGFHD for providing animals in a timely manner and maintain them in a healthy manner. Also appreciative to the IISER Engineering department for availing materials for the instrumentation. Finally, I would like to thank my parents and friends for their relentless support throughout the project duration.

1. Introduction

The ability to detect, discriminate, and respond to a particular stimuli marks the primary attributes required by a living organism for its survival. Different behaviours shown by animals are a true reflection of these very same processes. In their immediate environment, an animal is exposed with variety of sensory signals, e.g., visual cues like bioluminescence of the firefly (Marlene Dubuisson et al, 2004), auditory cues like alarm calls by a sentinel meerkat (R. Rauber & M. B. Manser., 2017), olfactory cues for territorial markings by a tiger (Burger B V et al., 2008) etc. Most of these naturally occurring stimuli have multi-sensory features. That is, animals can integrate congruent/incongruent information from different sensory systems together (Keil J et al., 2018). This attribute can potentially be beneficial for animals when the stimulus strength is weak in nature. For example, when animals cannot make a decision based on a single sensory stimulus, they take information from the other sense organ as complimentary and make an efficient decision leading to multi-sensory enhancement (Kerlin and Shapiro., 2015; Siemann et al., 2015). On the other hand, a multi-sensory stimulus can also lead to ventriloquist like effects when different stimuli are incongruent and contradictory (Teder-Sälejärvi et al. 2005). Hence, for the precise execution of a behaviour, an animal has to either integrate or exclude such multiple sensory cues.

Generally, different sensory systems are specialized to detect different kinds of sensory stimuli based on their physicochemical nature; however, the rodent olfactory system is known to process information of different types of stimuli. The olfactory system comprises four subsystems that can detect stimuli of various kinds, e.g., temperature, chemical cues (include volatile odours, volatile and non-volatile pheromones), and mechanical pressure as well (Minghong Ma 2008, Mamasuew, K et al 2008; Connelly et al., 2014; Grosmaitre et al., 2007)). This unique property of the olfactory system makes it a potential candidate to study the neural mechanisms of multi-sensory decision making using a single sensory system.

1.1 Rodent Olfactory system

Rodents rely on olfaction for many of their vital functions. It includes food evaluation and foraging, identification of potential mate, predator avoidance, and facilitating social interactions. The prime function of the olfactory system is thought to process the

chemical cues from the surroundings and make a perception of the chemical world around them. Anatomically olfactory system is subdivided into three parts - the nasal cavity (periphery), olfactory bulb (OB – a precortical region), and the olfactory cortex (Barrios et al., 2014).

1.2 Nasal cavity

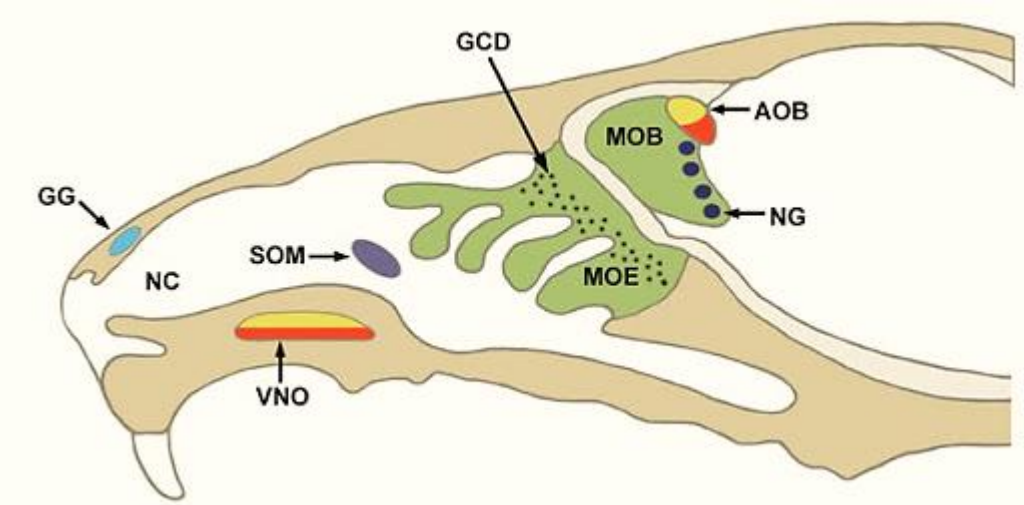


Figure1 *The sagittal section of mouse nasal cavity showing different olfactory subsystems. GG – Gruenberg Ganglion, NC – Nasal Cavity, SOM – Septal organ, MOE – Main olfactory epithelium, VNO – Vomronasal Organ, GCD – gunelyl cyclase receptors, NG – Necklace glomeruli. Adapted from Brennan and Zufall 2006;*

The functional diversity of the olfactory system reflected in the presence of various subsystems in the nasal cavity. The primary olfactory sensation takes place through the olfactory sensory neurons (OSNs) present at the main olfactory epithelium (MOE). The sensory neurons of both MOE and septal organ (SO) also shown to process the mechanical information apart from the chemical one. (Grosmaitre X. et al., 2007). Another interesting subsystem is the Grueneberg ganglion(GG) wherein the Grueneberg neurons could process cold ambient temperature in the air flux and specific alarm pheromone signals (Bumbalo et al., 2017; Mamasuew K et al., 2008). Also, the vomeronasal organ (VNO) the fourth subsystem, which is responsible for the detection of the pheromone like non-volatile cues (C J Wysocki et al., 1982; Julia

Moharhardt et al., 2018). Most of these subsystems have been studied separately; therefore, how information interaction from these subsystems effects the chemical perception still remains unanswered. In this study, we are trying to probe this issue by investigating how temperature modulates olfactory perception.

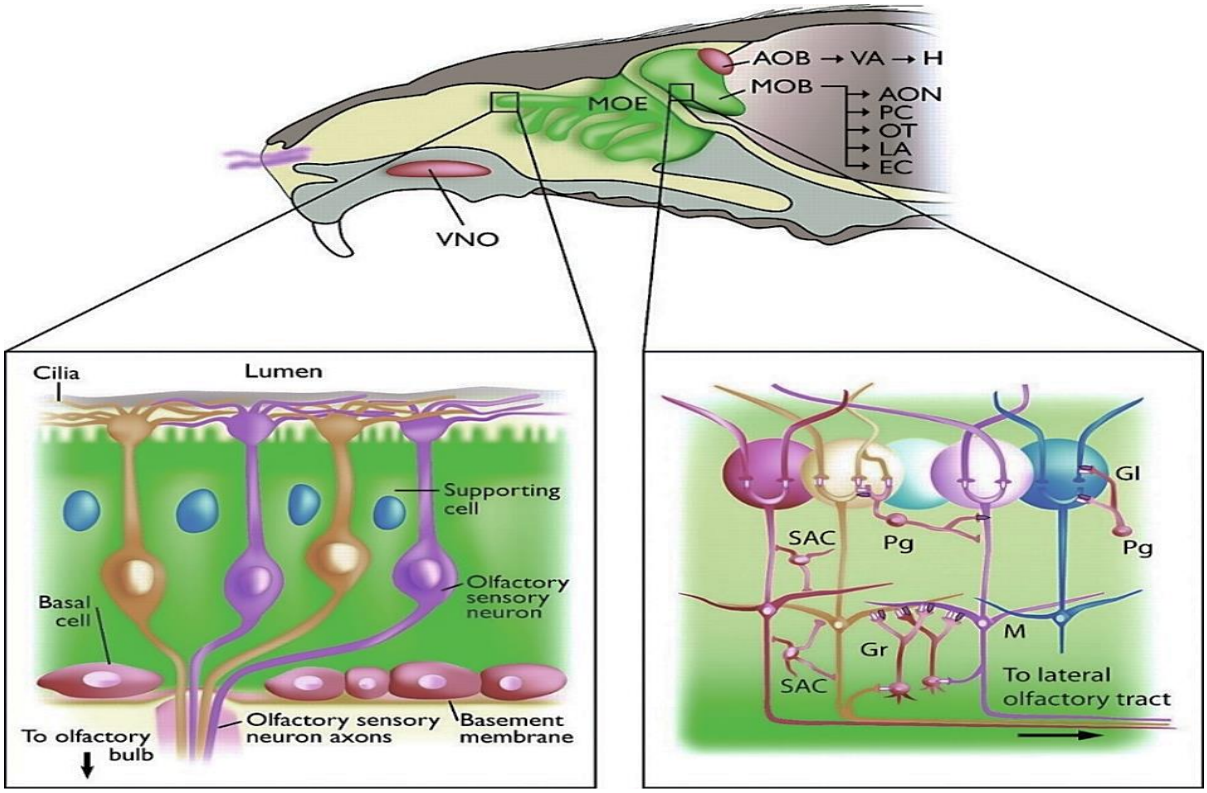


Figure 2 *Diagrammatic representation of the olfactory system (from Lledo et al., 2005). The lower left diagram depicts the signal reception apparatus and its components and the lower right diagram depicts the signal transduction in the olfactory bulb*

1.3 Main olfactory epithelium

As soon as animals sniff, a plethora of chemicals enter and travel through the nasal cavity. The odour molecules reach the end of the nasal cavity and bind to the olfactory receptors expressed on olfactory sensory neurons(OSNs). OSNs lies on the turbinates that make up the main olfactory epithelium. Each OSN expresses a single receptor, and each receptor can be activated by different odourants. These receptors are G protein-coupled receptors with seven-transmembrane domains expressed on the tip

of the cilia of receptor neurons (Buck and Axel., 1991; Ressler et al., 1994). The cilia extend into a mucous layer secreted by supporting cells in this region, which, with the help of further proteins and enzymes, facilitates accumulation, receptor binding and removal of excess odourant molecules in this region (Getchell et al., 1984). Once the odourant molecules bind to the receptor, a sequence of events takes place that ends with the depolarization in the OSNs, leading to the generation of the action potential. The OSNs then send signals to the first relay center of the olfactory pathway, which is the olfactory bulb (OB). Each type of sensory neuron projects to neuropil like spherical structure in the OB called olfactory glomerulus in a receptor-specific fashion (Greer et al., 2016). At the glomerulus, OSNs make synapses with mitral and tufted cells, the projection neurons of the OB. Inhibitory interneurons modulate the activity of the M/T cells at different levels. These interneurons are mainly periglomerular cells (PGCs), short axon cells (SACs), and granule cells (GCs). Their interaction with the M/T cells enhances and refines the output activity of the OB (Shin Nagayama et al., 2014). The M/T cells then transduce signals to the different places in the olfactory cortex leading to efficient olfactory perception.

1.4 Grueneberg ganglion (GG)

GG is a cluster of neurons present at the tip of the rodent nostril (Gru¨neberg., 1973). Anatomically it is a bilateral organ situated at two sides of the nasal septum (Gru¨neberg., 1973; Tachibana et al., 1990; Fuss et al., 2005; Koos and Fraser., 2005). Initially, GG was shown to be helpful in maternal detection as it is only activated while the mother was around the pups. However, further experiments showed that the activation of this ganglion is happening in response to colder temperatures. The GG showed higher activities below 22 degrees. (Maingret et al., 2000; Kang et al., 2005). Apart from this, GG neurons are also observed to be OMP+ (olfactory marker protein) that is a marker for mature OSNs. This suggests a role of GG in chemoreception as well (Koos and Fraser., 2005). Further anatomical studies explicitly targeting the GGs and their projections using different techniques revealed the details of this subsystem.

