

Role of ionotropic glutamate receptors in odour information processing through mouse olfactory subsystems

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Dedication

*'To my mummy and pappa,
who never stopped believing in me'*

Declaration

I declare that this written submission represents my idea in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I declare that I have acknowledged collaborative work and discussions wherever such work has been included. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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Certificate

This is to certify that the work incorporated in the thesis entitled '**Role of ionotropic glutamate receptors in odour information processing through mouse olfactory subsystems**', submitted by Shruti Dattatray Marathe was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

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Dr. Nixon M. Abraham

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Synopsis

Animals use sensory systems to detect and process various stimulus features from their surroundings to produce appropriate behavioural responses required for survival. For rodents, the sense of smell, (i.e., olfaction) is the primary sensory modality. Their daily tasks, such as foraging for food, avoiding predators, interacting with conspecifics, and finding potential mates, all depend heavily on their abilities to detect, discriminate, and memorize odours. Comprehending the process by which sensory systems interpret stimulus-induced neural responses to make decisions and execute suitable actions poses a major challenge in the field of neuroscience. The mouse olfactory system is an excellent model system for understanding the connection between stimuli, the dynamics of neural activity and decision-making behaviours.

The mouse olfactory system is broadly divided into three parts: Olfactory Epithelium (OE), Olfactory Bulb (OB) and Olfactory Cortex (OC). The odorant molecules first enter the nasal epithelium, where they travel through a mucus layer to bind to odorant receptors (ORs) present on the Olfactory Sensory Neurons (OSNs). OE include the Vomeronasal Organ (VNO), Septal Organ (SO), Gruneberg Ganglion (GG) and the Main Olfactory Epithelium (MOE) (Broman, 1921; Graziadei & Metcalf, 1971; Gruneberg, 1973; Harrison, 1987). Odorant molecules bind to the ORs in a combinatorial manner, meaning that one odour molecule can bind to multiple ORs, and one OR can be activated by multiple odorant molecules. Most OSNs express one type of OR, and all the OSNs expressing the same type of OR converge onto a neuropil-like structure called glomeruli, located in the Glomerular Layer (GL), which is the first entry point of OSNs inside the OB.

At the GL, OSNs establish excitatory synapses with the projection neurons of the OB, namely Mitral and Tufted Cells (M/TCs). M/TCs also receive inhibitory input from inhibitory interneurons in the GL, collectively termed Juxta-Glomerular Cells (JGCs). Interglomerular and intraglomerular inhibitory circuits regulate neuronal spike output to downstream networks (Shao et al., 2012). The interplay between JGCs and M/TCs may contribute to the balance between excitation and inhibition in response to various odours and may also modulate the temporal dynamics in the circuit (Shao et al., 2019).

M/TCs also receive inhibition from the interneurons, called Granule Cells (GCs), which constitute the Granule Cell Layer (GCL). GCs are the major inhibitory interneurons in the mouse OB. They form reciprocal dendrodendritic synapses with M/TCs. The ionotropic

receptors (iGluRs), namely NMDA and AMPA, present at the membranes of GCs, receive glutamate from activated M/TCs, causing Ca^{2+} influx. This further leads to the release of GABA into the M/TCs (Chen et al., 2000). When an M/TC activates a GC and it, in turn, releases GABA onto the same M/TC, this is termed recurrent inhibition (Isaacson & Strowbridge, 1998; Jahr & Nicoll, 1980, 1982a). If the activated GC inhibits another M/TC, this is named lateral inhibition (Isaacson & Strowbridge, 1998; Jahr & Nicoll, 1982a; Woolf et al., 1991). GCs also receive feedback projections from cortical regions (Price & Powell, 1970b). The overall processing at the GCL may provide a spatial contrast mechanism that helps segregate overlapping odour information, aiding in discriminating between odours (Abraham et al., 2010; Bhattacharjee et al., 2019; Stopfer et al., 1997). This indicates an important role of iGluRs of interneurons in odour information processing in the OB.

GABA is one of the most abundant inhibitory neurotransmitters released by OB interneurons. It is derived from L-glutamic acid through an enzymatic reaction involving glutamic acid decarboxylase (GAD). The mouse OB contains GAD65-expressing interneurons as a major intraneuronal class (Parrish-Aungst et al., 2007). Due to their abundance in mouse OB, we modified GAD65-expressing interneurons' functions by knocking out the NR1 subunit of the NMDA receptor, generating $\text{NR1}^{\text{GAD65}(+/-)}$ animals and removing the GluA2 subunit of AMPA receptors to obtain $\text{GluA2}^{\text{GAD65}(+/-)}$ animals using Cre-LoxP genetic approach. We confirmed the generation of heterozygous knockouts through genotyping and protein quantification. Approximately a 20 – 30% decrease in NR1 and GluA2 levels was observed in OB samples, confirming the generation of $\text{NR1}^{\text{GAD65}(+/-)}$ and $\text{GluA2}^{\text{GAD65}(+/-)}$ animals.

