Effect of environmental enrichment on complex olfactory behavioral readouts in male and female mice

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Certificate

This is to certify that this dissertation entitled "Effect of environmental enrichment on complex olfactory behavioral readouts in male and female mice" 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 Chadalavada Madhu Priya (20171117) at IISER Pune under the supervision of Dr. Nixon M. Abraham, Assistant Professor, Department of Biology, IISER Pune, during the academic year January-December, 2022.

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Declaration

I hereby declare that the matter embodied in the report entitled "Effect of environmental enrichment on complex olfactory behavioral readouts in male and female mice" 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

Chadalavada Madhu Priya

Dr. Nixon M Abraham

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Abstract

Brain plasticity is the phenomenon by which the neural circuits rewire due to the impact of various environmental cues and stimuli, which helps in adapting to the environment. From dendritic spine modulations to the birth of new neurons in adults, many processes contribute to the brain's plasticity. Adult neurogenesis is the phenomenon by which new neurons are differentiated from neural stem cells during adulthood. It is known to happen in the dentate gyrus in the hippocampus and the subventricular zone (SVZ). This study highlights the role of adult neurogenesis in both these regions, as the hippocampus aids in learning and adult-born neurons in the olfactory bulb refine responses to olfactory stimuli. Adult neurogenesis can be modulated by the environment and stress, and both the genders show difference in stress resilience. This project aimed to see how such factors would affect adult neurogenesis in mice and thereby their olfactory learning abilities in a complex behavioral task. A go/no-go 10-componenet mixture task was performed and the learning pace of the animals and time taken for each group to discriminate between rewarded and non-rewarded odors were compared. The learning is slower and discrimination time is longer when the mice perform complex 10-component task compared to that when they perform a simple task involving two odors as rewarding and non-rewarding. The number of adult-born neurons was quantified to correlate learning with adult neurogenesis. We found out that there is no gender-dependent variability in learning. No difference between male and female mice has been observed in the adult-neurogenesis in OB and DG or cell proliferation in SVZ. With limited number of animals, we didn't observe significant differences in learning abilities between enriched versus control mice. However, we need to repeat these experiments with more experimental animals to draw firm conclusions.

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Chapter 1

Introduction

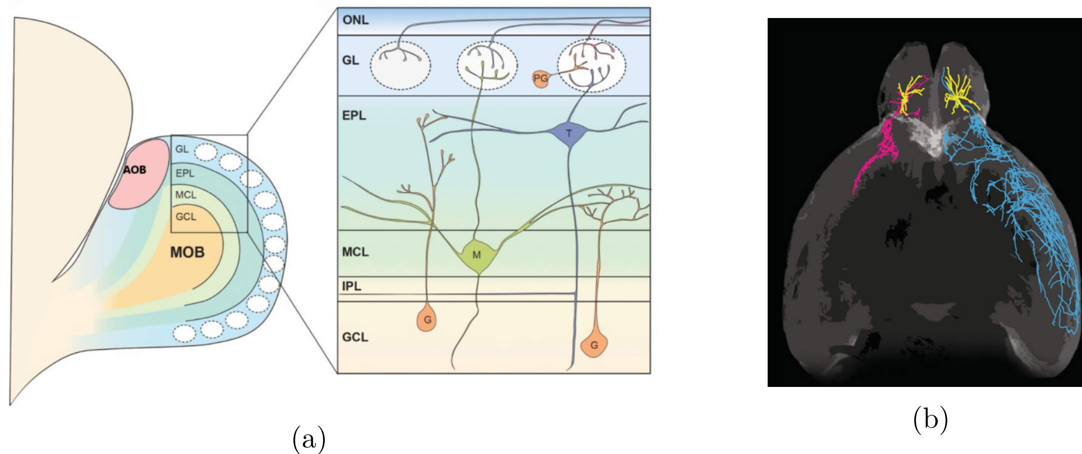
The ever-changing environment that we live in requires that we adapt to these changes. These adaptive changes also happen in the brain and can occur in various forms, from remodeling of dendritic spines to behavioral changes. This is termed as brain plasticity. For a behavioral change to come about, the brain and its neural circuits are modified by changing the dendritic spine density, formation and elimination of synapses, as well as birth of new neurons (Davidson, and McEwen, 2012). All these processes contribute to the plasticity of the brain, and the most complex of all is adult neurogenesis, which is the birth of new neurons after development of the individual. In adult neurogenesis the differentiation of neural stem cells into neurons, the maturation, migration, and synapse formation of these adult-born neurons make adult neurogenesis the most complex form of brain plasticity.

The differentiation of neural stem cells into neurons, glia, astrocytes or oligodendrocytes is called neurogenesis. When in the embryonic stage and during development the rodent brain exhibits most plasticity, and neurogenesis occurs in all brain regions. But post-development, neurogenesis occurs only in certain regions of the adult brain and it varies between species. In rodents, adult neurogenesis can be seen only in the dentate gyrus of the hippocampus and the sub-ventricular zone, and it continues to occur throughout their lifetime.

1.1 Olfactory bulb adult neurogenesis

The olfactory epithelium is made of olfactory sensory neurons (OSNs), basal and supporting cells. Upon inhalation, the odor molecules bind to the olfactory receptors (OR) present on the cilia of OSNs and activates a G-protein (Golf). This Golf binds to guanosine triphosphate (GTP) to activate adenylyl cyclase III, which helps in the production of cyclic AMP (cAMP).

cAMP binds to cyclic nucleotide gated channels which open to give rise to an influx of $Ca^{+}2$ and Na^{+} ions, followed by Cl^{-} efflux. This generates an action potential. The OSNs then repolarize as the cAMP decays and the ion channels close (Ghatpande et al., 2011). The OSNs terminate in circular neuropil-like structures called glomeruli and make synapses with projection neurons of the olfactory bulb (OB). Depolarization of OSNs lead to the excitation of specific glomeruli. Mitral and tufted cells are the two types of projection neurons. Mitral cells project to various anterior and posterior regions in the olfactory cortex whereas tufted cells only reach the anterior parts of the olfactory cortex.[5] The projection neurons also synapse on inhibitory interneurons, which mostly inhibit the projection neurons from which they receive excitatory inputs as well as the neighboring projection neurons. This is called lateral inhibition which is important in reducing the signal to noise ratio in the primary areas of olfactory information processing. Hence the response to an odor molecule depends on the excitation of both projection neurons and inhibitory interneurons, by the OSNs, and the network motifs that connect these three.



(a) (b)

Figure 1.1: a.) Illustration of OB circuit, where MOB is main olfactory bulb, ONL is olfactory nerve later, GL is glomerular layer, EPL is external plexiform layer, MCL is mitral cell layer, IPL is internal plexiform layer, GCL is granule cell layer and AOB is accessory olfactory bulb (Image source : Imamura F., 2020) ; b.) Representation of projections of a single tufted cell (left) whose axons project only to the anterior parts whereas the axons of a mitral cell (right) terminate on almost all areas of the olfactory cortex (OC). (Image source : Mori et al., 2014)

Inhibitory interneurons get continuously replaced throughout the life of the animal. The neuroblasts from the SVZ migrate tangentially for a distance of about 5-8mm each day via the rostral migratory stream (RMS) through glial tubes. It takes about 2 weeks for the neuroblasts to reach the OB, after which they mature and become functionally active (Petreanu et al., 2002). The cells are connected to each other, with the aid of neural cell adhesion

molecule (NCAM) and form chains. The preceding cell can be thought of as a guiding cell for the following cells in the RMS. Once their journey through the RMS is over, they show radial migration which is not guided by the radial glia, and reach the OB. Approximately 94% of these neurons become granule cells, periglomerular cells and astrocytes make 4% and 2%, respectively. They get integrated into the OB either in the granule cell layer (GCL) or in the periglomerular layer. In the GCL, the adult-born interneurons form synapses with the projection neurons. These interneurons help in synchronization of the projection neurons (Lledo and Lagier, 2006). They are GABAergic and inhibit the projection neurons, mostly by lateral inhibition and thereby help in refining the response of mitral and tufted cells. Since the projection neurons transduct the information to higher areas (olfactory cortex), their response to olfactory stimuli becomes important. Thus, more the number of adult-born neurons in the OB, more refined is the response.

The survival of mature adult-born neurons depends on the activity that they receive (Petreanu et al., 2002; Yamaguchi et al., 2005). Sensory deprivation reduces adult neurogenesis in mice due to the lack of activity in these adult-born neurons. Most of the adult-born neurons in the OB die due to sensory deprivation, approximately 14-20 days after the birth of these neurons. Hence, exposure to olfactory stimuli during this period is critical for the survival of adult-born neurons in the OB (Yamaguchi et al., 2005). Associative learning during this critical period shows improved plasticity and alters neurogenesis by increasing the survival rate of adult-born neurons (Anderson ML et al., 2011). Pheromonal cues from male mice show enhanced adult neurogenesis in both OB and hippocampal regions in female mice (Mak et al., 2007; Nunez-Parra, Alexia et al., 2011).

Studies show that environmental enrichment aids in the survival of these adult-born neurons, rather than enhancing the proliferation. A large number of the adult-born neurons do not get integrated into the existing neural circuit and even among those that are functionally active, 50% of them die after about a month (Petreanu et al., 2002; Lledo et al., 2006). Hence, an increase in abGCs in OB and DG of enriched animal brains implies that more number of adult-born neurons have survived.

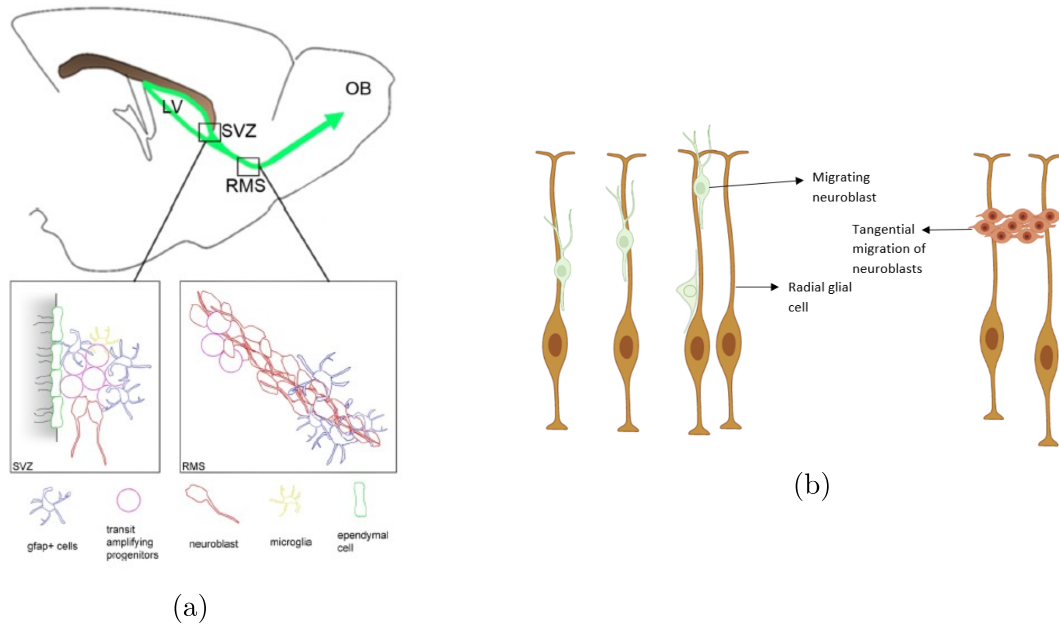


Figure 1.2: a.) Representation of chain formation in the rostral migratory stream which migrates tangentially and reaches the OB. (GFAP : glial fibrillary acidic protein which stains astrocytes and glia) ***ImageSource*** : James et al., 2011; b.) Illustration of radial and tangential migration of neurons

1.2 Adult neurogenesis in Dentate gyrus

Unlike the adult neurogenesis in the OB, only one type of neuron- granule cells (GCs) are generated from the stem cells present in subgranular zone (SGZ) of the dentate gyrus. Studies show that these adult-born GCs play a role in learning and memory formation even when they are immature and not integrated into the circuit (Deng, Wei et al., 2009). It is believed that the unique physiological properties of these immature neurons, such as increased long-term potentiation and insensitivity to GABA (due to lack of $\alpha 1$ GABA receptor subunit) make them important in the context of learning, memory and perception (Lledo et al., 2006; Lods M et al., 2021). Transgenic mice (Nestin-tk) whose adult neurogenesis could be modulated temporally induced contextual fears, in the absence of immature GCs, among few other cognitive defects.