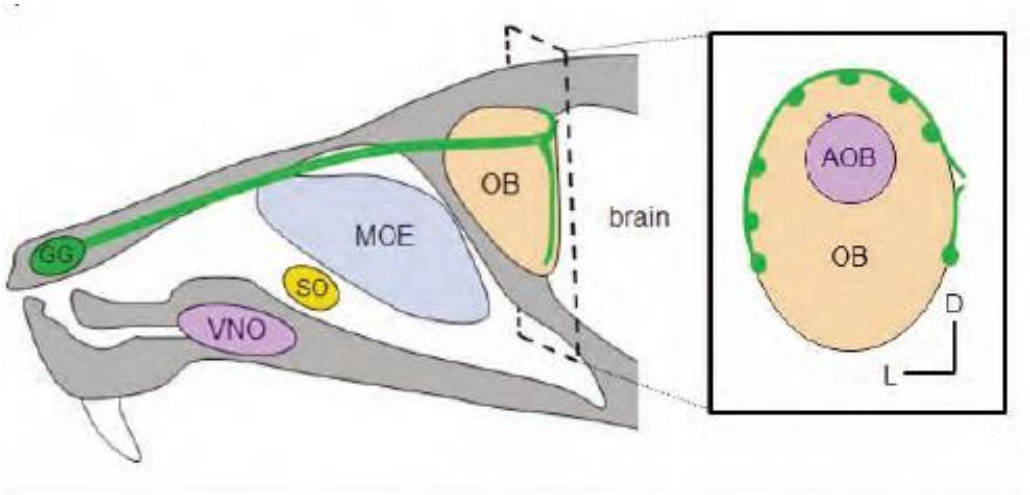


Figure 3 *left side -Grueneberg ganglion and its axonal projection to the olfactory bulb(green). Right side – axonal projections from GG in the caudal side of olfactory bulb forming necklace glomeruli. Adapted from (Rosolino Bumbalo et al., 2017)*

The receptor cells of GG send their axonal projection to the Olfactory Bulb(OB) as the normal olfactory sensory neurons do (Koos and Fraser., 2005). Specifically, GG cells project their axons to the caudal side of the olfactory bulb. There they branch out to two lateral sides and form spherical structures that are interconnected with axons. This creates the famous beads on a string array, therefore, being called as the necklace glomeruli (Rosolino Bumbalo et al., 2017). It has been already reported that some of the specialized olfactory sensory neurons expressing guanylyl Cyclase-D receptors project their axons to this very same region (Juilfs et al., 1997). Despite the independent findings of odour and temperature stimulating sensory neurons projecting to the necklace glomerular area, the possibilities of interactions between these two systems are not well studied so far. Since these two systems are projecting to the very same region, the cross-talk between these two systems is more likely to happen.

1.5 Our work

So far, we know that the olfactory system is peculiar in nature as it can sense a wide variety of physicochemical stimuli. This makes the rodent olfactory system a robust candidate to study multi-sensory interactions and decision-making. As in the environment, physical parameters like airflow associations and temperature variations can affect the dynamics of the odour plumes; It becomes necessary and ethologically

essential to study the effects of these variations on olfactory perception. In our lab, we are already focusing on the modulation of olfactory perception by varying airflows. In this study, we are trying to address how temperature variations can alter olfactory perception.

To start with, we first built and standardized a behavioural setup that is capable of delivering odour stimulus coupled to different temperatures as stimuli. The behavioural paradigm was based on the previously used Go/No-Go based operant conditioning paradigm (Abraham et al., 2004). We challenged the animals to discriminate different stimuli 1) with varying temperatures in the absence of odourants, 2) changing odours by keeping the temperature constant and 3), by coupling the odourant to the temperature. We observed that without any odour cues, the trained animals were able to perform with an accuracy of 90% and above in response to cold temperatures (18°C vs 22°C). However, the same group of animals was also capable of discriminating against the odourants at 22°C. Apart from this, when the animals were trained to discriminate different odourants coupled to different temperatures, the learning pace was significantly faster than that observed in temperature discriminations. This clearly suggests that both of these stimuli are interacting and helping animals to make a decision; however, further studies are required to find out the interactions at the level of detection thresholds and also to address the neural mechanisms underlying such interactions.

2. Materials and methods

2.1 Subjects

A total of 31 male C57BL6/J animals were used in this entire study. Subjects were 6-8 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. Animals were provided with ad libitum food and were kept on a water-deprived schedule no longer than 12 hours during the training period. The weights of animals were monitored daily during the experimental period. Animals with weight less than 80 percent of the original weight were immediately taken off from the water deprivation.

2.2 Field studies – Temperature measurements

The temperature measurements were done from the potential rodent burrows. Since the environmental changes largely influence the temperature conditions, we planned to take seasonal temperature readings from the burrows. All of these shown measurements were carried out in Wayanad, a place that lies in the southern part of India. All the observations were made during the monsoon season. Approximately 30 sq. Km area and 34 potential rodent burrows were sampled to have a statistically relevant idea of the temperature distribution in the monsoon season. The choice of burrows was made by the criteria of people spotting rats recently close to it.

The temperature readings were measured with a probe from the outside and inside (50cm) of the burrow. Multiple readings were taken from each burrow. The temperature was recorded for 5mins with a temporal resolution of 800ms with the help of an automated data acquisition software. Higher values were mostly found inside the burrows. However, in a few burrows temperature was higher on the outside. Hence the mode is taken to plot the positive variation between two sides.

2.3 Behavioural training

2.3.1 Apparatus

In order to perform temperature discrimination tasks, we custom-built a two-channel thermo-olfactometer, which is controlled by custom-written scripts in Igor Pro (Wave-

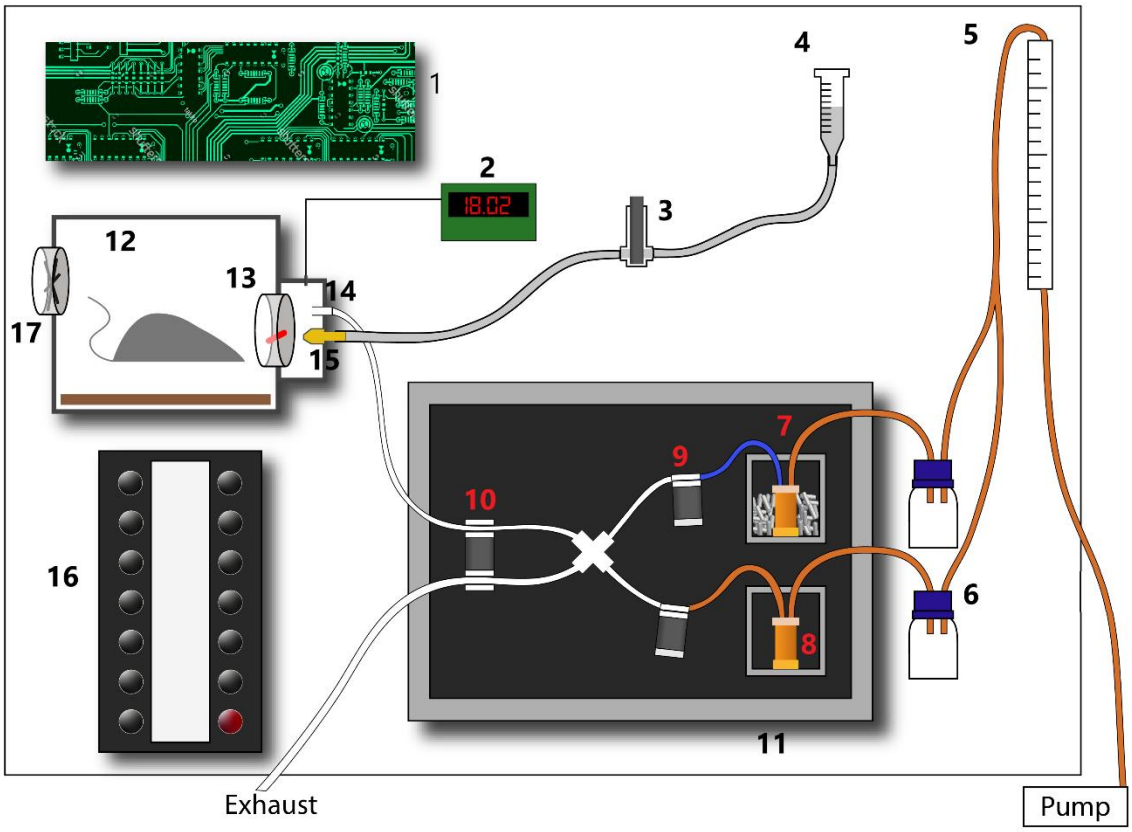


Figure 4 *– Diagrammatic representation of the behavioural training apparatus and the odour/cold air delivery system*

1) Circuit board. 2) Thermometer. 3) Water valve. 4) Water source. 5) Flow meter. 6) Odour bottles. 7) Cooling unit. 8) Copper tube. 9) Valves. 10) Two-way valve. 11) Insulated box. 12) Freely moving animal holding chamber. 13) Entry to sampling port =guarded by IR beam. 14) Stimulus delivery tube. 15) Lick port/water delivery tube. 16) Manual controlling unit. 17) Exhaust line.

metrics). Figure 4 shows the structure and function of the apparatus. The influx of airflow to the device is made possible by the use of an air pump wherein the airflow rate is controlled by the airflow meter. The controlled air flows into the two channels initially via the odour bottles attached, as shown in the diagram. There it mixes with the odourant and proceeds to the cooling chamber wherein the temperature of the output air is reduced. The whole setup is enclosed in an insulated box to minimize the heat exchange with the environment. The onset and offset of stimulus delivery are precisely controlled by a set of solenoid valves and are validated by PID profile analysis.

The stimulus is then delivered to the animal, which is kept inside an animal holding chamber. One side of the chamber has a sampling port which is guarded by an IR beam. As soon as the animal pokes his head into the sampling port, the IR beam is broken, and the trial is initiated. Soon a stimulus is provided to the animal. A thermometer probe monitors the temperature of the stimulus (odorized air/normal air) kept near to the stimulus delivery port. Based on whether the stimulus is rewarded or non-rewarded animal has to lick on the tube placed parallel to the sampling port. During the temperature discrimination task, the odour bottles are kept empty and the temperature of air through one channel is kept lower (18°C) and from the other channel air with relatively high temperature (22°C) is delivered.

2.3.2 Pre-training

Initially, a pre-training task is carried out with the experimental animals in order to familiarize the animals with the setup. Animals that were at ≥80% of their body weight after 2-3 days of water deprivation were used for the pre-training task. The pre-training task was divided into 9 phases (phase 0 to 8) with an increasing level of complexities. In the first stage of the pre-training, animals get water reward merely by poking their head into the sampling port. After completion of this phase, the animals know where the water source is. In the second stage, animals only get a reward if they register at least one lick. From the third stage onwards the duration for which animals have to lick in order to get the reward is gradually increased. The reward criteria for the eighth phase matches with the reward criteria during the discrimination training. Animals completed this pre-training within 3-5 sessions of 30 minutes each.

2.3.3 The go/no-go paradigm

The odour and temperature discrimination abilities of an animal were tested using a go-no-go operant conditioning paradigm as previously employed (Abraham et al 2004). During the behavioural task, the water-deprived animal has to poke its head into the combined odorized air/ sample port. Disruption of the IR beam leads to trial initiation. One of the valves from individual channels opens during this time and the odorized air starts to flow through the corresponding channel. One of these channels

is associated with the water reward (S+), and the other channel is neither associated with a reward nor with punishment (S-). The animal has to discriminate between the S+ and S – stimuli and respond to the reward criterion accordingly.

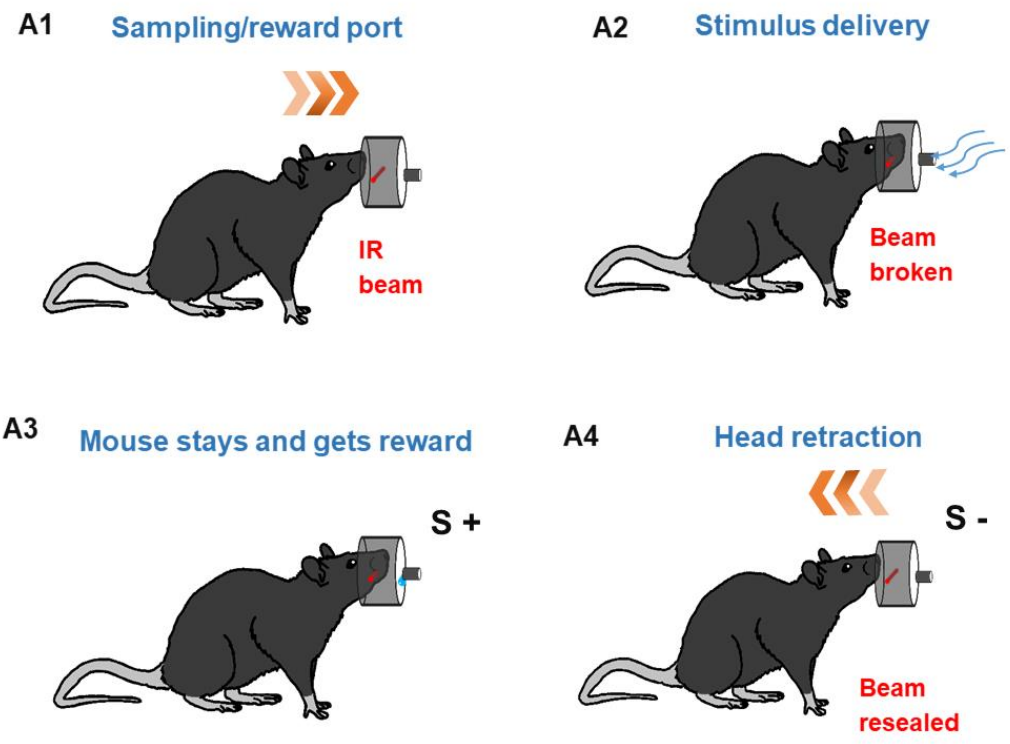


Figure 5 *– Diagrammatic representation of the go/no-go paradigm.*

A1) Sampling port guarded by an IR beam. A2) Animal pokes the head and breaks the IR beam leading to trial initiation and stimulus onset. A3) S+ stimulus delivery, animal stays, meets the reward criteria by licking the tube and gets the reward. A4) S – stimulus delivery, animal retracts the head and IR beam is resealed.

2.3.4 Reward criterion

The total duration of an actual trial is 2000ms and it is divided into four bins of 500ms. Once the animal pokes the head, for the initial 500ms (pre-trial period) the odorized chilled air will flow through the exhaust of the final valve (two-way valve) Figure6a. This has been done to ensure the proper stimulus delivery time and odour mixing with the cold air. After the first 500 milliseconds of the onset of the pre-trial period, the

exhaust valve will close; simultaneously, the valve to the sampling port will open (Figure 6b). For the S+ stimulus to be correct, the animal has to register a single lick in three out of four 500 ms bins (2000 ms of stimulus duration is divided into four bins of 500 ms). The reward given is water in this case, and each correct rewarded trial finishes with a release of 3-4 µl of water. For S- trials to be accurate, the animals cannot lick, or they can lick maximum in 2 of such bins.

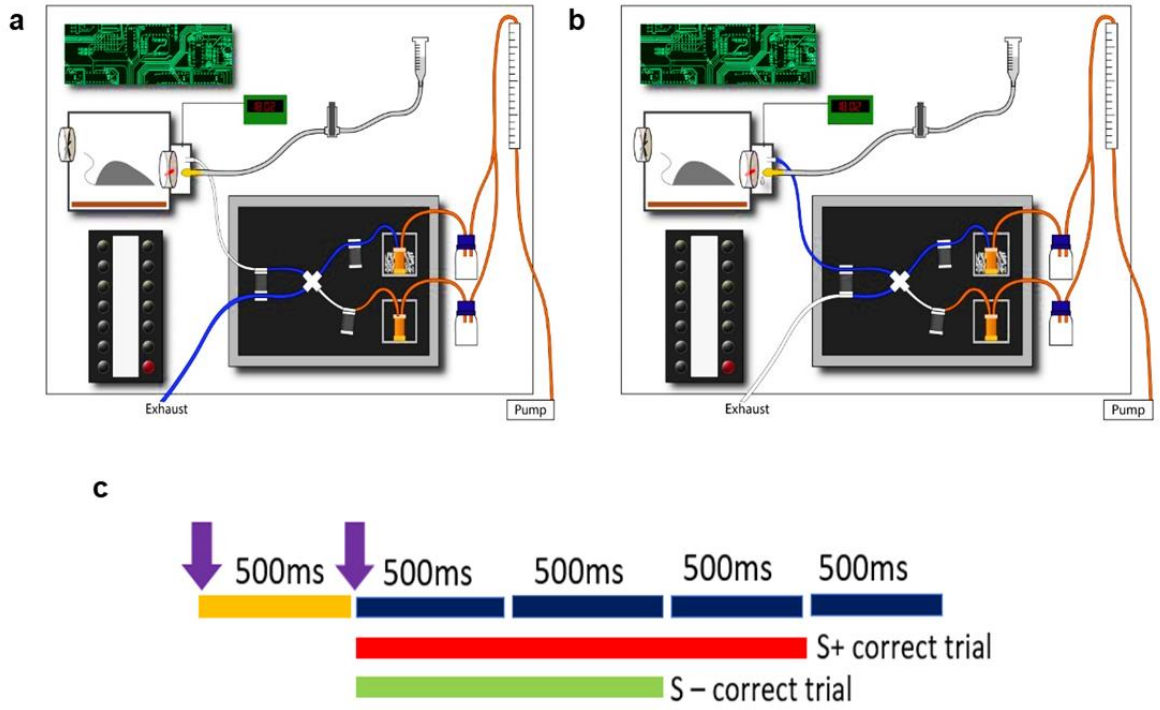


Figure 6 *– Stimulus onset and reward criteria*

a) Initial 500ms of the trial where the exhaust valve is open b) Post 500ms of the trial onset final valve is open for 2000ms. During this time, the animal receives the stimulus c) Reward criteria for S+ and S- trials. Animal has to register a lick in at least three out of four bins for S+ trial to be correct. For S- trial to be correct the animals cannot lick or they can lick maximum in 2 of such bins

The animal has to perform 900 to 1200 trials to reach the asymptotic performance levels. A task consists of 300 trials and is subdivided into blocks of 20 trials. In every block, half of the trials are S- (non-rewarded) and the other half is S+ (rewarded). S+ and S- trials are pseudorandomized in a way that not more than two trials of the same reward contingency will be provided consecutively.

2.4 Instrument standardization

2.4.1 Airflow standardization

We used an anemometer to check the consistency of airflow throughout an entire task. We optimized the flow to minimize the variations in the desired temperature and was kept constant between 3.5 – 3.7 LPM (liters per minute).

2.4.2 Temperature standardization

Temperature measurement was done throughout a task to rule out the changes in the temperature as the task proceeded. We found minimum variations for the two temperatures 22°C and 18°C used for discriminations. Both of these temperatures were chosen based on the fact that these temperatures lie in the range that activated the GG.

2.4.3 Photoionization detector (PID) measurements

PID measurements were performed to characterize the odourant profiles. In this method, high energy photons on collision with vaporized molecules would elicit a voltage change in the photo ionic detector. The PID profiles would be helpful in order to check the dynamics and concentration of molecules in the odorized air flux (stimulus). PID measurements were carried out using a PID probe for 11 different odourants. For each odourant, the PID measurements were carried out for two different temperatures (18ºC and 22ºC). For each odourant, the profile represents the average of 20 measures. Odourants with similar readouts at different temperatures were taken for further studies.

2.4.4 Odours

The odours used for the initial test were limonene, ethyl butyrate, acetophenone, nonanal, amyl acetate, valeraldehyde, benzaldehyde, butyraldehyde, methyl benzoate, hexanal and hexanone. Odours were diluted to 1% of their stock in mineral oil. Out of the ten odours, we got four odourants that show similar PID profiles at the desired temperatures. Those were valeraldehyde, butyraldehyde, hexanone and benzaldehyde. These odourants were used for further studies wherein they were coupled with different temperatures.

2.5 Behavioural task readouts

The **percentage accuracy** for the behavioural tasks is calculated from the behavioural response of all animals. Successful responses towards the S+ and S- trials represent the learning behaviour of an animal. The learning curves of different behaviour tasks were plotted as a measure to check the learning progress of animals. Each point in the learning curve represents the average accuracies of 100 trials (50 S+ and 50 S-). Initially, the animal starts with a chance level learning of 50%, where it used to lick for everything. Later through the progress of further trials, they will reach an accuracy of 90% and above wherein they successfully differentiate between the S+ and S- trials. Generally, the learning pace shown by animals varies with the complexity of behavioural tasks employed.

The **lick percentage** gives the time duration in which an animal keeps on licking onto the lick tube throughout the entire period of a 2-second trial. Whereas the result section is provided with the average of this lick percentage for a task (300 trials).in the first task, for the naïve animals, the percentage will be 80-90%, suggesting that animal is licking for all S+ and S- trials irrespective of the reward contingency. However, as animals start to learn to discriminate the stimuli, this percentage will reduce to 50% in the final task, suggesting that the animal licks only for S+ trials (Figure S1).

The **sample pattern** gives an estimate of the time that an animal spends inside the sampling port during a trial. The temporal profile of the broken LED beam measured as a proxy for this. Binary values were given to the broken IR-beam and intact one. For each trial, the sampling behaviour of animals is measured across 125 bins. Each

bin corresponds to 20ms. It is averaged separately for S+ and S- trials of an entire task and plotted against the duration of the trial (figure 11). The naïve animals lick for all trials irrespective of S+ or S-. Hence the beam will always be broken in this scenario, showing higher sampling rates for both types of the stimuli. However, once the animal has learned the paradigm successfully, then it will stay longer for S+ trials and an S- trial, it will retract his head, causing a deflection among the sampling profiles of a rewarded and non-rewarded stimuli.

Lick pattern is another parameter that tells us about the licking behaviour towards a particular stimulus. Similar to the sample pattern, For each trial, the licking behaviour of animals is measured across 125 bins. Each bin corresponds to 20ms. Binary values were given to the lick, and non-lick responses.this is plotted against the duration of the trial (figure 10). for the naïve animals, the lick patterns are higher and non-divergent for both types of stimuli. However, as the learning progresses, the animal will preferentially lick for the S+ trials and avoid licking in the S- trials causing a divergence in the lick patterns.

Discrimination time (DT) is the first time point when the animal learns to distinguish between a rewarded and a non-rewarded stimulus. In the paradigm that we used, DTs can be calculated by both lick and sample patterns. Figure 10 and Figure 11 shows the evolution of sample and lick patterns as the learning progresses. A p-value curve can be obtained by the statistical comparison of S+ and S- sample/lick patterns. In the p-value curve, the last time point where $p<0.05$ is considered as the discrimination time (Figure S2).

Inter trial interval (ITI) is the time taken by an animal between the two consecutive trials. This parameter has more importance in this behavioural task due to the choice dependent nature of the task. The ITI is dependent on the motivation level of an animal. If the animal is over-motivated, the ITI will be very less and despite the learning the animal lick towards all S+ and S- trials. Likewise, under-motivation leads to more head retraction; therefore, false positives for S- trials would happen more likely. Since we do not have a readout for the false-positive trials, we are judging it by looking at the responses towards S+ trials. Under-motivated animals retract their head for both S+ and S-.

2.6 Data analysis

The behavioural task data were analyzed in a custom-written program in Igor. All the parameters mentioned above were analyzed using the same. All the plots were made using Graph Pad Prism 8.