Neural stem cells in the SGZ extend their axons through the GCL and reach the molecular layer. They divide and give rise to identical cells, which acts as intermediate progenitor cells (IPCs). These then proliferate into immature adult-born granule cells (abGCs), which migrate into the GCL but do not yet form synapses. They eventually differentiate into a mature granule cell, which then forms synapses with the neurons in the CA3 (cornus ammonis) region of the hippocampus.

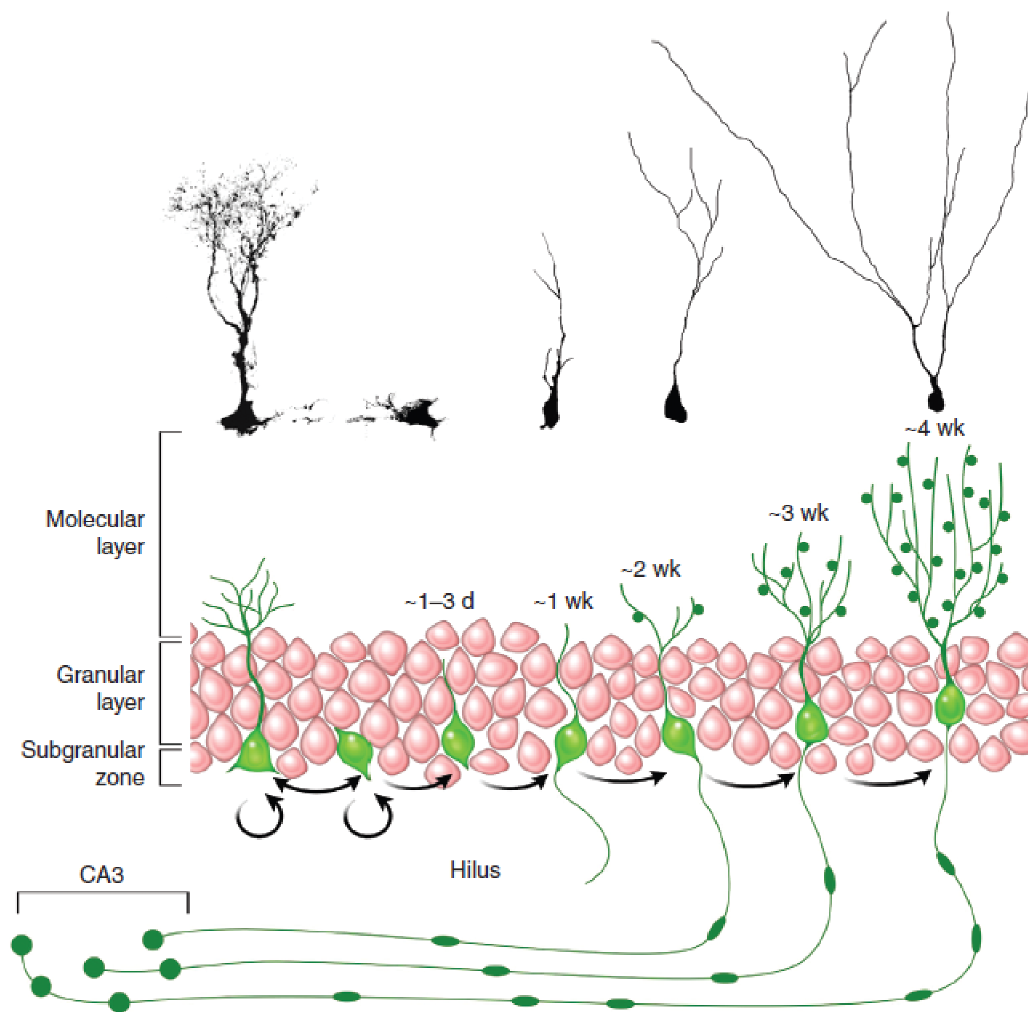


Figure 1.3: Differentiation, migration and maturation of neural stem cells present in the subgranular zone ***Imagesource*** : Toni N, Schinder AF, 2015

1.3 Effect of enrichment and stress on adult neurogenesis

Adult neurogenesis can be modified by physical activity, environmental enrichment, early life adversity (ELA), maternal separation or deprivation, social interactions, etc. Even gender can be a factor that modulates adult neurogenesis since males and females respond to stress differently. Learning and memory is shown to be improved when animals are raised in an enriched environment. In an enriched environment, the animals are provided with a large area to explore, toys for voluntary exercise, social exposure as a greater number of animals are housed together. All of these factors are shown to improve adult neurogenesis in the dentate gyrus, but not in the olfactory bulb. Olfactory enrichment can be observed when the animals are exposed to different odors. Therefore, we added odor boxes in which natural odorant

such as spices are kept. This ensures passive exposure to odors. Olfactory enrichment is shown to improve adult neurogenesis in the OB.

In rodents, following their birth, the brain becomes less plastic gradually as the animal grows, and is at its peak during early development, which lasts till about 4 weeks after birth. Therefore, stress or enrichment during this period would drastically change the learning and corresponding behavior of the animals. Animals which experience early life adversity exhibit depression and anxiety-like behaviors such as excessive grooming, avoiding open and brightly lit spaces. They show less successful escape strategies like moving away from danger, instead of moving to a safer place or less limb movement in tail suspension test (Vale, Ruben et al., 2017). Early life stress can be induced in mice by early weaning, early maternal deprivation for a few hours a day from postnatal day 3 to 14 followed by early weaning, less bedding and nesting which changes the dam's behavior towards the pups and causes increased anxiety (Murthy, S., & Gould, E. (2018)). Anxiety and depression-like phenotypic mice also show lesser cognitive capabilities (Darcet, Flavie et al., 2014). Such learning deficits have been observed in the context of olfactory learning as well and can be rescued by treating the animals with anti-depressants such as fluoxetine, further providing evidence about the association of depression and anxiety with olfactory deficits. It has also been observed that both olfactory learning abilities and adult neurogenesis is seen in these mice models that exhibit anxiety-like behavior (Siopi, Eleni et al., 2016).

5-bromo-2'-deoxyuridine (BrdU) is analogous to the nucleoside thymidine (Fig.). Hence, when a cell proliferates BrdU molecules compete with thymidine to get incorporated into the DNA during DNA replication (Taupin P., 2007). Therefore, BrdU is injected in to the animal intraperitoneally (I.P) which then marks all the adult-born cells which can be quantified by staining the BrdU expressing cells with anti-BrdU antibody. Staining brain sections by immunohistochemistry (IHC) method is discussed later in the methods and materials section.

There are 4 groups of animals whose learning is analyzed in go/no-go behavioral paradigm- environmentally enriched (EE) females, EE males and normally weaned females and males as control groups. Normally weaned animals are subjected to maternal separation 28 days after their birth, after which they are continued to be raised in standard cages. The EE groups were also normally weaned but are raised in enrichment cages. To see if there is a difference in neurogenesis between males and females, we started with the control group. Before starting the training, BrdU (Bromodeoxyuridine) was injected intraperitoneally (I.P). BrdU is an analog of thymidine and therefore, during DNA replication BrdU gets incorporated into the DNA. Thus, BrdU is used to mark adult-born neurons.

These animals perform a complex task called 10-component mixture task. This is an

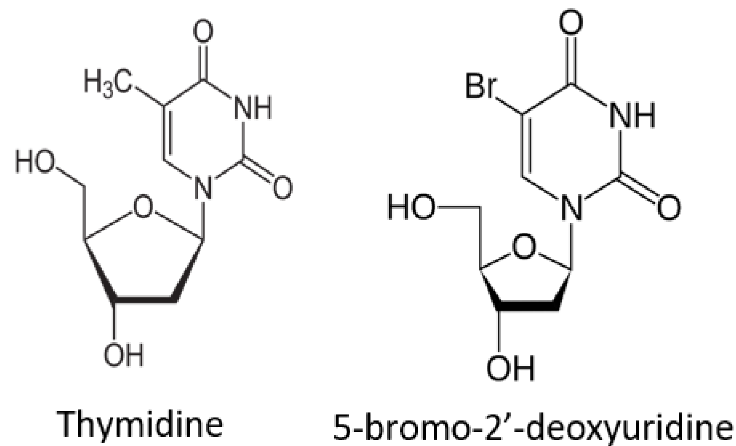


Figure 1.4: BrdU is analogous to thymidine and hence gets incorporated into the DNA of new born cells

olfactory go/no-go operant conditioning in which the animal associates the olfactory stimulus with the water reward. This was done in a head-restrained paradigm which is shown to not affect the performance and learning of the mice, compared to freely moving mice (Abraham, Nixon M et al., 2012).

Immunohistochemical analyses was done for NW male and female mice. In IHC staining, primary antibodies specifically bind to some proteins called antigens. Then the primary antibody is tagged with a fluorescent secondary antibody for visualization. So, all the cells expressing a particular protein of interest can be identified using IHC staining. In BrdU positive cells, BrdU molecules present in the DNA are tagged using anti-BrdU antibody. The OB and DG sections were stained with anti-BrdU antibody and activity-regulated cytoskeleton-associated protein (Arc) antibody. Active adult-born neurons can be marked by Arc antibody, since Arc is an immediate early gene, which is synthesized rapidly when the neuron is active. The expression of Arc would display the involvement of active neurons while the animal performs olfactory task. The adult-born neurons that express Arc are those that are being activated during task performance.

The SVZ sections were stained with Ki67 which is a proliferation marker. This marks all the neurons in the G1 phase, S phase and G2 phase. Olfactory cortex (OC), comprising of piriform cortex, anterior olfactory nucleus and olfactory tubercle were marked with Arc antibody. This was done to see the activity in the OC induced by the activation of projection neurons, whose activity is in turn modulated by the interneurons.

Mitral and tufted cells are second order neurons which form a connection between the OSNs and higher parts of the brain. The tufted cells fire action potential at higher rates compared to the mitral cells (Nagayama et al., 2004). Other ongoing work in the lab (done

by Sanyukta Pandey and Arpan Kumar Nayak) suggests the possibility of a window in which decision reversal happens in mice when they perform olfactory go/no-go discrimination task. The reversal window of non-enriched male and female mice has been compared.

The aim of this project is to see how enrichment can affect olfactory discrimination abilities of male and female mice, when given with a complex discrimination task.

Chapter 2

Materials and Methods

2.1 Animals

A total of 19 animals were used which were divided into four groups - normally weaned (NW) females and NW males, environmentally enriched (EE) females and EE males were used during the experiment. Different mouse lines in CB57BL/6J genetic background, from Jackson Laboratories were used for the experiments. Mouse strain expressing Enhanced Yellow Fluorescent Protein (EYFP) in somatostatin (SST) positive neurons were used for the NW male group, and mice expressing Green Fluorescent Protein (GFP) in SST positive neurons were used for the NW female group. The NW animals were separated from the dam on postnatal day 28 and were kept in standard cages and when they were 10-12 weeks old, were trained in the head-restrained go/no-go paradigm for olfactory discrimination. For the enriched animals, a pregnant female was used, and the brood was used for olfactory discrimination when they were 10-12 weeks of age. There are 5 NW females and 5 NW males, 5 EE females and 4 EE males. The animals were provided with food ad libitum and were kept in individually ventilated cages whose temperature was maintained at 25-27°C and a relative humidity of 50-60%. A 12-hour light/dark cycle has been maintained. The animals were kept under a 12-14 hour water-restricted condition so that they have the motivation to perform the task accurately to get water reward. For all the procedures involving animals, the Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines were followed.

2.2 Enrichment Cages

A large cage with toys to incentivize voluntary exercise, materials with various textures

for tactile stimulation and natural odorants such as spices (bay leaves, cloves, chocochips, almond, nutmeg, cumin, cardamom, cinnamon) for olfactory enrichment is called an enriched environment. A pregnant female mouse was kept in the enrichment cage and then was separated from the dam on postnatal day 28 to provide normal weaning to the pups. The pups were caged separately as males and females in enrichment cages and some nesting material that was used before maternal separation was added in both cages. The pups were then allowed to grow in the enriched environment till they were 10-12 weeks old and their weight was a minimum of 20g, after which they were implanted with a headpost to be trained in a head-restrained go/no-go paradigm to dsicriminate between rewarded and non-rewarded odors as a learning task.