3. Results

3.1 Optimization of experimental parameters

3.1.1 Temperature differences measured from rodent burrows

Temperature readings were measured from natural rodent habitats. We sampled a total of 34 burrows, observation of 10 burrows excluded from the analysis as a consequence of lack of consistency. Since the burrow are where the animals home themselves and go out into the environment in search of food and potential mates, we measured the temperature difference between inside and outside of the burrows Figure 7a.

a

b

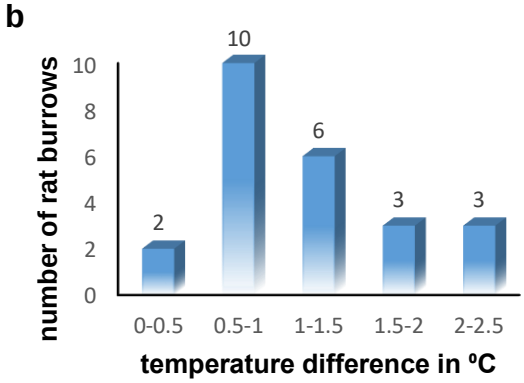


Figure 7:

a: Rat burrows where field studies carried out

b: Frequency distribution of the temperature differences observed between inside and outside of rat burrows

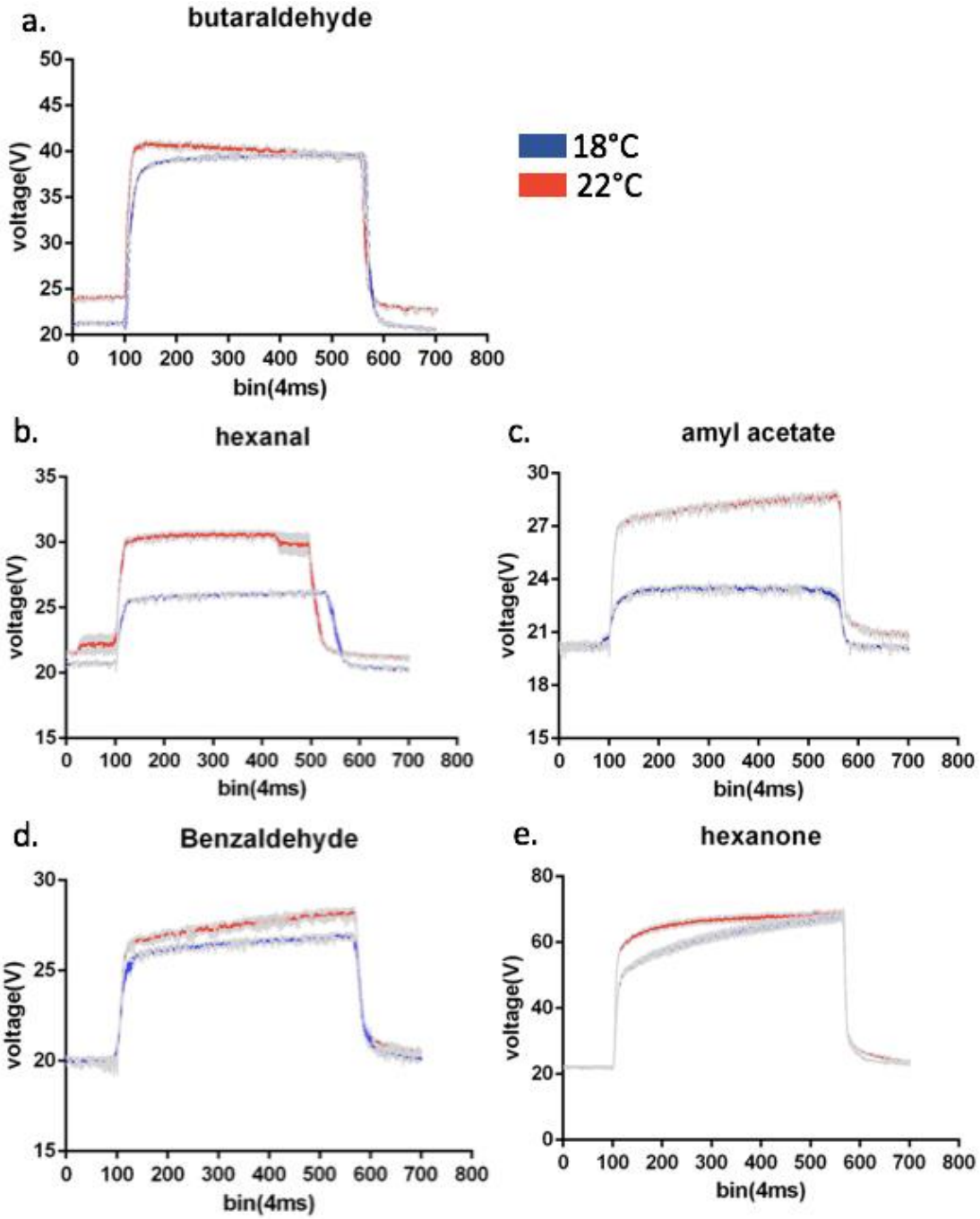
As the temperature is highly prone to change with the season and different times of the day, we measured the temperatures during the monsoon season at a particular time in the day in order to minimize the variations. From the measurements, we observed that temperature difference falls in a range of 0.5 to 1.5˚C for the most number of the burrows Figure 7b. As this temperature difference is consistent among 50 % of the burrows we sampled, we further asked a question of whether animals can sense this difference. To start with, we planned to train the animals to discriminate different temperatures, higher than what we have observed in the natural habitat. We are planning to take readings from similar burrows during different seasons to check the seasonal variability.

3.1.2 Effect of temperature on odourant characteristics

Odourant profiling was carried out using a PID (Photo-ionization detector) probe. We did profiling for 11 different odourants representing different classes (Alcohols, aldehydes etc.), under two different temperature conditions (room temperature approximately - 22°C and a lower temperature - 18°C). Therefore, for each odourant, there were two sets of PID measurements. Measurements averaged for 20 trials for each temperature and odour with the duration of stimulus kept constant as 2s, as this is the same duration used in behavioural tasks. The PID readouts used to assess the stability of the odour pulses at different temperatures. For most of the odourants, the onset/offset dynamics were overlapping and consistent at two different temperatures, Figure 8. However, the temperature variations did affect the concentration profiles of a few odourants. From Figure 8, most of the odourants are showing a noticeable difference in their PID voltage during the task for different temperatures (blue-18°C, red-22°C), with odourants at 18°C having lower amplitudes except methyl benzoate and acetophenone. Apart from this, odourants like benzaldehyde, butyraldehyde, hexanone and valeraldehyde showed similar temporal dynamics and amplitude characteristics even when the temperature varied. As we aim to study how temperature variations can alter the olfactory perception in mice, these results helped us to select the odourants.

The effect of temperature on olfactory perception is studied by challenging the animals on a multisensory based discrimination task wherein one odourant is coupled with one

temperature. For the same purpose, we planned to use these odourants that showed similar PID profiles for both the temperatures. By doing so, we will be able to cast aside the biases in the learning due to concentration variation at different temperatures. Before proceeding to understand the effect of temperature on the olfactory perception, we first needed to answer if and how animals can perceive the temperature differences?



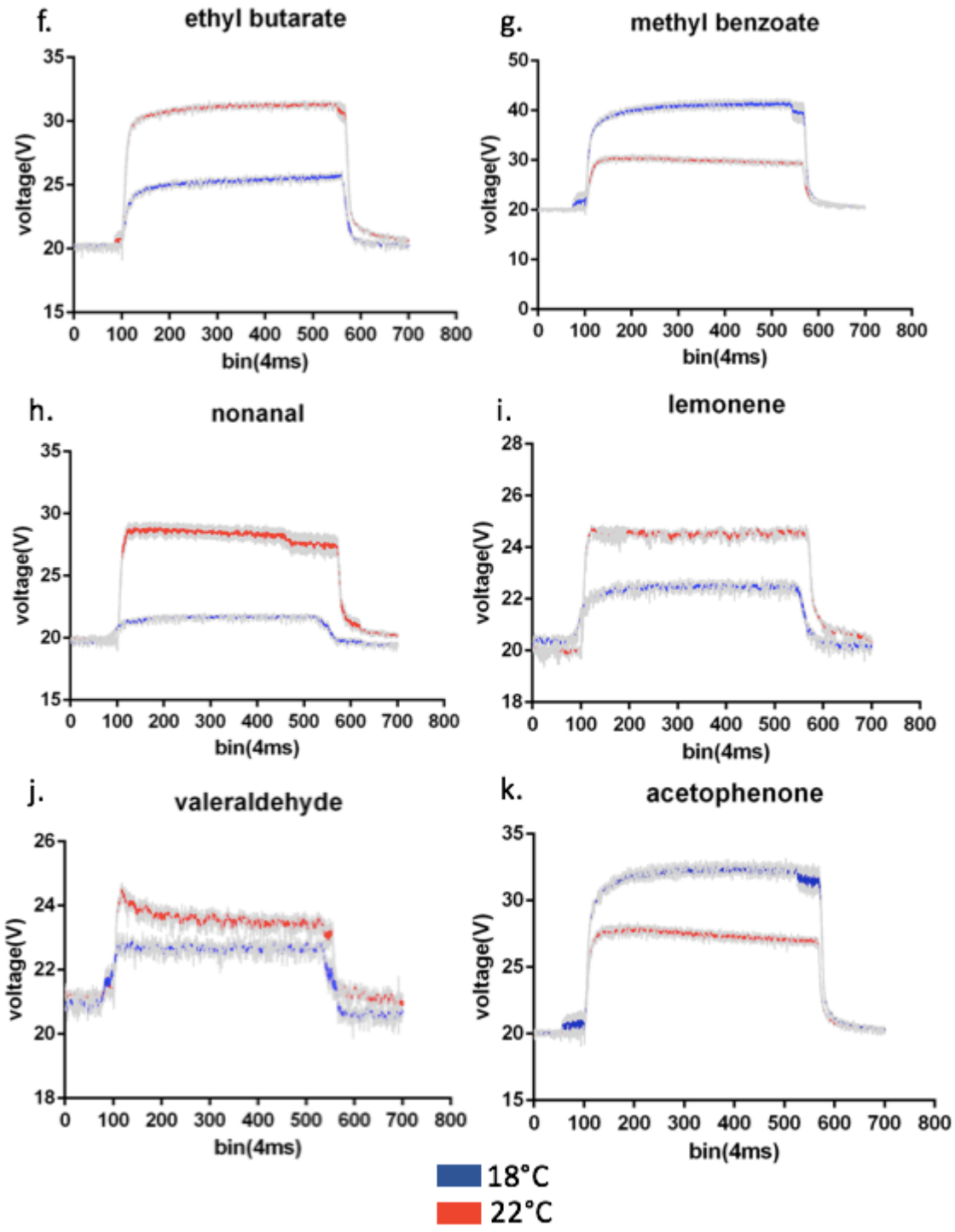


Figure 8: *Small temperature differences cause variations in the concentrations of few odourants*

a-k: PID measurements for different odourants under room temperature-22˚C and cold temperature-18˚C

a,d,e,j: odourants with same amplitude for different temperatures

3.2 Mice can learn to discriminate temperature differences in a go/no-go Paradigm

In order to understand whether animals can pick up the temperature differences that we observe in the environment, the animals were trained to perform a temperature discrimination task with Δtemp. (Δt) as 4 degrees Celsius. The idea was to train the animals on a higher temperature difference first and then lower the Δt to a range as observed in the habitat. The animals began with a chance level performance in which the percentage accuracy was 50%, suggesting that animals were sampling and licking for all the trials irrespective of the reward contingency (Figure 9a,10a and 11a). Later on, the animals started to pick up the S+ and S- cues, and they began to perform accordingly by licking and sampling on the S+ trials and retracting their heads for the non-rewarded ones. Different parameters such as lick percentage, lick patterns, and sample patterns were measured (see methods), and all of these parameters showed a significant decrease for S- trials compared to S+ trials (Figure 9b, 10, and 11). Animals learned to discriminate the temperatures within three tasks (each task has 300 trails with 150 S+ and 150 S-) and reached the asymptotic performance levels in a total of 900 trials. Mice were counterbalanced to avoid any bias towards a particular temperature stimulus used for rewarded and non-rewarded trials (Figure 9). At the end of the 4^{th} task, animals were able to perform with an accuracy of above 90% and licked only for S+ stimulus as the lick percentage reduced to 50% (Figure 9b). The percentage lick shown by animals (50%) proves that their motivation levels were optimum and all behavioural readouts were unaffected by their motivational status.

Once the animals learned to perform the task above 85% accuracy in the final task, we switched the valves for every 100 trials by keeping the stimulus cue the same. No change was observed in learning behaviour, suggesting that the valve clicking sound is not used by animals as a learning cue (Figure S3). These observations indicate that animals can discriminate temperature differences very efficiently. Therefore, rodents might be able to make decisions based on temperature variations they encounter in their natural habitats. These results call for further studies to elucidate the neural mechanisms underlying temperature detection and discriminations in mice.

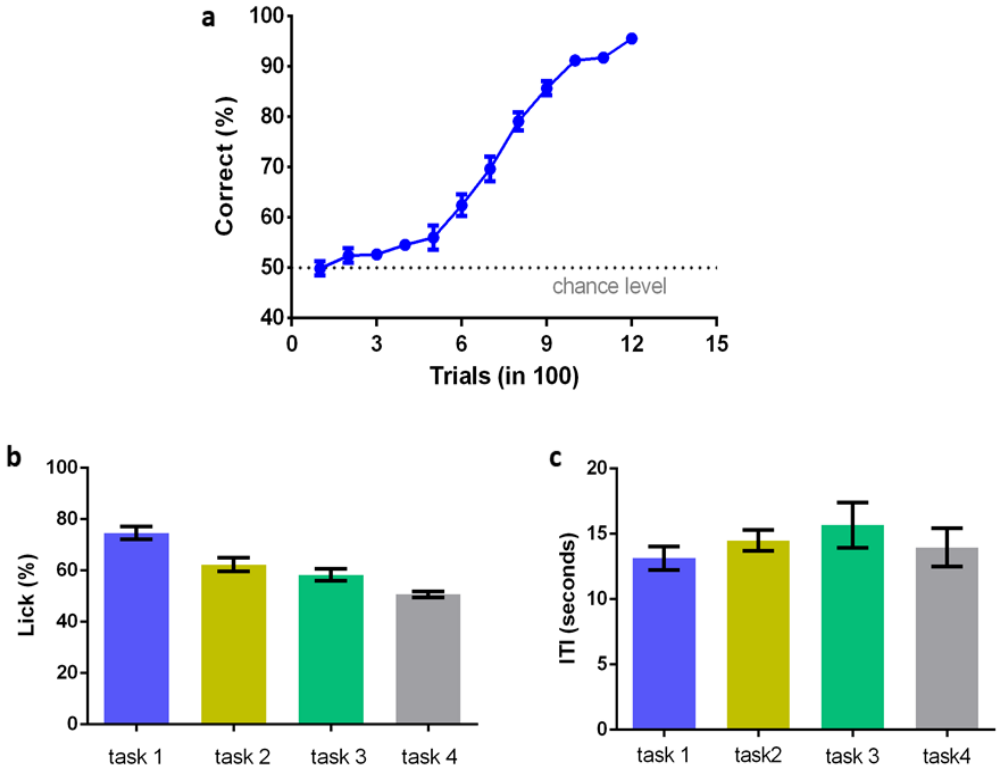


Figure 9: *Mice can learn to discriminate temperature differences (N=9)*

a: Percentage correct shown by mice in a temperature discrimination task. Each data point is the average of 100 trials (average ± SEM).

b: lick percentage for different tasks, 50% lick indicates optimal motivation levels and licking for only S+ trials c: inter-trial interval between two consecutive trials as a proxy to check the animal's motivation level across different tasks (average ± SEM).

3.2.1 Monitoring the motivation levels of animals

Since the conditioning task is a choice dependent one, the motivation level of an animal would have a more substantial influence on the results. The inter-trial interval (ITI) is generally used as a proxy to check the motivation level in animals. ITI is the duration between two consecutive trials. If the ITI is very large, then the animal is considered to be not/under-motivated. Conversely, if ITI is very less, then the animal

is supposed to be over-motivated (water deprivation schedule of the animals strongly affects the motivation levels). Ideally, it lies between 10-15 seconds, the time we measured from all earlier experiments done in the lab. This parameter is critical to have control over the motivation levels of animals and to make sure that all behavioural readouts are unaffected by the motivational status of experimental animals. Figure 9c depicts the ITI for all four tasks. For all tasks, the ITIs lies in the ideal range, and no difference was observed (Figure9c: one-way ANOVA F- value =0.6, p-value = 0.56, Tukey's multiple comparison test). Shows that animal's performance levels were unaffected by their motivational status.

3.2.2 Discrimination time– from sampling behaviour and lick patterns

When animals are subjected to a decision making task, the decisions are generally made within a time range. This time range is regarded as the reaction time. However, in this case, as we are challenging animals to discriminate different stimuli, the reaction time gives an idea about how much time animals needed to discriminate different stimuli under different contexts. Hence here we call the reaction time as discrimination time.

In our paradigm, reaction time is the time taken by the animal to discriminate between the S+ and S- stimuli. Typically, when the animal is performing at chance level accuracy, it would lick for both stimuli. As the learning progresses, the animal learns to retract its head from the sampling tube for S- stimulus while still licking to get the reward for S+ stimulus. We take into account this sampling and licking probabilities for the stimuli to calculate the discrimination time.

In the case of calculating DT through sampling probabilities, we assess the sampling difference between the two stimuli once the animal has learned the task. For S+, as the animal continues to keep its snout inside the sampling tube during the stimulus duration, the probability hovers around 1. However, the probability decreases sharply for S- odour. This difference is used to assess discrimination time. Similarly, the licking probability for S+ and S- differs sharply after the animal has learned to discriminate and thus, this parameter was also used to calculate DT. The observed discrimination times calculated from both sample and lick patterns lied in the range of 400-600 ms for a temperature-based discrimination task (Figures 10 & 11).

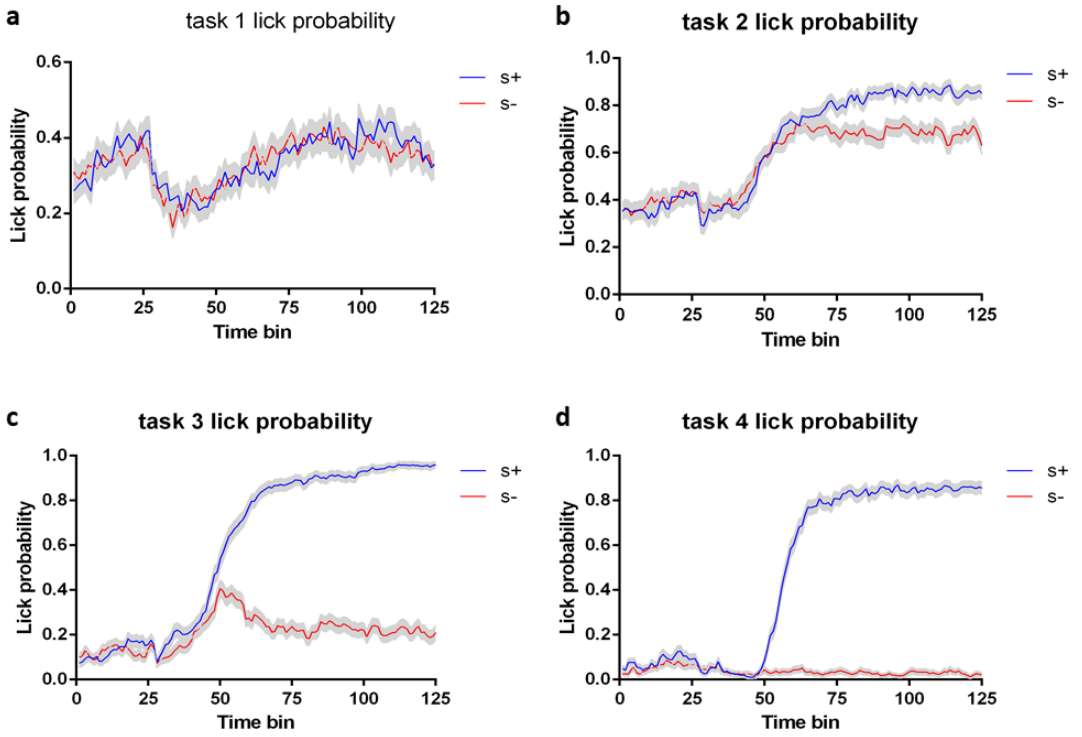


Figure 10: *Lick probabilities for S+ and S- stimuli across four tasks of discrimination learning (average+SEM) a: equal response to S+ and S- (no discrimination) b: lick probability reduced for S- (started to discriminate) c,d: licking for rewarded trials & low or no-lick for non-rewarded trials (animals discriminated between S+ and S- trials)*

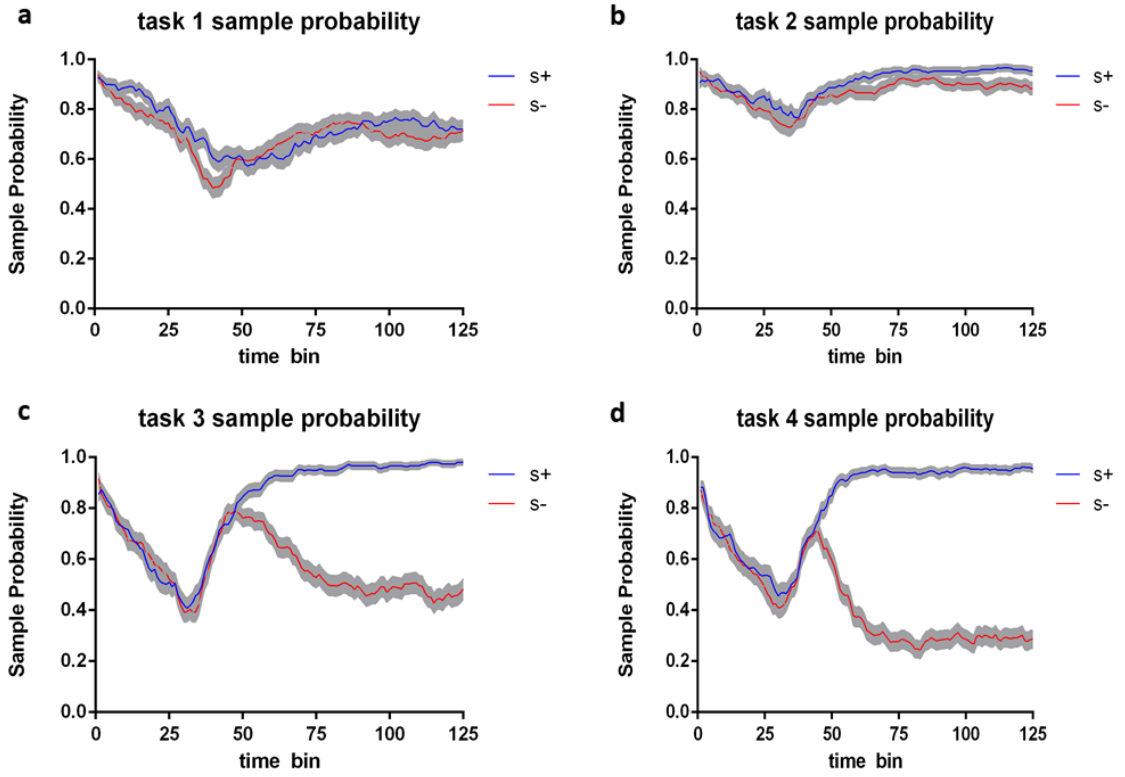


Figure 11: *Sampling pattern in response to rewarded and non-rewarded trials as the learning progress (average+SEM) a: equal response to S+ and S- (no-discrimination) b: lick probability reduced for S- (started to discriminate) c,d: licking for rewarded trials & low or no-lick for non-rewarded trials (animals discriminated between S+ and S- trials)*

3.3 Similar behavioural readouts for multisensory decision making and odour-dependent discriminations

Our experiment displayed that the animals were able to discriminate between two differentiating temperature cues using the instrumental conditioning paradigm. The same batch of mice were used for further studies. They were challenged to differentiate between two different odourants, butyraldehyde and hexanone. The choice of odourants made as the intensity amplitudes of these odourants (measured

using PID) remained unaffected by the temperature differences. Animals were asked to perform the task where S+ and S- stimuli are two odourants, and both provided at a temperature of 22 °C. The experimental animals were counterbalanced across rewarded and non-rewarded stimuli. Butyraldehyde and hexanone were used as the odourants for discrimination task. These animals learned to discriminate between odourant cues very quickly and reached an accuracy of about 95%, then maintained the accuracy throughout the training period. The quick learning behaviour shows significant difference from the temperature discriminations. We can not rule out the possible role of procedural learning during the temperature discriminations as the reason for the better performance with the odour discriminations. Therefore, we repeated these experiments with different batches of mice (see later).