The early weaned animals were separated from the dam on postnatal day 14 and were kept on a heating pad for 20 minutes, twice everyday so that they do not become hypothermic. They were given wet food and slurry during PN-14 to PN-28.

Figure 2.1: Enrichment Cage with toys for voluntary physical activity, items for tactile stimulation, natural odors for olfactory stimulation and maze.

2.3 Headpost Implantation

Once the animals were about 20grams weight, they were surgically implanted with a headpost. A stereotaxic apparatus is used to perform the surgery, which was sterilized using 70% ethanol. To anaesthetize the animal, a volume of 2μl/g of body weight of ketamine and xylazine (12:5 ratio) was injected intraperitonially, which keeps the animal anesthetized for about an hour. The animal was checked for reflexive movements by the toe-pinch. To avoid choking, the tongue of the animal is pulled out using debakey forceps. The right and left ear

bars and bite bar of the stereotaxic apparatus were aligned to the same height. Following that the animal is mounted where the ear bars are inserted into the ears and the upper teeth of the animal is placed in the bite bar, and the nose bar is placed upon its snout.(Fig. 2.2)

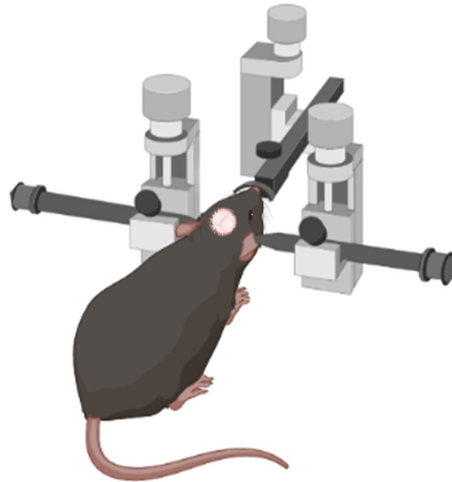


Figure 2.2: Illustration of the stereotaxic apparatus

A sheet of tissue paper is laid on the base of the stereotaxic apparatus to provide insulation to the animal during the surgery. Eye drops() were used to keep the eyes lubricated. To reduce any chances of infection, an antiseptic ointment, cipladine is applied using an earbud on the area between the ears of the animal. All surgical instruments used during the procedure were sterilized.

Using a sterile surgical blade an incision was made on the skin on top of the skull, 1-2 centimeters posterior from the eyes. To make a window, a curved iris scissors is used and starting from the incision, a semi-circular cut is made. Immediately after the window is made artificial cerebrospinal fluid (ACSF) is applied on the window to prevent the skull from drying. The pericranium, which is a connective tissue on the skull is removed by tilting the surgical blade and gently scraping the tissue to the centre of the window. The tissue is then lifted off using a forcep. The area is then air dried followed by phosphoric acid application. Phosphoric acid makes the skull porous, so that the adhesion of the implant is strong. The phosphoric acid is allowed to react for 5-7 seconds and then cleaned with a clean earbud and then with ACSF. To avoid excessive damage and bleeding, the duration of phosohoric acid application is optimized to 5-7 seconds. A primer is applied on the skull, which when exposed to UV light for about 30-45 seconds, forms crosslinks and solidifies. A thin layer of white cement is applied on the primer, and the layer is made more textured by nudging the cement using forceps. It is then exposed to UV light for about 15-30 seconds after which it hardens. A headpost with a circular hole and two legs is then placed in the

centre of the base layer using some more white cement. The white cement is infused into the base layer for more stability. UV light is used to harden the cement and fix the headpost in place. Finally, the window is closed using acrylic cement and is allowed to set.

Once the acrylic cement sets, the nose clamp is removed, followed by the bite bar and ear bars. To bring back the metabolism of the animal to a normal state, it is kept on a heating pad at optimal temperature for 20 minutes.

The water restriction was started 1-2 days after the surgery to ensure full recovery. To acclimatize the animals with deprivation, they were initially deprived for 6 hours, and then for 12-14 hours. This keeps them motivated for water reward as well as maintain their weights in a healthy range.

2.4 Head-restrained go/no-go testing

In the head restrained setup, there is a PVC tube of about 8cm diameter, with a base plate fixed in it, where the animal sits and performs olfactory discrimination task (Fig. 2.3). The headpost of the animal is screwed onto a custom-made slot, which is fixed on the tube. This restricts any head movement. Once the animal gets used to the setup, it is restrained and is introduced to the licktube, and then to the odor port during pre-training.

2.4.1 Pre-training

Pre-training helps the animal associate olfactory stimulus from the odor port with the water reward from the licktube. In the first stage of pre-training, the animal gets 5µl water immediately after a 200ms tone, with no delay. The tone is to make the animal alert. After 20 trials, the water delivery is delayed by 1000ms after the tone for the next 40 trials, and by 2000ms for the next 30 trials. The animal is then introduced to the odor port in the next stage of pre-training. The animal detects airflow from the odor port and learns to respond by licking, which results in a water reward. In this stage, the number of licks required for the animal to be rewarded with water increases gradually from 120ms to 240ms within 200 trials. Following the pre-training, a detection task is performed in which instead of airflow, the animal is presented with methyl benzoate (MB) for 2000ms. This is called the reward window. The reward window is divided into 500ms segments called bins, and if the animal licks during the reward window in at least 3 out of 4 bins or a minimum of 500ms in total, it is rewarded with water. The lick pattern is recorded and later analyzed to find out the discrimination time.

During the training, a rewarded (S+) and non-rewarded (S-) odor stimuli are presented

to the animal in a pseudo-randomised fashion. The animal has to lick for a minimum of 240ms within this window when S+ odor is delivered to get 3μl of water reward. S- odor stimulus is a non-rewarded neutral stimulus. When S- odor is delivered, the animal is not supposed to lick, but there is no punishment or reward even if it licks.

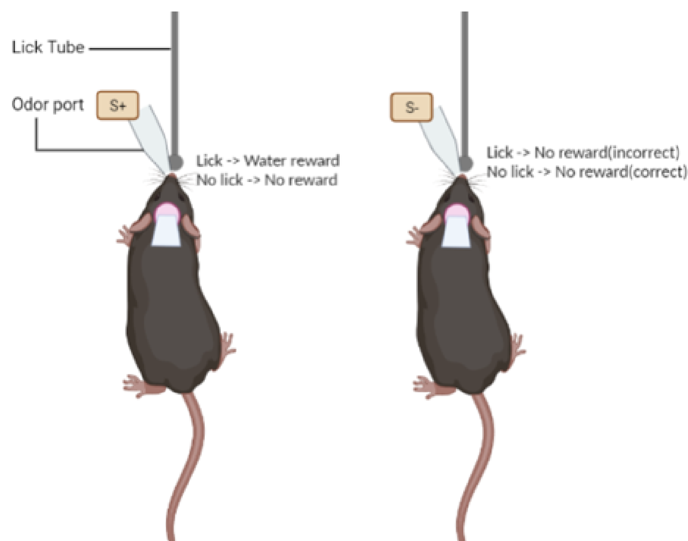


Figure 2.3: Illustration of head restrained set-up

2.4.2 10-component Mixture Training

The animals performed a 10-component mixture task under the head-restrained paradigm. In the 10-component mixture, there are 9 odors in both S+ and S- stimuli (4μl each), and there is one differentiating odor- Acetophenone or 1-Hexanol (Fig 3). 4μl of each of the 10 odorants are added to 4ml of mineral oil, making it 1% concentration (v/v). The mixture was stirred everyday before the experiment to make sure there is no sedimentation since there are many odors present. To avoid any bias towards one odorant, the groups were divided into 2 sub-groups in which 3 animals form a sub-group that were trained to learn that the odor mixture containing Acetone is S+. Rest of the animals from the group were rewarded (S+) if they lick when the odor mixture containing Hexanol was delivered.

The lick pattern of the animal throughout the trial is recorded for further analysis. The base plate on which the animal sits during task performance and the lick tube are made of conducting metal. Whenever the animal licks, the electric circuit is closed which is recorded as a lick. Later, the lick pattern during the reward window is analyzed.

Volume Added	S+ Odor Mixture	S- Odor Mixture
4µl	Ethyl Butyrate	Ethyl Butyrate
4µl	Benzaldehyde	Benzaldehyde
4µl	Cineole	Cineole
4µl	(+) Limonene	(+) Limonene
4µl	Methyl Benzoate	Methyl Benzoate
4µl	Nonanol	Nonanol
4µl	Octanal	Octanal
4µl	1-Pentanol	1-Pentanol
4µl	Nonanal	Nonanal
4µl	Acetophenone	1-Hexanol
Total Volume = 40µl	**Acetophenone + 9 Background Odors**	**1-Hexanol + 9 Background Odors**

Figure 2.4: Odors present in rewarded and non-rewarded odors in the 10 component mixture

2.5 Decision Reversal

In a go/no-go operant conditioning paradigm, during initial phase of learning, the animal licks for both S+ and S- trials, since it is water-restricted, and thus is motivated to lick to recieve water. But as it learns to discriminate between S+ odor and S- odor the number of licks for S- trials decreases. A large fraction of the licks for S- trials lie in a particular window of time. Based on unpublished work from our lab, this window usually lies in the first half of the reward window i.e, 400ms-1000ms.

2.6 Data Analysis

Learning accuracy plots were generated by taking average correct responses in every 100 trials. The lick state within the reward window were analysed manually to plot the task-wise lick pattern. All the graphs were plotted using Graphpad Prism 8. Mean of the data along with standard error mean is shown in the graphs.

Decision reversal plots were generated by a custom written program on python, by Arpan Kumar. All the S- trials in blocks (20 trials = 1 block) with more than 80% accuracy were taken into account. Then all those S- trials with non-zero licks were filtered for analysis. If any of the licks in a trial were longer than 80ms, they were excluded since they might be erroneous trials in which the animal holds on to the lick tube when overmotivated. The lick state of all the animals in a group are averaged and normalized to get a cumulative lick plot of the group. From the cumulative lick plot, the reversal window in which maximum number of licks lie is obtained. This is done by plotting a p-value curve of the cumulative lick plot by comparing each bin in the reward window with the first 25 bins, called the baseline licks.

Two-way ANOVA test is used to compare the data between groups and are treated to be significantly different if $p<0.05$

2.7 Quantification of Neurogenesis

BrdU was injected intra peritoneally on the first day of training. 4 injections were given at 100mg/kg dosage, with a 2-hour gap between each injection. Studies show that maximum number of BrdU-expressing cells can be found the brain between 14-28 days after injecting with BrdU (Yamaguchi et al., 2005). 28 days after the injections, the animals were made to perform the olfactory task and then were sacrificed within 30minutes. Perfusion was done within 30minutes to get maximal Arc activity. The brains were extracted after they were perfused and were stored in 4% (w/v) paraformaldehyde (PFA) overnight. They were later transferred to 30% sucrose solution overnight or till the brain sinks to the bottom. The brain absorbs sucrose and prevents the tissue from freezing when it is stored at -20° Celsius for sectioning the brain. After fixing the tissue in cryo embedding medium coronal sections of 50μm were cut using the cryotome. The sections were then stained using immunohistochemical (IHC) technique and were observed under Leica SP8 confocal microscope at 40x magnification; oil immersion.

2.8 Immunohistochemical Analyses

2.8.1 Olfactory Bulb Sections

The OB sections were stained with anti-BrdU antibody. They were incubated in HCl at 37°C for 45 minutes followed by 3 washes in 1x PBS. They were then kept in blocking solution (BS) (5% normal goat serum (NGS) and 1% Triton-X in PBS). The primary antibody for BrdU used is anti-BrdU raised in rat (1:1000 dilution) in 1% NGS and 0.1% Triton-X solution. The OB sections were incubated in primary antibody for 22hours at 4°C. They were then given 3 PBS washes, after which secondary antibody was introduced. Anti-Rat Alexa Fluor 488 was used as secondary antibody which is diluted in 1% NGS to 1:500 dilution. The sections were kept in secondary antibody for 2 hours. Then after 3 PBS washes, DAPI diluted to 1:500 in 1% NGS was added and then the sections were mounted onto glass slides. Vectashield was used to keep the fluorescence unaffected with time.

2.8.2 Dentate Gyrus Sections

For quantifying the neurogenesis in DG, some sections were stained with Ki67 and some were stained with anti-BrdU and Arc atibodies. The number of Ki67 expressing cells is lower than the number of BrdU expressing cells, hence like the OB sections, DG sections were also stained with anti-BrdU and Arc, following the same procedure as above.

2.8.3 Sections containing the Sub-ventricular Zone

The SVZ and hippocampal sections were stained with Ki67 antibody, which is a proliferation marker. All the adult-born neurons can thus be identified. The sections were kept in BS for 2hours and then the primary antibody was added (1:500 dilution in 1%NGS and 0.1% Triton-X). After 3 PBS washes, the sections were kept in secondary antibody (anti-Rabbit Alexa Fluor 594; 1:500 dilution in 1%NGS) for 2 hours. 3 PBS washes were given and then they were kept in DAPI before mounting them onto glass slide.

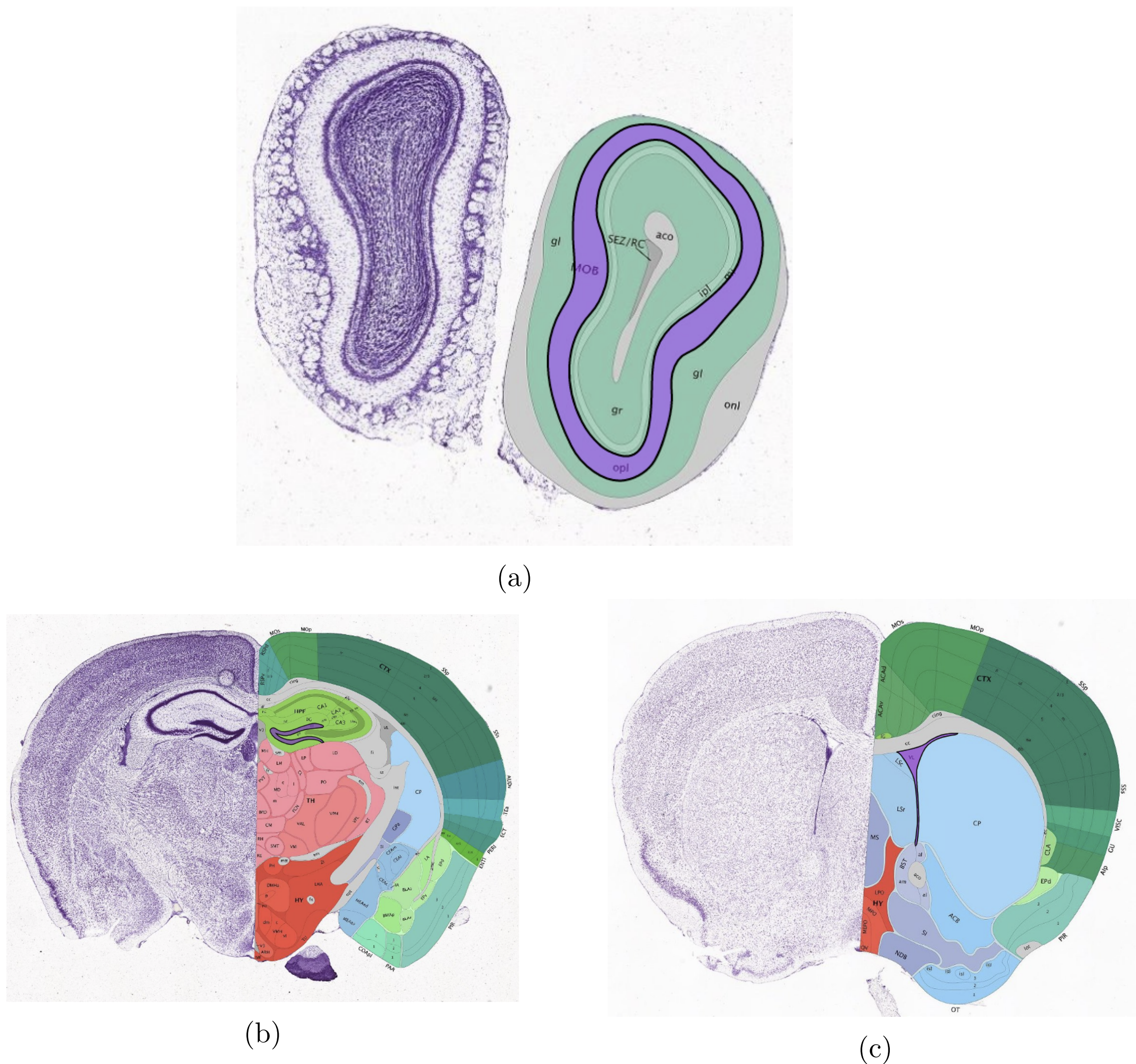
2.8.4 Olfactory Cortex Sections

Since projection neurons synapse on the neurons present in the regions of the olfactory cortex (OC) such as piriform cortex and olfactory tubercle, the OC sections were also stained with Arc to see their activity. The blocking solution for Arc is made of 5% Bovine Serum Albumine (BSA) and 1% Triton-X. Arc primary antibody was diluted to 1:1200 in 1% BSA and 0.1% Triton-X. Following the primary antibody incubation, 3 PBS washes were given. The sections

were then kept in secondary antibody (1:500 dilution of anti-rabbit 594 in 1%BSA) for 2 hours, followed by DAPI staining and mounting.

2.9 Imaging

The following figures are all taken from the Allen brain atlas of mouse coronal sections. On the left is DAPI stained sections of OB, DG and SVZ and on the right is the atlas of various regions identified in these sections. Images of the areas of interest were taken while a particular laser was used to observe the fluorescence of secondary antibody.



(a)

(b)

(c)

Figure 2.5: (a) The area highlighted in purple (right), showing MOB region of the olfactory bulb; (b) Granule cell layer of the DG in which adult-born neurons are generated; (c) The lateral side of the lateral ventricle serves as a niche for neural stem cells called the subventricular zone *Imagesource* : *AllenBrainAtlas*

2.9.1 Image Analysis

The images of the sections were analysed using ImarisX64 software. They were processed in both Imaris and ImageJ. While imaging, each antibody expression is imaged in separate channels, sequentially. Each channel was analysed separately if there are more than two channels, excluding DAPI channel. By clicking on 'add spots' and optimizing the diameter of the cells according to the zoom level, the software detects all points that show significantly higher intensity and marks them as cells. These are the cells that are expressing the antibody that is present in the channel being analysed. The cells were counted manually as well to make sure there are no cells that are false positives and add antibody expressing cells that could be disregarded by the software.

Chapter 3

Results

To test the olfactory learning abilities, we used an olfactometer to perform an operant go/no-go task in which the mice learn to elicit two different responses to two different olfactory stimuli. When the animal responds accurately i.e, licks on the lick tube when it is presented with a rewarded (S+) odor and does not lick when a non-rewarded (S-) odor is delivered, it is considered as correct response. The animals performed a complex 10-component task in which there are 9 odors present in both S+ and S- odor mixtures and one differentiating odor is present in S+ (Acetophenone) and S- (Hexanol) odor mixtures. The accuracy of 1500 trials was recorded and is divided into tasks of 300 trials each. And each task consisted 15 blocks, where 20 trials make a block. Each block has equal number of S+ and S- trials which are pseudo-randomly delivered. The accuracy of every 100 trials is averaged and plotted to find out how the animals learn to discriminate between rewarded and non-rewarded odors and elicit lick responses accordingly. This is called the learning curve. We also assessed the lick pattern in each task, for S+ and S- odors. The discrimination time (DT), which is the time point at which the lick probability of S+ is significantly different from the lick probability when S- odor is delivered. We compared the DTs of all the groups.

Learning curve represents the progression of the ability of the animals to discriminate stimuli during the training phase. Each animal performs 5 tasks, each task containing 15 blocks with 20 trials in each block. In the learning curve, each data point represents the average accuracy of 100 trials.

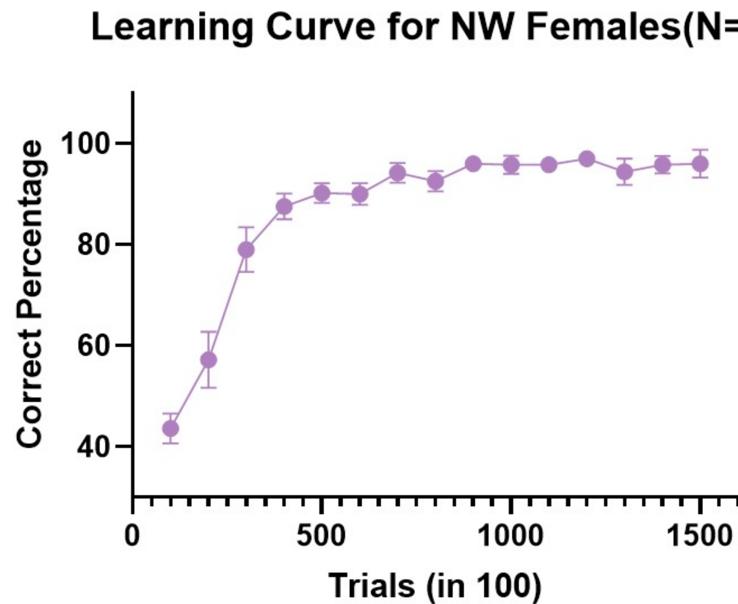


Figure 3.1: Learning curve of normally weaned females that performed a complex 10-component mixture discrimination task (N=5)

A representation of the learning curve of normally weaned females (N=5) is shown in Fig. 3.1. In the initial tasks, the animals lick during both S+ and S- trials. But as they learn, their accuracy increases as they lick only when S+ odor is presented.

The average licking when S+ and S- odors are delivered is plotted as lick probability curve or lick pattern. This shows how the lick responses are varied for rewarded and non-rewarded odors when the animal accurately performs the go/no-go task. The lick pattern for each task is plotted by taking the bin-wise lick state for rewarded and non-rewarded trials separately (Fig. 3.2). The lick probability curve is generated in which the x-axis is the reward window (2000ms), which is divided into 1000 bins of 2ms each. This lets us visualize in which task the animal has learnt to discriminate between rewarded and non-rewarded odors (Fig. 3.2 (a) to (e)).

A task-wise discrimination time plot for normally weaned males (N=5), for representation, shows that the DT decreases from task-1 to task-5 (Fig. 3.2 (f)). DT in task-3 is significantly lesser than that in task-2 and it shows that this set of animals have learnt to discriminate by the third task.