These animals were later trained to differentiate the multisensory stimulus. For this purpose, Valeraldehyde was coupled with 18°C and benzaldehyde were coupled with 22°C as multisensory stimuli. Animals were counterbalanced across the cues. Animals were faster than the previous discrimination task and started to perform with higher accuracy and maintained the learned behaviour throughout three tasks (Figure 12b). The learning curves across these different conditions were compared. Animals were significantly slower on a temperature-based discrimination task as compared to the other two conditions (Figure 12b) (Two-way ANOVA p-value <0.0001, F- value =843.7(column factor), Tukey's multiple comparison test). The phenotype observed was not a result of alteration in the motivational states as ITI for all groups was similar (Figure 12c) (one-way ANOVA p-value = 0.28, F – value = 1.34 Tukey's multiple comparison test). Also, there was a significant difference between the initial learning pace between odour and odour-temperature coupled group. Faster learning for the second discrimination pair could either be a reflection of the procedural learning that happened as the experiment progressed or as a result of multisensory enhancement. Therefore, an ideal way of comparison will be to train different sets of animals under these different conditions. However, from this set of experiments, we can reliably claim that animals can discriminate among different temperatures, odourants and temperature coupled to odourants.

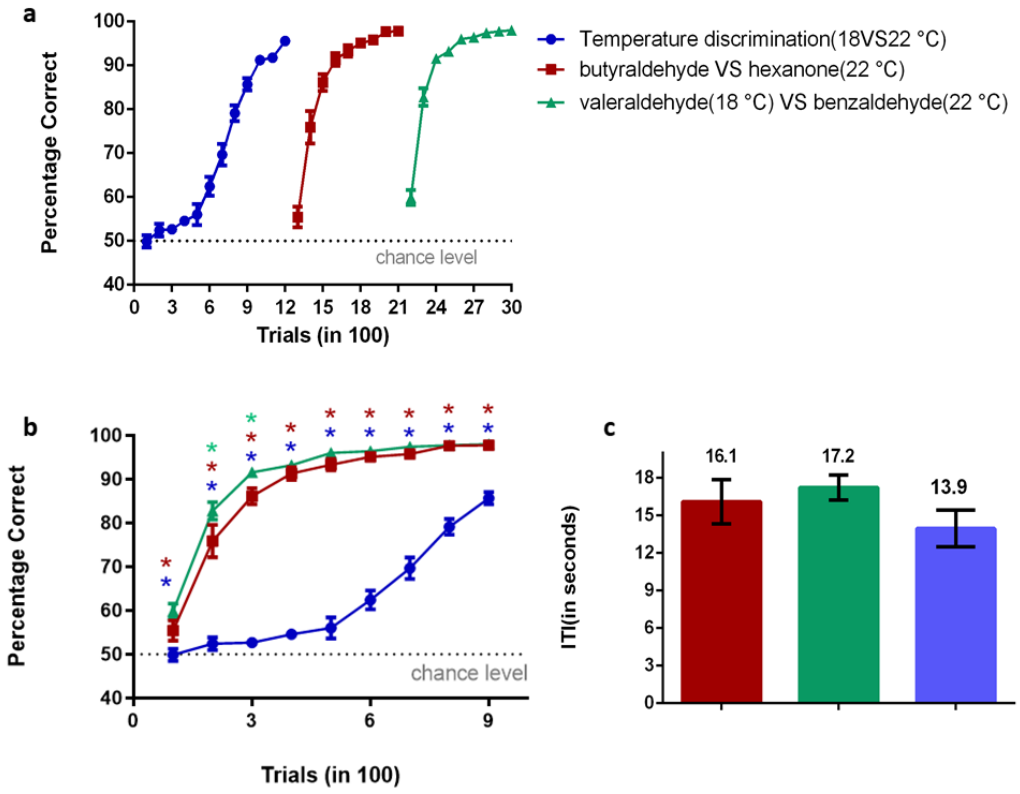


Figure 12: *Percentage correct response to different sensory cues throughout the trials (N=9). a: learning towards progressive experiments by same batch of animals (average ± SEM). b: difference in the learning progress between experiments showing significant difference between temperature discrimination and other experiments (average ± SEM). * two-way ANOVA p-value <0.0001, F-value =843.7(column factor), tukey's multiple comparison test, * temperature18 ˚C vs 22 ˚C V/S valeraldehyde(18 ˚C) vs benzaldehyde(22 ˚C), * temperature18 ˚C vs 22 ˚C V/S butyraldehyde(22 ˚C) vs hexanone(22 ˚C), * valeraldehyde(18 ˚C) vs benzaldehyde(22 ˚C) V/S butyraldehyde(22 ˚C) vs hexanone(22 ˚C)*

c: ITI values of the 4th task for different sets of experiments showing non-significance in their difference C one-way ANOVA F-value = 1.34, p-value = 0.28, tukey's multiple comparison test (average ± SEM).

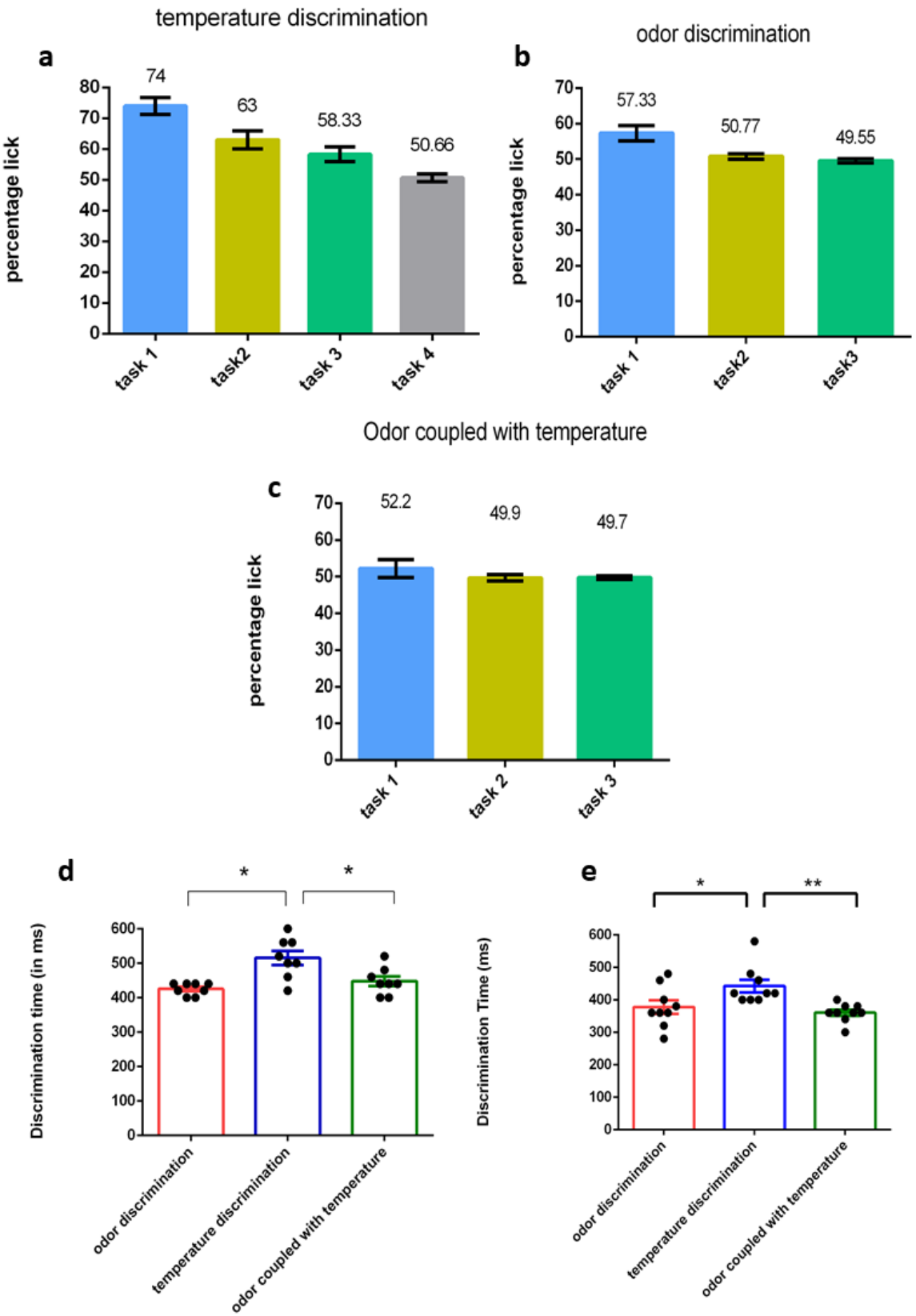

a
temperature discrimination
percentage lick
74
63
58.33
50.66
task 1
task2
task 3
task 4
b
odor discrimination
percentage lick
57.33
50.77
49.55
task 1
task2
task3
Odor coupled with temperature
c
percentage lick
52.2
49.9
49.7
task 1
task 2
task 3
d
Discrimination time (in ms)
odor discrimination
temperature discrimination
odor coupled with temperature
e
Discrimination Time (ms)
odor discrimination
temperature discrimination
odor coupled with temperature

Figure13: *a-c: Percentage lick responds for different set of experiments across different tasks showing the consistency of motivation levels along with the learning (average ± SEM). d: discrimination time for different experiments calculated from lick pattern * one-way ANOVA F- value =10.4, p-value = 0.0021, Tukey's multiple comparison test, e: discrimination time for different experiments calculated from sampling response one-way ANOVA F- value =6.17, p value = 0.0069, Tukey's multiple comparison test, (average ± SEM).*

(both cases shows significant difference between temperature discrimination and other odour- and odour-temp discriminations)

3.4 Higher discrimination time for temperature dependent discriminations

Discrimination times for all three groups were plotted together to compare their reaction times shown towards different discriminations. DTs were calculated for individual mice and averaged over the entire group. The DTs of temperature discrimination group was significantly higher than that of the odour discrimination and odour- temperature coupled groups Figure 13d (*one-way ANOVA F- value =10.4, p-value = 0.0021, Tukey's multiple comparison test*). No difference was observed in the DTs for odour only and odour-temperature coupled groups. A similar trend was observed for the DTs calculated from the sampling patterns Figure 13e (*one-way ANOVA F- value =6.17,* p-value = 0.0069*, Tukey's multiple comparison test,*).