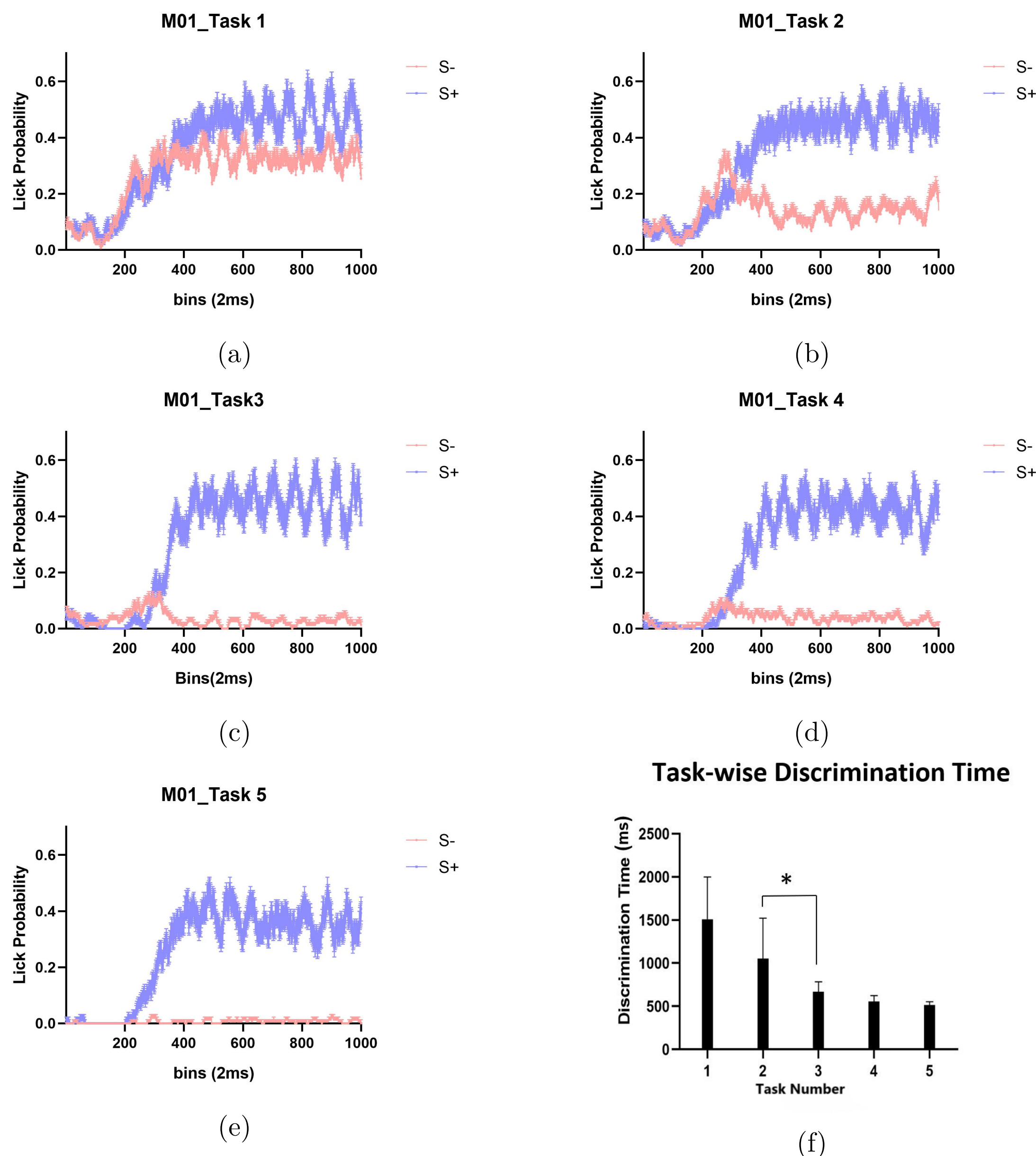


Figure 3.2: (a)-(e) Task-wise lick probability of a NW male, shows that the amount of licks gradually decrease when S- odor is presented, as the animal learns to discriminate between S+ and S- odors (Lavendar : lick probability in S+ trials; Peach : lick probability in S- trials); (f) Task-wise DT shows decrease in discrimination time from task-1 to task-5, and also that DT in task-2 is significantly different from DT in task-3

3.1 Simple versus 10-component mixture task comparison

Studies done on perception of odor mixtures indicate that the efficiency of identifying target odor from background odor depends on the extent of overlap in the glomerular activation pattern (Rokni D et al., 2014). This ability to identify target odor among other background

odors also depends on the number of background odors (Rokni D et al., 2014). Odorants with similar chemical structure can evoke a similar activation pattern in the OB. The spatial pattern in the olfactory bulb changes dynamically in order to produce different patterns, to discriminate odors that evoke similar patterns (Gschwend O et al., 2015). While performing the 10-component task, to perform the task correctly, the animal has to treat all the 9 same odors (see section 2.6) as background odors and the differentiating odor (Acetophenone or Hexanol) as the target odor, and accordingly has to make a decision to lick or to not lick. Therefore, this task is considered to be a complex one. To see if this difficulty results in a delay in learning we compared the data with simple odor pair (Acetophenone Vs Octanal). This comparison between SSTxEYFP males' learning pace when performing a complex 10-component task and simple odor pair (unpublished data from Meenakshi Pardasani) shows that the learning happens faster for simple odor discrimination task (Fig. 3.3). The discrimination time for 10-component task is longer compared to simple odor pair (Fig. 3.4 (a)) and this has been statistically tested using unpaired t-test with a p-value of 0.0361 (Fig. 3.4 (b)).

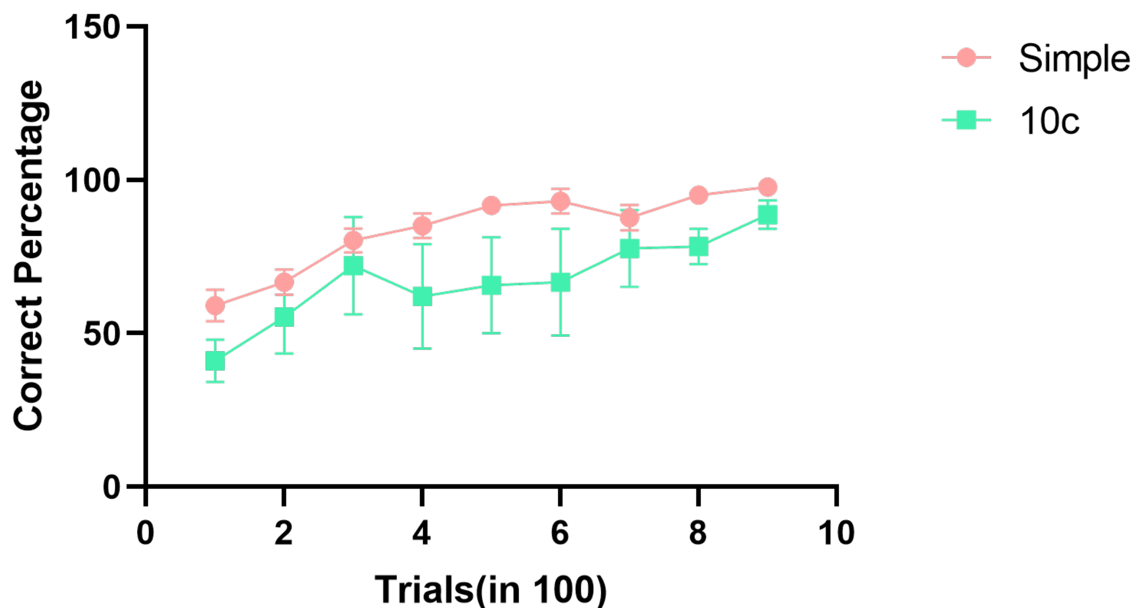


Figure 3.3: Learning curve comparison between simple odor pair (pink) task and 10 component task (green). The learning is significantly slower when normally weaned males perform 10-component task, when compared to a simple odor pair - Acetphenone vs Octanal (p-value=0.0007, F=13.73), Two-way ANOVA

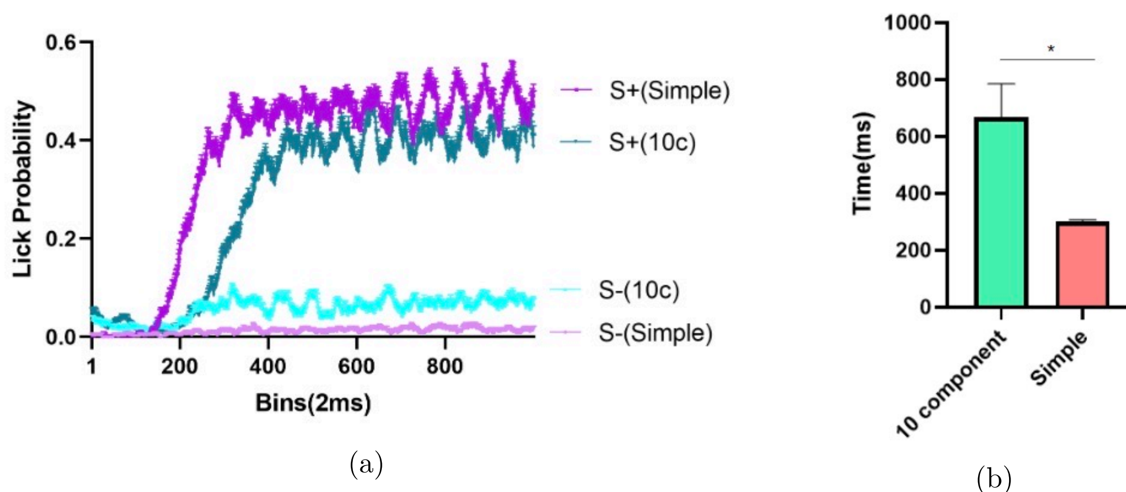


Figure 3.4: (a.) Lick pattern comparison between simple task and 10-component mixture task. (b.) The discrimination time for 10-component mixture task is significantly longer than that for simple task.

3.2 Gender-dependent variability is not observed in both enriched and non-enriched animals

Most of the studies done on mice are done with male mice. Here, we compared the learning behaviors between male and female mice that are normally weaned and also between enriched males and females. Fig. 3.5 and 3.6 show the learning curve, lick probability (Fig. 3.6 (a)) and DT (Fig. 3.6 (b)) between NW males and females. The p-value when non-enriched male mice learning was compared to females was 0.051. So, to confirm that there is no difference in learning between non-enriched male and female mice, more number of animals have to be tested in the paradigm. Learning curve (Fig. 3.7), lick probability (Fig. 3.8 (a)) and DT (Fig. 3.8 (b)) of enriched males and females have been compared.

Though enriched males showed more radical changes in behavior such as aggression, their learning abilities did not vary much compared to the enriched females. Similar to NW males, EE males showed more variability within the group (longer error bars; SEM).

Gender-dependent learning or DT variabilities have not been observed in both NW and EE animals.

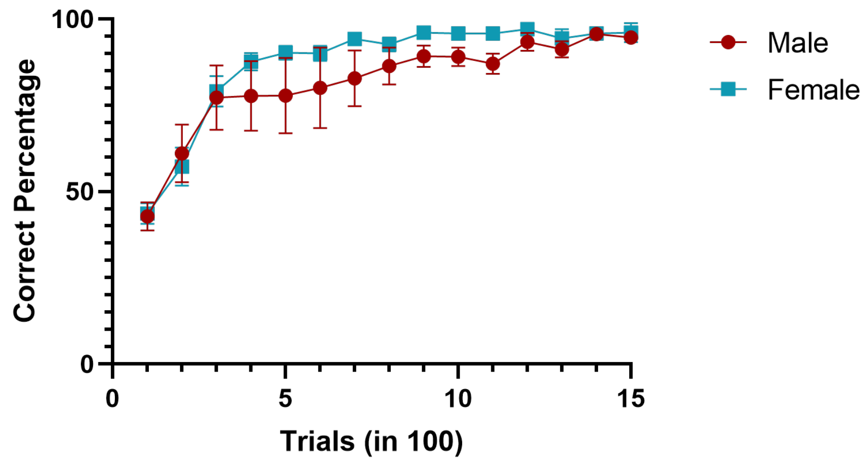


Figure 3.5: Learning curve comparison between males (red) and females (blue). Males showed a trend of slower learning compared to females (P=0.051, F=8.151, Two-way ANOVA (n=5))

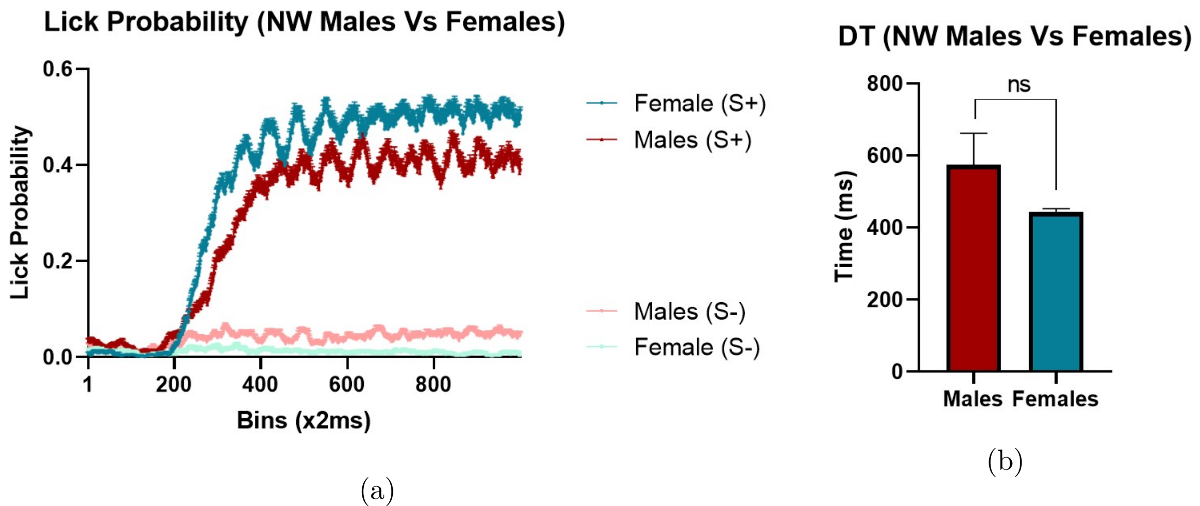


Figure 3.6: (a) Lick probability comparison between males (red) and females (blue). Males showed a trend of slower learning compared to females but not significant (P=0.051, F=8.151, Two-way ANOVA (n=5)); (b) Discrimination time shows no significant difference. This experiment needs to be repeated with more experimental mice.

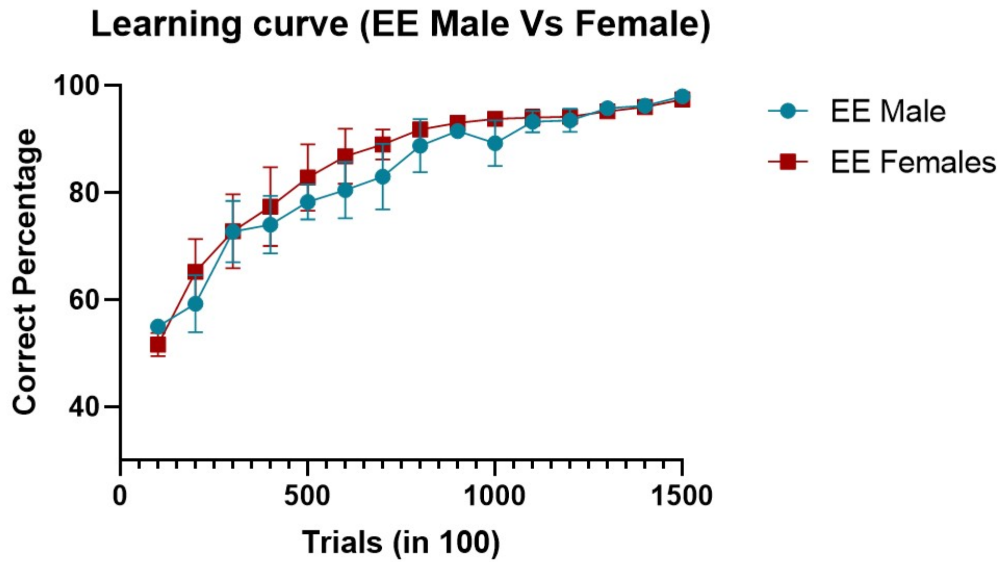


Figure 3.7: Learning abilities of enriched male and female mice showed no variability. (N=4 males, 5 females)

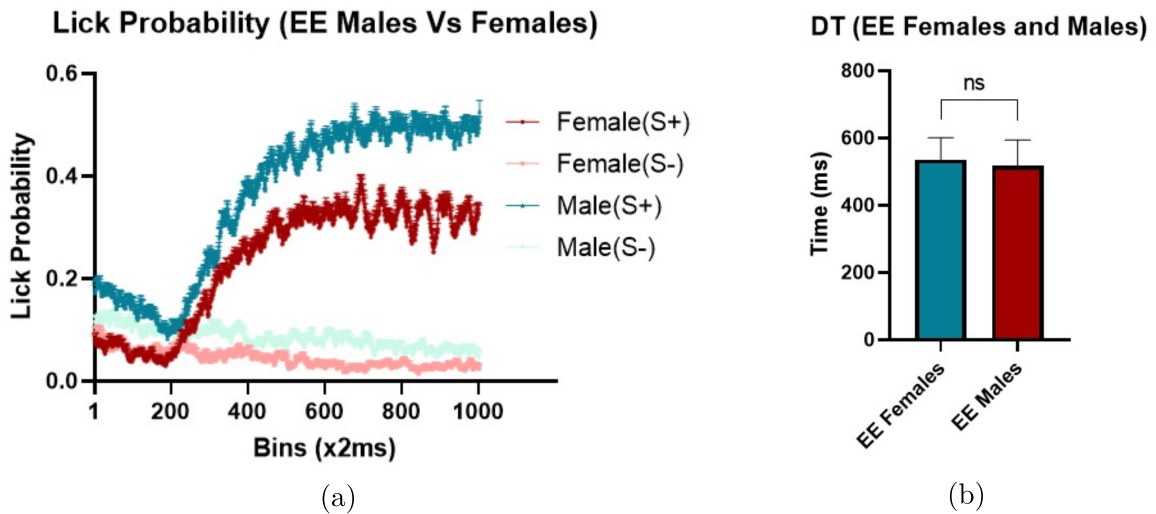


Figure 3.8: (a) Lick probability of enriched males and females when they were in task-3 (N=4 males, 5 females); (b) Discrimination time of enriched males and females show stark similarity (N=4 males, 5 females)

3.3 Effect of environmental enrichment on olfactory learning

Voluntary exercise is shown to improve learning and memory significantly. Based on previous literature, it is hypothesized that faster learning and more neurogenesis happens in enriched mice.[9] But no such significant improvement has been observed. However, the results reported here are from the limited number of mice. Therefore, we need to repeat these experiments with more number of experimental mice.

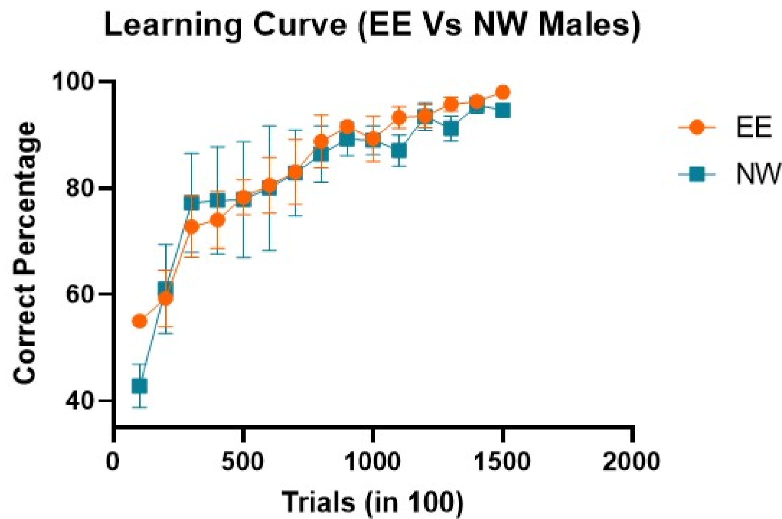
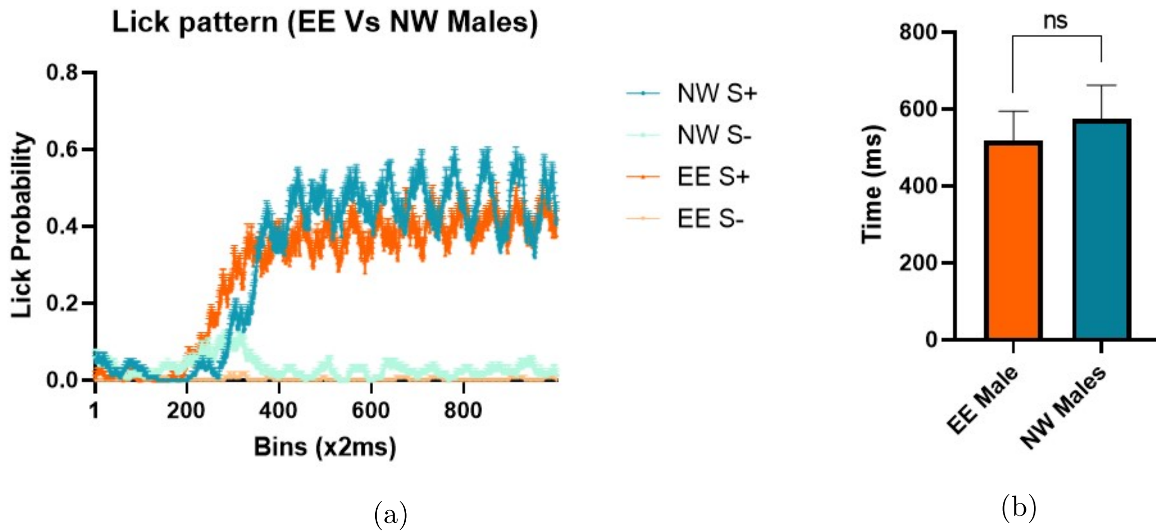


Figure 3.9: Comparing learning curves of enriched and non-enriched males shows no difference in learning pace. (N=4 EE males, 5 NW males)

Fig. 3.9 shows learning curve comparisons between enriched and control males.Fig. 3.10 shows lick pattern and DT comparisons between EE and control males. The effect of enrichment can be different on males and females. Yet, when compared to NW males and females, neither of the genders showed any significant difference (Fig. 3.11, 3.12).



(a) (b)

Figure 3.10: (a) Lick pattern of enriched and non-enriched male mice; (b) Discrimination time of enriched males is similar to the DT of non-enriched males (N= 4 EE males, 5 NW males)

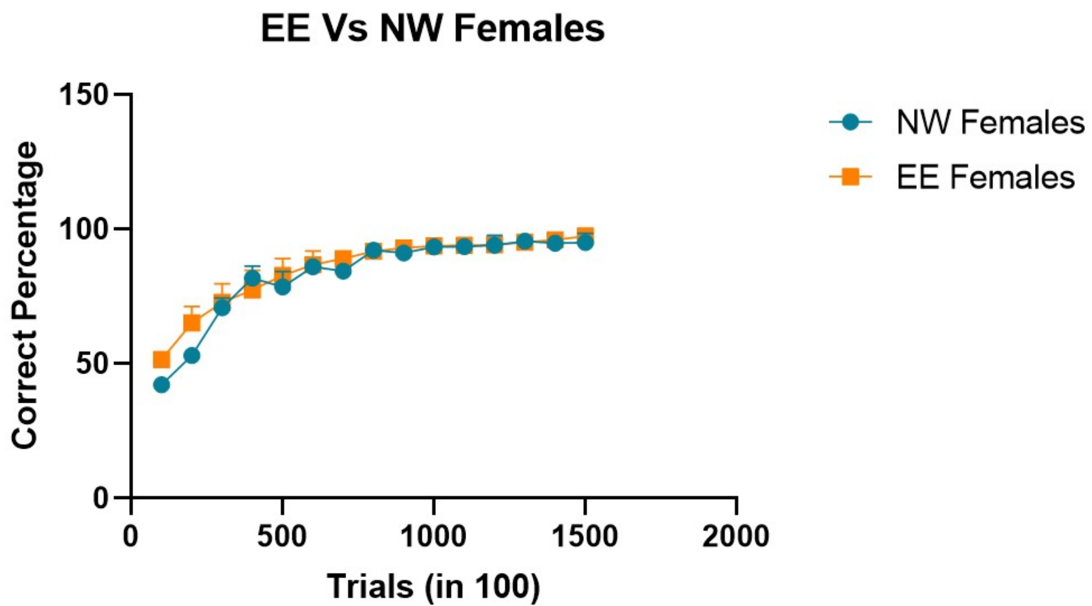
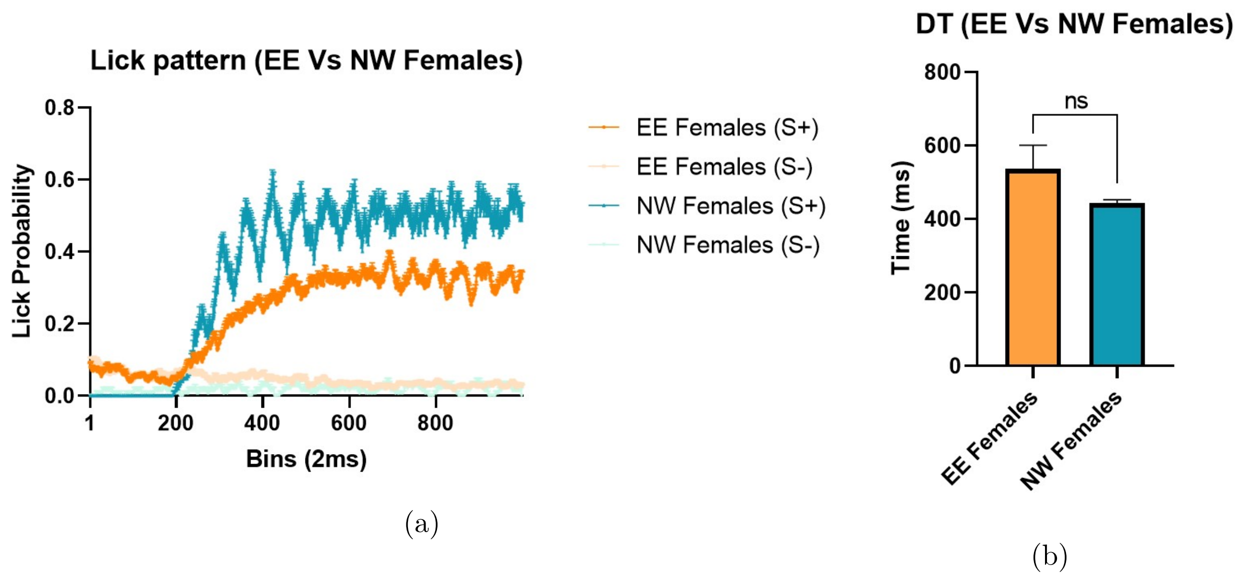


Figure 3.11: Comparison of learning curve of enriched females and non-enriched females (N=5)



(a)

(b)

Figure 3.12: (a) Lick pattern comparison of enriched females and non-enriched females; (b) Discrimination time of enriched females is not significantly different from that of non-enriched females, when in the third task. (N=5)

3.4 Decision reversal in olfactory learning

When the odor is delivered, the tufted cells being the fast-firing neurons, produce a faster response (Nagayama et al., 2004; Mori et al., 2014). The initiation of fast lick responses, which are stopped in a few milliseconds, might occur due to the signalling through tufted cells. The stopping of licking might be due to the signalling through the mitral cells. However, we need to verify the underlying neural mechanisms of these behavioral responses.