DT analysis, along with learning pace, suggests that despite the multisensory stimuli presentation, the animals may rely more on odourant stimuli as compared to the temperature cues. However, further experiments are required to characterize this behaviour in detail.

3.5 Slower learning pace and DTs were observed for temperature discriminations even with a different batch of mice

Temperature based odour discrimination is a novel paradigm that we developed. To rule out the possibilities of procedural learning modulating the behavioural readouts, we repeated these experiments using different batches of mice.

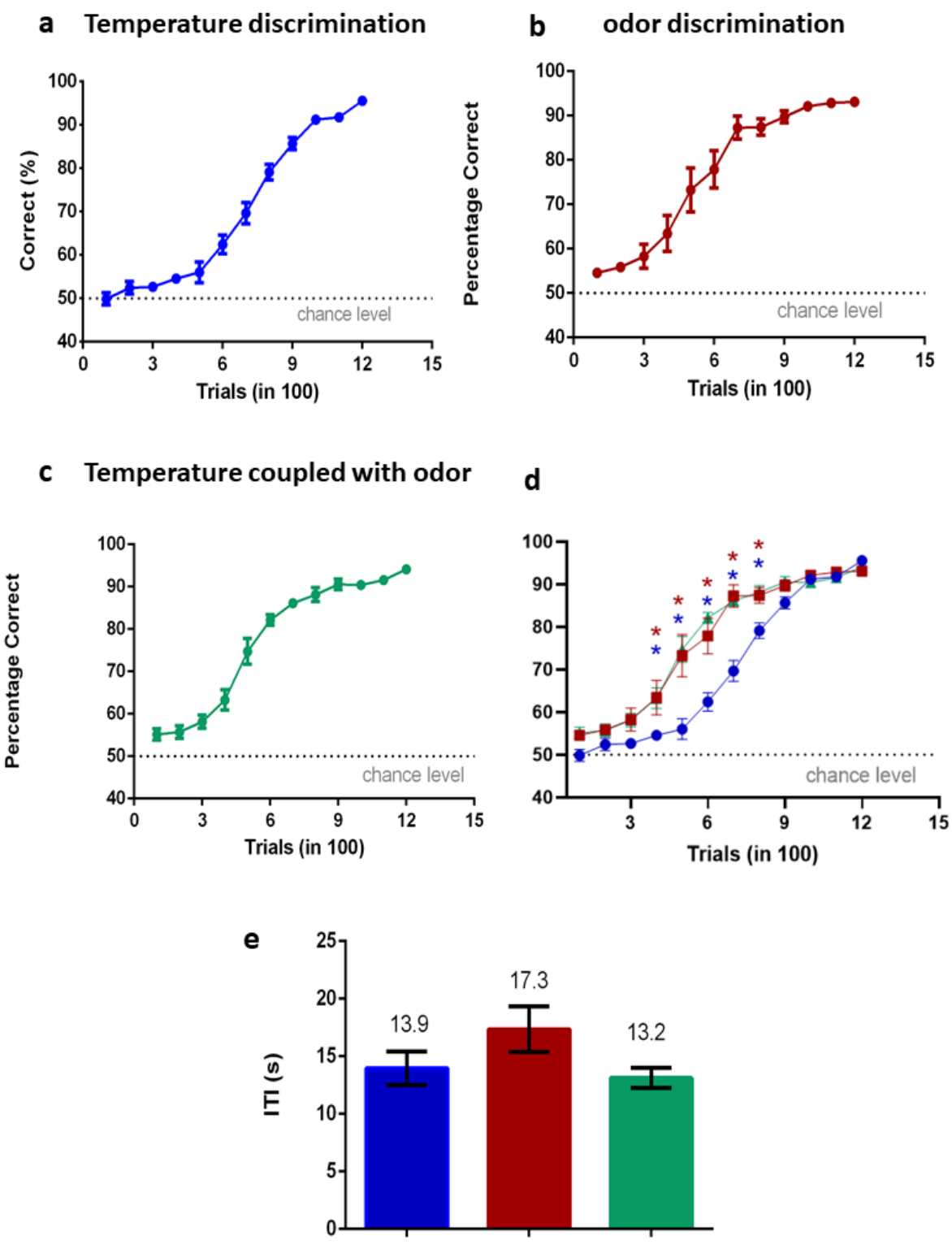

a Temperature discrimination
Correct (%)
chance level
Trials (in 100)
b odor discrimination
Percentage Correct
chance level
Trials (in 100)
c Temperature coupled with odor
Percentage Correct
chance level
Trials (in 100)
d
chance level
Trials (in 100)
e
ITI (s)
13.9
17.3
13.2

Figure 14: *Mice showed slower learning pace for temperature discriminations. Each point in the learning curve corresponds to 100 trials (average ± SEM). a: Temperature discrimination task (18˚C vs 22˚C and N=9) b: Odour discrimination task (valeraldehyde vs benzaldehyde and N=7) c: Odour-temperature coupled task (valeraldehyde 18˚C vs benzaldehyde 22˚C and N=7)*

*d: Plot depicting all three learning curves (average ± SEM). (temperature discrimination pace is significantly different from other two sets of experiments two-way ANOVA p-value <0.0001, F- value =59.97(column factor), Tukey's multiple comparison test, * temperature18˚C vs 22˚C V/S valeraldehyde(18˚C) vs benzaldehyde(22˚C), * temperature18˚C vs 22˚C V/S valeraldehyde(22˚C) vs benzaldehyde(22˚C),)*

e: ITI of all three sets of experiments (average ± SEM). (the difference is non-significant suggesting that motivation level is consistent across all experiments e-one-way ANOVA F-value = 1.34, p-value = 0.28, Tukey's multiple comparison test)

Independent groups were trained on temperature-, odour- and multisensory discrimination tasks. Valeraldehyde and Benzaldehyde were used as the odour pairs. Since the groups were different, the same odour pair was used in all discrimination tasks. The animals started with a chance level of performance and reached the asymptotic performance level in about 600 trials and maintained the accuracy throughout the learning (Figure14 b & c). Strikingly, no difference in the learning pace was observed between odour-only and odour-temperature coupled discriminations (figure 14 d).

However, when compared with the temperature discriminations, the learning pace for the other two groups was significantly higher (figure 14d (two-way ANOVA p-value <0.0001, F- value =59.97(column factor), Tukey's multiple comparison test). The difference observed was not a result of alteration in motivation levels as ITI among different groups was non-significant and lay in the desired range (Figure 14e One-way ANOVA F-value = 1.34, p-value = 0.28, Tukey's multiple comparison test). Also, lick percentage followed similar trends, i.e., it was high during the initial tasks and towards

the end, it reduced to 50 percent; hence the motivation level is consistent with the learning abilities of animals (Figure 15 a & b)

As observed before, the discrimination time required for temperature discrimination was significantly higher than that of odour only and odour coupled temperature (figure 15 c: one-way ANOVA F- value =8.59, p-value = 0.0022, Tukey's multiple comparison test). Also, no difference in DTs was observed between the odour and odour-temperature coupled group (Figure 15 d one-way ANOVA F- value =0.9, p-value = 0.4 Tukey's multiple comparison test). These results show that animals might be relying on odour cues mostly as compared to temperature cues when they are challenged by combining these two sensory stimuli. However, the odour concentration and the temperature difference that we have chosen could be much higher than the detection thresholds of the animal. Therefore, optimizing the detection threshold and further experimenting the same temperature odour coupled discrimination tasks would require to confirm the observed behavioural readouts.

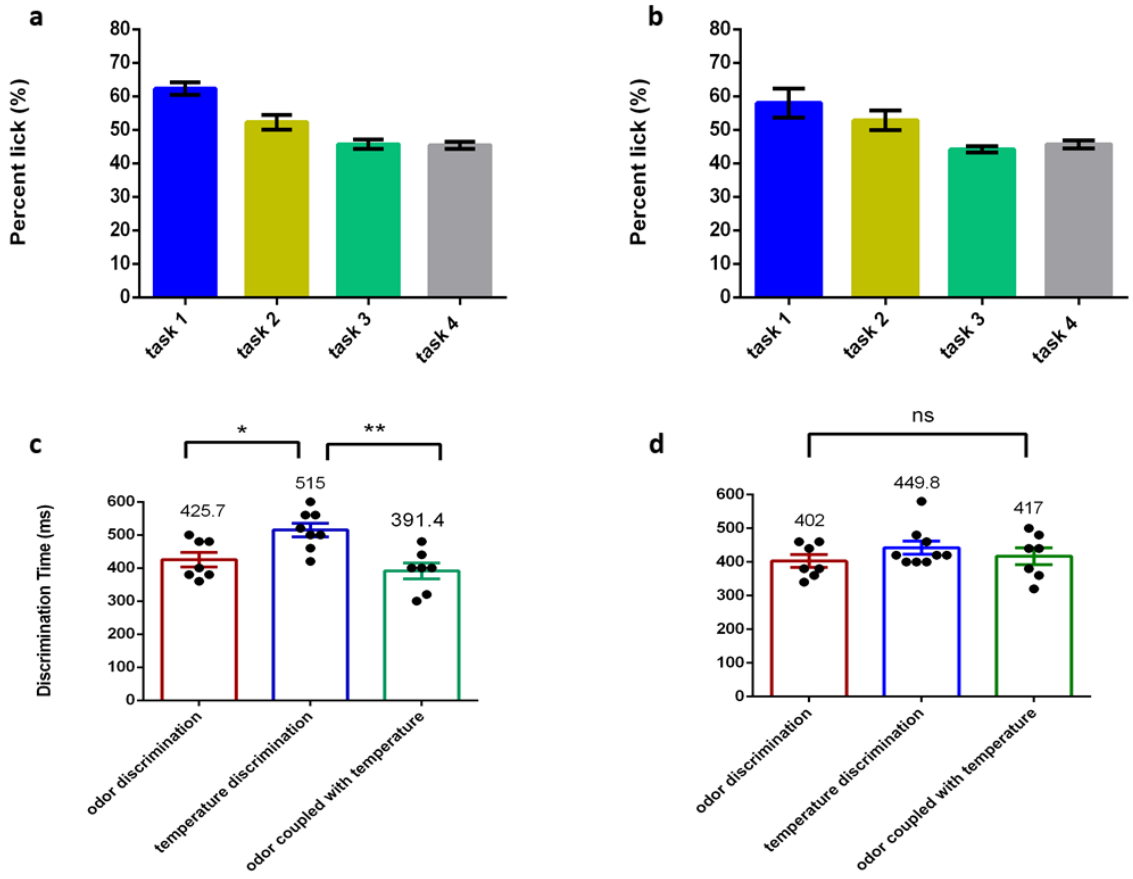


Figure 15: *Mice show slower DTs for temperature discrimination task.*

*a-b: Lick percentage throughout different tasks showing consistency in the motivation level along with the learning(average ± SEM). c: discrimination time calculated from the lick pattern for the final task (temperature discrimination read out is significantly different from other two sets of experiments *(significant), one-way ANOVA F- value =8.59, p-value = 0.0022, Tukey's multiple comparison test,) d: discrimination time calculated from the sampling pattern for the final task (non-significant difference ns(non-significant) one-way ANOVA F- value =0.9, p value = 0.4 Tukey's multiple comparison test,) (average ± SEM).*

4. Discussion

Our sensory systems usually encode various features of single sensory stimuli. The signals from different sensory systems either converge together (multisensory enhancement) or are eliminated (conflicts among sensory systems) to form an active percept. However, the rodent olfactory system is unique as it is capable of processing different sensory signals such as volatile and non-volatile odours, temperature cues as well as mechanical stimulations through the subsystems (Minghong Ma et al., 2008; Grosmaitre X. et al., 2007; Bumbalo et al., 2017; Mamasuew K et al., 2008). How do the information processing of such variety of signals through different olfactory subsystems interact and affect the olfactory perception remains elusive. Here for the first time, we tried to address the effects of temperature on the olfactory perception by training the animals to discriminate unisensory (temperature and odours) and multisensory (temperature coupled to odourants) stimuli.