When a cumulative plot of lick responses during the reversal trials that are filtered out (see section 2.5), a peak was observed. This peak could indicate decision reversal. To pinpoint to the time window in which this peak lies, a p-value curve of the cumulative lick is generated by comparing the lick responses in the reward window with the lick responses during the first 50ms of the reward window (Fig. 3.13 (c)).

3.4.1 Reversal Window in non-enriched males

The plots above show the reversal trials in non-enriched control males. Fig. 6a is a representation (of one of the five animals) of the normalized lick plot. Here, the number of licks in each bin from all the reversal trials is summed up and normalized. Figure 6b is the cumulative lick plot for all five control males. A time window can be identified in which a maximum number of licks fall. By taking the p-value curve of the cumulative lick plot and setting a threshold of 0.05, the maximum lick window can be obtained (see section 2.8). This window lies in the first half of the reward window for the non-enriched males i.e., 168-473 bins or 336-946ms (Fig 6c).

3.4.2 Reversal Window in NW females

Fig. 3.14 shows the cumulative lick plot and the p-value curve of five normally-weaned females. The reversal window lies between 364-758ms. Compared to control males, in control females, the reversal window is more narrow by approximately 240ms. The reversal window in control females begins after about 30ms compared to males, but the decay of the peak is delayed.

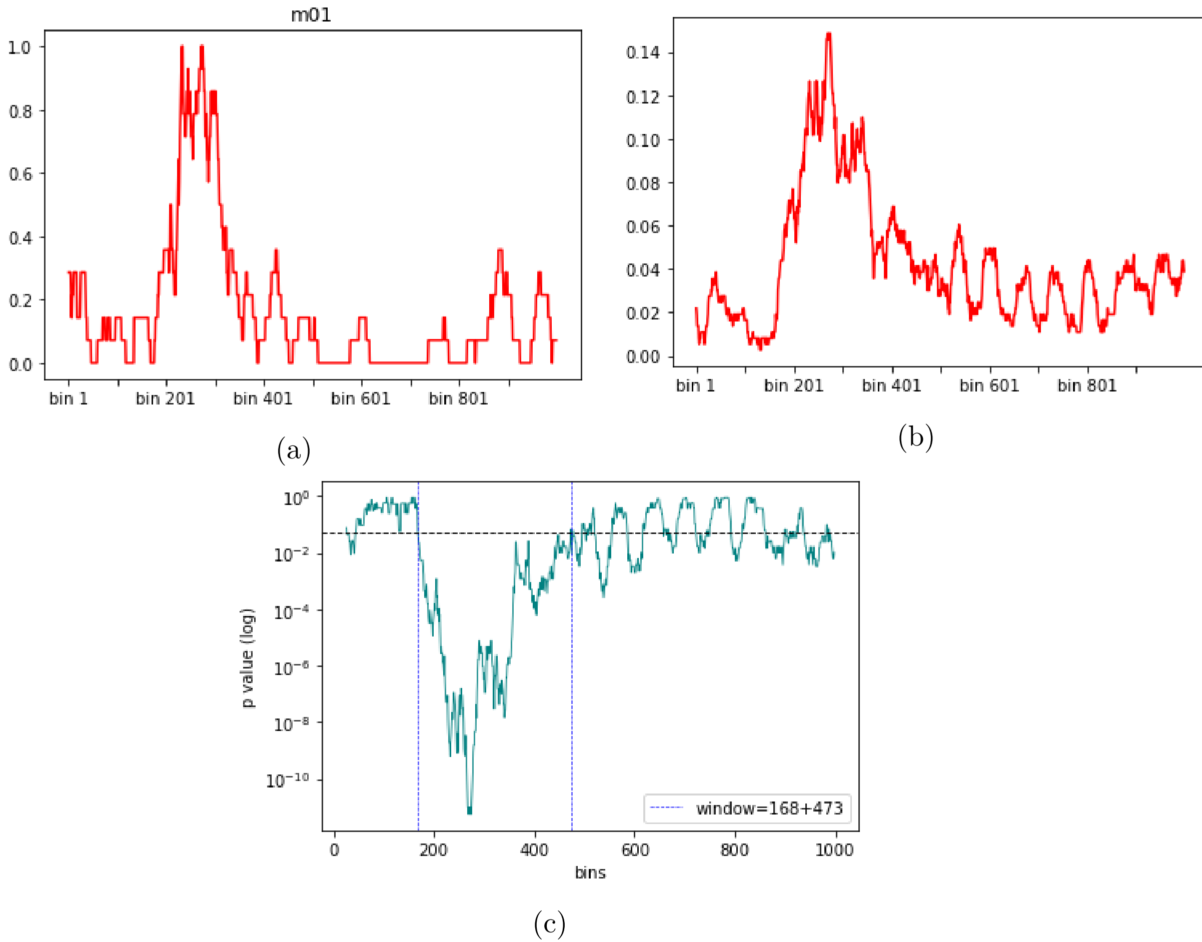


Figure 3.13: (a) Representative image for normalized lick pattern of one animal from NW male group; (b) Cumulative lick plot for NW male group (N=5); (c) The p-value curve of cumulative lick plot. Defining a threshold gives the window (in bins of 2ms) in which maximum licks fall.

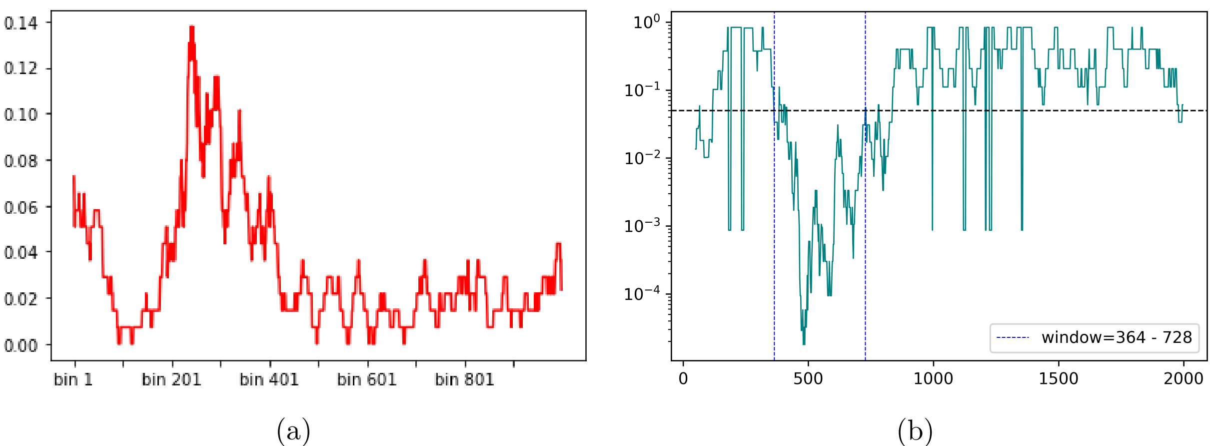
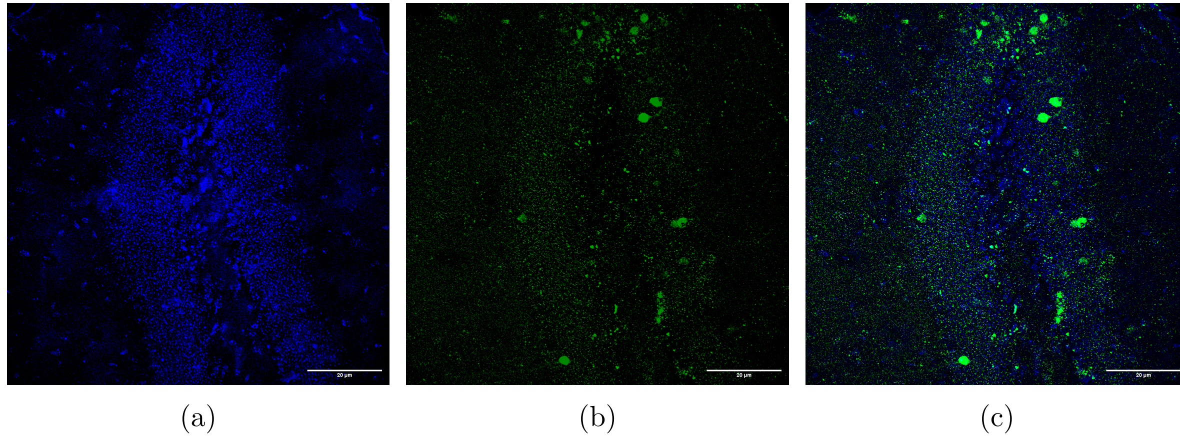


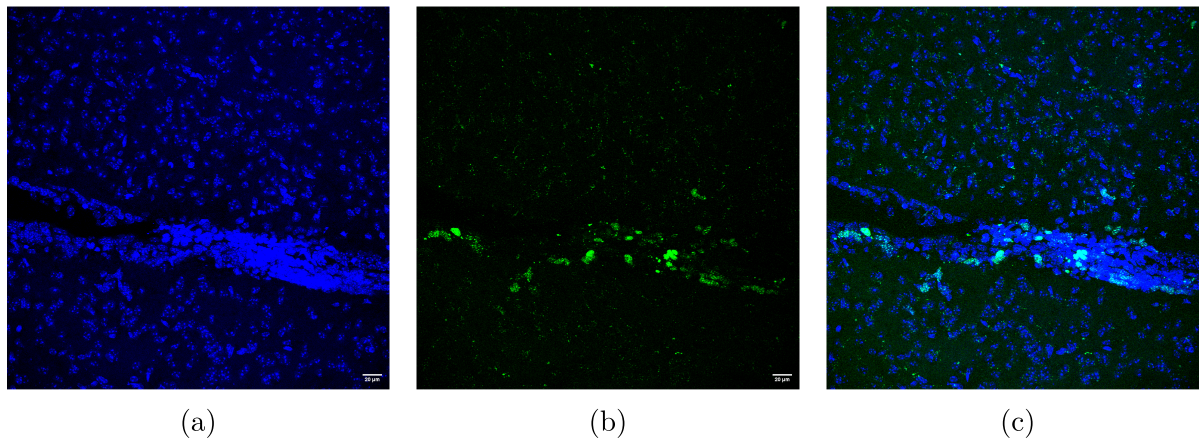
Figure 3.14: (a) Cumulative lick plot of five non-enriched females; (b) The p-value curve of the cumulative lick plot of non-enriched female mice.

3.5 No gender-dependent variability in neurogenesis and cell proliferation



(a) (b) (c)

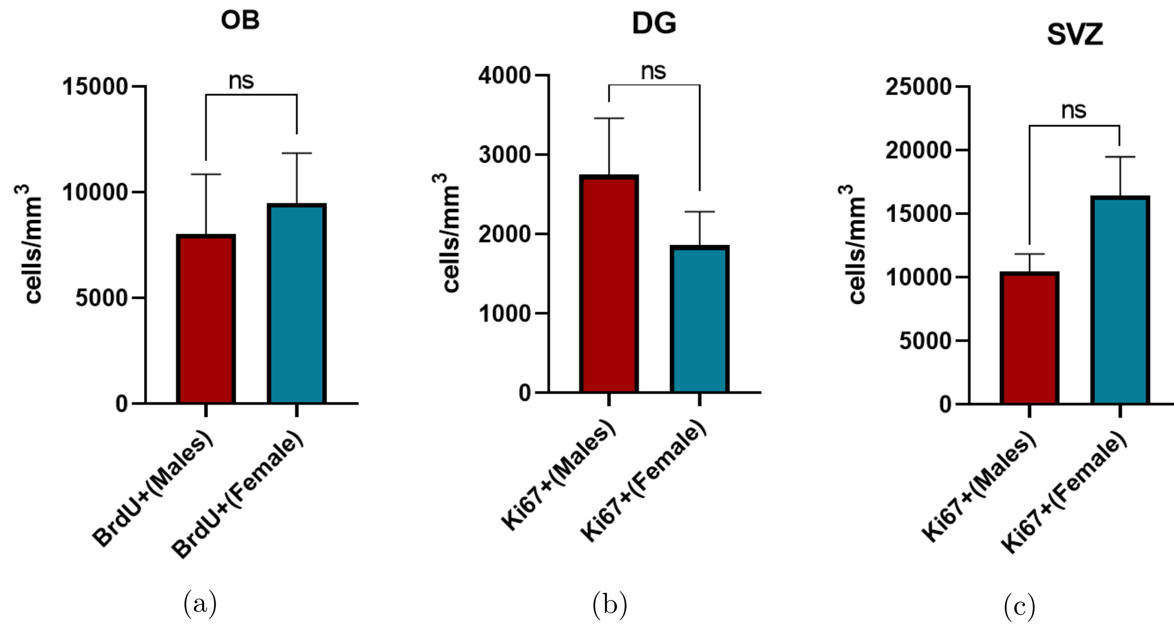
Figure 3.15: (a) Region of DG stained with DAPI; (b) and with Ki67; (c) merged image of DAPI and Ki67 expressing cells; scale = 20μm



(a) (b) (c)

Figure 3.16: (a) Region of sub-ventricular zone stained with DAPI; (b) and with Ki67; (c) merged image of DAPI and Ki67 expressing cells; scale = 20μm

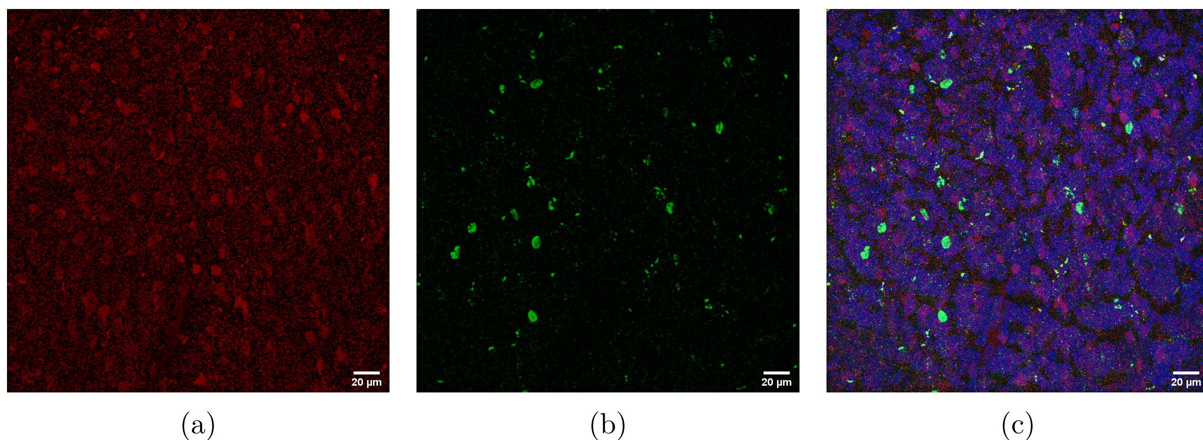
A representation of the stained sections of OB is shown in Fig. 3.18. Images of DG and SVZ, stained with DAPI and Ki67, are shown below (Fig. 3.15 and Fig. 3.16). To quantify the number of BrdU-positive cells in order to estimate the number of adult-born neurons, image analysis of sections of the olfactory bulb (main olfactory bulb) was done. The number of proliferating cells in the dentate gyrus of the hippocampus and the subventricular zone is estimated by cell counting and is shown below (Fig. 3.17). The plots shown in Fig. 3.17 compare the expression of BrdU in OB and Ki67 in DG and SVZ between non-enriched males and females. No significant difference in expression has been observed in all three regions.



(a) (b) (c)

Figure 3.17: (a) Number of BrdU+ cells in OB is not variable between NW males and females (N=2); (b)-(c) Ki67 expression in DG (N=3, 2) and SVZ (N=3) show no significant difference in expression between NW males and females

3.5.1 Activation of immediate early gene Arc in adult-born neurons of the olfactory bulb



(a) (b) (c)

Figure 3.18: (a) Arc^{+} cells at 40x magnification; (b) $BrdU^{+}$ cells; (c) Colocalization of Arc and BrdU cells in MOB. Blue: DAPI, Green: $BrdU^{+}$, Red: Arc^{+}; Scale bar=20μm

After IHC staining, cell counting (done using IMARIS software) reveals that there is colocalization of $BrdU^{+}$ cells and activity-regulated cytoskeleton-associated protein expressing cells (Arc^{+}) in the MOB. The activation of adult-born neurons is known to improve task

performance (Alonso, Mariana et al., 2012; Gheusi, G et al., 2000). The study done by Alonso et al. shows that the activation of adult-born interneurons is important when the animals perform a complex olfactory discrimination task. They also show that the number of adult-born neurons in the OB correlates to the learning ability of the animal. Hence, to quantify the adult neurogenesis in enriched and non-enriched animals, their brains have to be analyzed to check how much colocalization happens in these groups. This quantitative analysis may provide evidence to our hypothesis that enrichment enhances adult neurogenesis, and stress reduces neurogenesis and can be correlated with olfactory learning abilities.

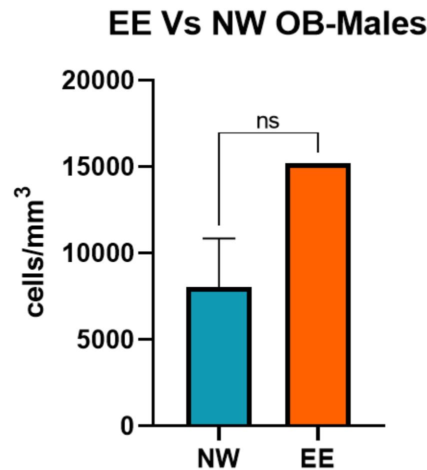


Figure 3.19: Adult neurogenesis quantification in non-enriched (NW) and enriched male mice (N=2, 1)

Adult neurogenesis in enriched male is comparatively higher than in males grown in standard cages (Fig. 3.19). But this difference is not significant. This has to be confirmed with more animals.

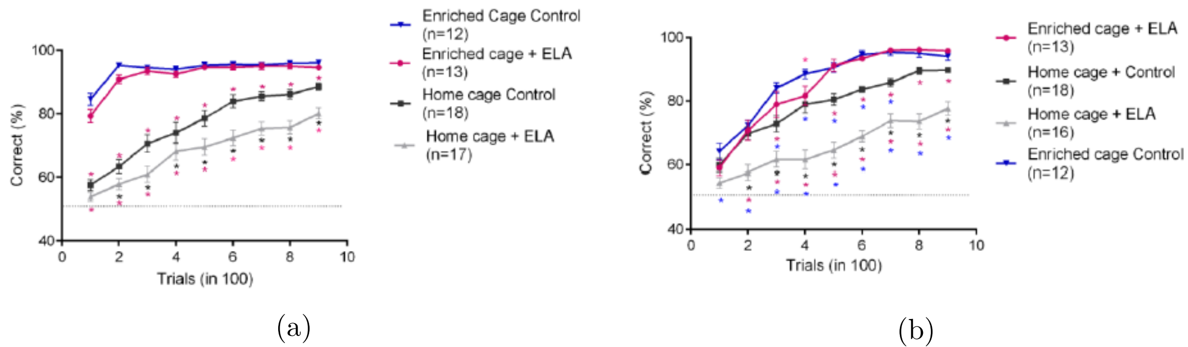
Chapter 4

Discussion

Environmental enrichment is shown to improve the brain's overall plasticity by altering the existing neurons physiologically or structurally or by addition of new neurons. In terms of behavior, enriched mice are shown to learn various paradigms significantly faster than control mice grown in standard cages (Doulames, Vanessa et al., 2014; Gheusi, G et al., 2000; Korkhin, Anna et al., 2020; He, Chuan et al., 2017). Previous studies from the lab showed significant improvement in learning olfactory discrimination of a simple odor pair- Acetophenoene (AP) Vs Octanal (ON) (Work done by Eleanor McGowan Meenakshi Pardasani). They also performed a complex task in which a mixture of acetophenone and octanal in 60:40 ratio is treated as a rewarding odor, then 40:60 ratio of AP:ON is treated as non-rewarding. Enhancement of learning abilities due to environmental enrichment has been observed in both the tasks. The 10-component mixture discrimination is much more complex than the two-component complex mixture. The complexity of the task could be too much for enrichment to enhance learning abilities. This could be the reason why no difference in learning was observed in any of the groups. The number of animals in each set is low, which is another limitation due to which we could not draw conclusions.

A future prospective could be to increase the complexity of odor mixtures in a step-by-step manner, so as to discern the extent of effect of enrichment on learning abilities of mice.

There could be other factors such as the stress induced due to surgery. Especially enriched males showed aggressive behavior right after their recovery after the headpost surgery was performed on them. To avoid any serious injuries, due to fighting between the animals, they were separated and kept in standard cages with small toys, odor boxes and Lego pieces for enrichment, but this lacked any scope for social contact and exposure to large spaces with multiple options of exploring different enriching materials. Hence, these animals may not be properly enriched.



(a) (b)

Figure 4.1: (a) Learning curve of enriched males (blue) and home cage control males (black) differ significantly when the animals perform a simple discrimination task; (b) In a two-component complex mixture task, the difference in learning pace is reduced. (Image Source : Eleanor, Masters' Thesis)

Moreover, when the enriched animals were restrained in the head-restrained set-up, they were more agitated than control animals. When they are too agitated, there is a chance that the headpost detaches from the skull, sometimes even causing damage to the skull. Many of the enriched animals were rendered futile to be re-implanted with another headpost, since their skull would be damaged. This pushed us to use these animals in the freely moving set-up which does not require any headpost implantation surgery or additional stress due to the restraining. Hence, three out of the four enriched males and three out of five enriched females were trained in the freely moving set-up. In the freely moving paradigm, the animal is placed in a chamber which has a port guraded by an infrared (IR) beam. When the animal enters the port, the beam breaks and water reward is given in the habituation task which is similar to the pre-training phase in the head-restrained paradigm. Following the pre-training phase, when the animal enters the port and the IR beam breaks, rewarded and non-rewarded odors are delivered in pseudo-random fashion. Since the licktube and the base of the chamber are made of metal, whenever the animal licks on the licktube, the circuit is closed and every lick is registered.

The observation that enriched males, when confronted with stress, such as stress due to surgery or restraining, show radical change in behavior, compared to females. This again points out the gender-dependent variability in stress resilience.

Early life adversity(ELA) has a great impact on the behaviour and learning of the animals. Studies have also shown that environmental enrichment enhances brain plasticity and hence adult nuerogenesis. Another future direction could be to use early life adversity model animals, such as early maternal separation. Further, quantification of neurogenesis in these ELA models would help us correlate adult neurogenesis with olfactory discrimination abilities.

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