The animals trained in temperature discrimination task showed a slower learning pace as compared to odourant discrimination and multisensory discrimination tasks, whereas no difference in learning was observed between odour and multisensory discrimination (figure 12b). This betterment of learning was observed in both experiments we conducted (1. When the same animals were trained to discriminate temperatures first followed by odourant discrimination and at last, the multisensory discrimination. 2. When different groups of animals underwent training for all the conditions mentioned above), suggesting that procedural learning may have a minimum influence on the unisensory vs. multisensory discrimination learning (figure

12b, 14d). To address if this is a multisensory enhancement, we need to do further experiments in a more controlled way (using both sensory stimuli close to their detection thresholds). We also observed faster DTs for odourant-temperature and odourant-only discriminations as compared to temperature-only experimental conditions. The phenotype observed here was not as a result of alteration in the motivation levels of the animals as in the learned animals, lick percentage and ITI levels were found to be similar across all experiments (Figure 12c,13 a, 13b, 13 c, 14e, 15a, 15 b). The following possibilities may explain why no difference was observed in the learning of odour-temperature coupled discrimination and odour discrimination tasks.

4.1 Multisensory enhancement takes place at sub-threshold levels.

Previous reports suggest that animals challenged to discriminate multisensory sensory stimuli show better performance levels compared to single sensory stimulus discriminations. This phenomenon is generally termed as a multisensory enhancement (Diederich and Colonius., 2004). Keeping the same principle in mind, we would have expected to see faster learning in temperature/odour discrimination as compared to temperature-alone and odour-alone discrimination tasks. We did observe that temperature discrimination was slower, but odour discrimination and odour-temperature coupled discrimination tasks showed no-differences in the learning (Betterment in learning was observed only at two points in the learning curve and only in the first group of mice). The chances for multisensory integration and enhancement is more when the information from a single sensory stimulus is weak, like our visual system get support from auditory system under dim light conditions to identify objects. It might be possible the odour concentration that we used in this paradigm is enough for animals to make a correct decision relying on single sensory stimulus and animals may not need any additional temperature cues for making the decisions. Hence, multisensory integration of information will not lead to multisensory enhancement explaining why the odour discrimination and odour/temperature coupled discrimination exhibited similar learning curves. Therefore, it will be essential to train the animals on the subthreshold odour concentrations and then address the effect of temperature on olfactory perception.

4.2 A stable odour coding strategy

Since the dynamicity of odour space is too high due to different influencing factors like airflow, vapour pressure, concentration etc., animals use stable odour coding strategies to preserve the odour quality/identity. For example, the dynamicity of airflow in the environment varies the concentration of odourants. Animals use phase coding as a strategy to overcome such challenges to detect the same odour despite of the concentration variation (R Iwata et al., 2017). As the PID measurements suggest that temperature variations affect the odour profiles, it is possible that animals probably use a different odour coding strategy for efficient olfactory perception at different temperatures. If this is the case, at different temperatures, the performance of animals will remain the same. This calls for further experiments to understand the behavioural relevance and mechanisms underlying interactions of temperature and odour molecules and their effect on odour perception.

4.3 Laboratory housed animals are less exposed to temperature variations.

The animals used in this study were bred and grown under laboratory conditions. Hence they were kept at optimum temperature conditions in separate air-conditioned cages. Because of this reason, they were not exposed to any temperature variations in their entire life. At the same time, they were exposed to different kinds of olfactory cues such as food odour, pheromones, bedding odour etc. Due to these reasons, their olfactory ability might be highly sensitive as compared to their ability to sense different temperatures. This can be a possible explanation of why mice are focusing more on odour cues compared to temperature cues while they are challenged with the combination of these two stimuli. These findings suggest that the commonly exposed stimuli possibly would have more effect on any behaviour than the less exposed sensory stimuli.

To summarize, the experiments reported in this thesis provide a piece of clear evidence that mice can be trained to learn temperature/odourants differences under Go/No-Go paradigms. All the animals were able to sense and discriminate unisensory as well as multisensory stimuli efficiently. These findings call for further experiments to confirm the involvement of GG in the temperature discrimination learning we described in the thesis. Further, mechanisms underlying multisensory integration and learning mediated by the olfactory subsystems will be studied in the future.

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6. Supplementary materials

S1 Lick percentage

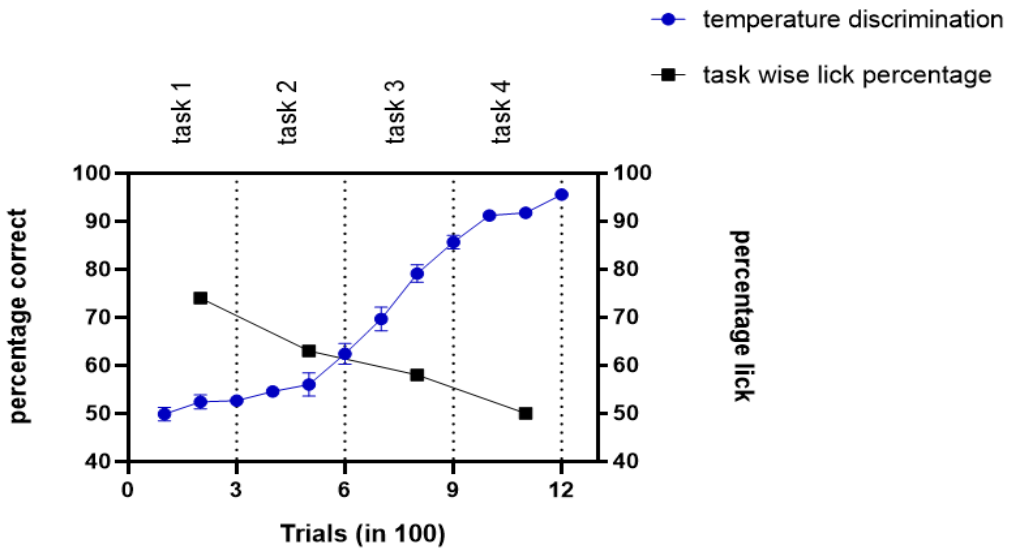

Figure S1: *Task wise lick percentage reduces as the animals learned to perform accurate responses (N=9)*

Blue curve: Percentage correct shown by mice in a temperature discrimination task. Each data point is the average of 100 trials (average ± SEM).

Black curve: lick percentage for different tasks, 50% lick indicates optimal motivation levels and licking for only S+ trials (averaged over a task).

S2 discrimination time (from lick pattern it is similar for sample pattern)

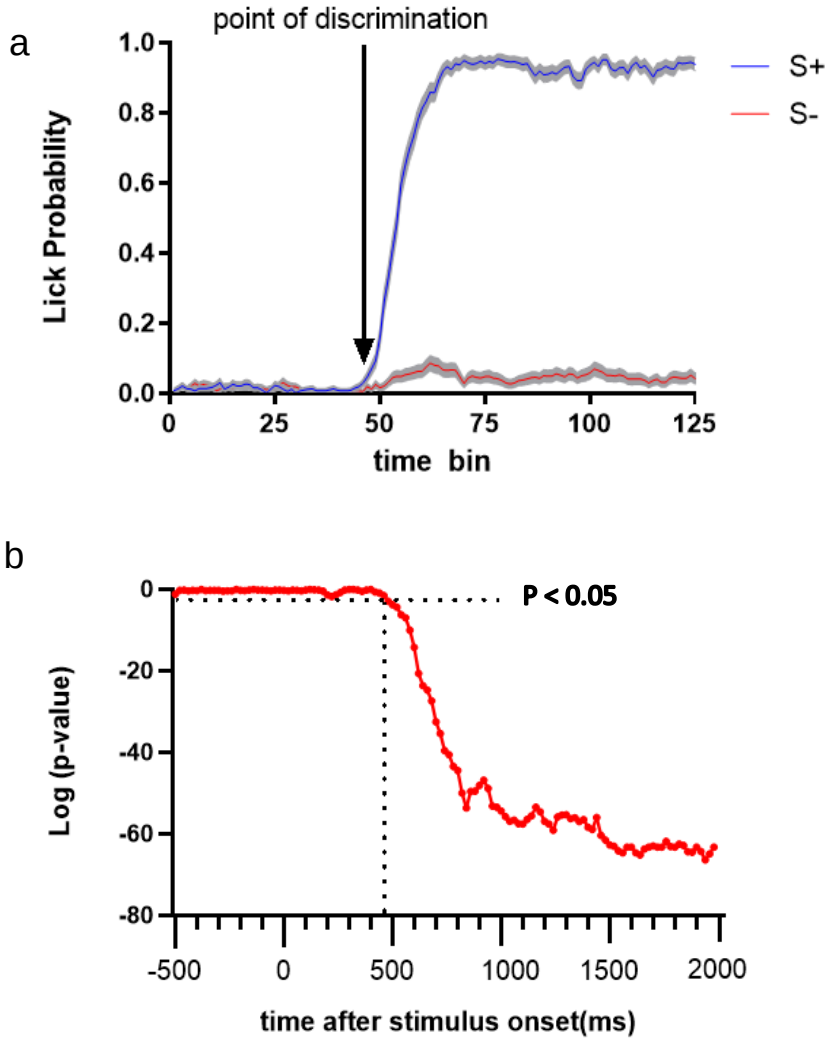

Figure S2: *discrimination time calculated from lick pattern (representative animal at the final task of temperature discrimination)*

a*: lick pattern of the animal at the final task, the point of discrimination between S+ and S- is depicted*

b*: P-value curve obtained from the statistical difference between S+ and S- trials, the last time point where p<0.05 is considered as the discrimination time*

S3 control for valve clicking sound

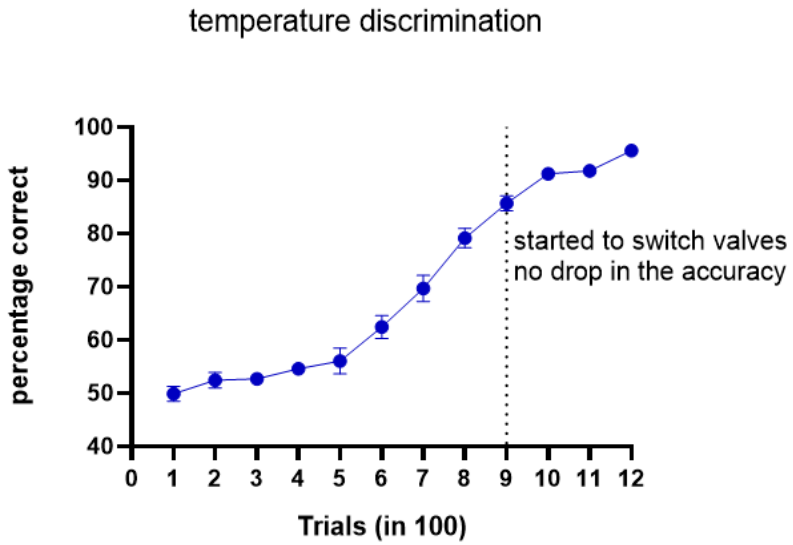

Figure S3: *valve clicking sound does not influence on the learning behaviour (N=9)*

Percentage correct shown by mice in a temperature discrimination task. Each data point is the average of 100 trials (average ± SEM).