While the majority of neurons expressing GAD65 are found in the OB, genetic modifications were achieved in the GAD65-expressing neurons throughout the brain. Therefore, a battery of tests was performed to observe any changes in mood disorder-related behaviours. Open field test, elevated plus maze test, forced swim test and tail suspension test confirmed the absence of anxiety or despair in the knockout animals. We trained these animals in different paradigms such as pheromonal place preference assay, and Go/No-go behavioural tasks to understand the role of iGluRs in odour information processing through olfactory subsystems.

Olfaction plays a crucial role in driving major decisions in social interaction among rodents. Social communication and cognition are essential for mate choice processes. Male mice release pheromones in the form of bodily fluids such as urine, and saliva. In nature, these pheromones are encountered on stones, foliage of various sizes, and shapes. Previously in our lab, it has been demonstrated that female mice remember the location of pheromones and this

learning is multimodal, involving olfactory and whisker systems in the formation of pheromone location memory (Pardasani et al., 2021). To investigate the neural correlates behind multimodal learning of pheromone locations, we subjected the NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals to a pheromone place preference assay and assessed their memory of the location of male urine on post-training day 15 (as explained in Chapter 2). The parameters used were time spent and number of active attempts made by the animals towards male urine and water zone. NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} female mice showed no preference toward the male urine zone on memory day 15, as indicated by similar time spent and active attempts made toward the male urine and water zone. This suggests no memory toward the pheromone locations in animals with altered iGluR subunit composition in GAD65-expressing interneurons. To further assess the memory toward pheromone locations during early recall periods, a new set of animals was trained for the pheromone place preference assay, and their memory was quantified on post-training day 7. During the early recall period, the animals did not show any preference towards the male urine zone. This demonstrates a lack of multimodal learning of pheromone locations in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals. Previous study has shown that the OB, dentate gyrus and somatosensory cortex were activated in females displaying multimodal memory for male pheromones. There was differential activation of the Activity-regulated cytoskeleton protein (Arc), an immediate early gene product used as a proxy for neural plasticity (Pardasani et al., 2021). We observed lower expression of Arc in MOB, AOB, somatosensory cortex and dentate gyrus in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals compared to control animals. The differential expression of Arc corroborates with the phenotype observed during memory days (7 and 15) of the pheromone place preference assay. To study the behaviour displayed by NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals during the training phase, we quantified the time spent and the number of active attempts made towards male urine chamber on days 1, 7 and 15. We observed similar sampling behaviour by NR1^{GAD65(+/-)}, GluA2^{GAD65(+/-)} animals compared to controls. However, when the expression of an immediate early gene was quantified across regions, we found higher expression of c-Fos in the dentate gyrus of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals. Higher c-fos expression in the dentate gyrus of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals, during the last day of the training phase suggests the lack of learning-dependent refinement in the neural circuitry compared to control animals. This may imply their inability to learn associations between different modalities.

Further, we sought to deduce the role of olfactory subsystems in multimodal learning of pheromone locations. Mouse OB contains MOB which receives projections mainly from MOE and AOB which gets input from VNO. Traditionally, it has been thought that AOB is

responsible for processing social odour cues such as pheromones and kairomones (Belluscio et al., 1999; C. Jia & Halpern, 1996). However, now it is known that MOB and AOB have overlapping functions (Mucignat-Caretta, 2010; Xu et al., 2005). We aimed to investigate the role of iGluRs in different subsystems of mouse OB in multimodal learning of pheromone locations. To modify the circuitry in mouse OB and AOB separately, we delivered Cre-viral particles in NR1^{2Lox} and GluA2^{2Lox} animals via a stereotaxic approach. Firstly, we subjected NR1^{ΔOB} and GluA2^{ΔOB} animals to a pheromone place preference assay and observed their memory towards male urine location on memory days 7 and 15. We did not observe any preference towards the male urine chamber on memory days, demonstrating a lack of pheromonal location memory. Next, we modulated AOB microcircuitry, which plays an important role in processing social odour cues. NR1^{ΔAOB} and GluA2^{ΔAOB} animals also did not display any preference towards the male urine chamber on memory days 7 and 15, suggesting the vital role of the iGluRs in the AOB during multimodal learning of pheromone locations. Social odour cues such as pheromones, are processed through MOB and AOB (Restrepo et al., 2004), displaying overlapping functions of MOB and AOB in pheromonal information processing.

In nature, odorant molecules exist in various physiochemical entities such as monomolecular odours or a mixture of different odorant molecules. To deduce the role of iGluRs of GAD65-expressing interneurons, we trained NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals in odour discrimination tasks. For a monomolecular odour discrimination task at supra-threshold concentrations, NR1^{GAD65(+/-)}, and GluA2^{GAD65(+/-)} animals performed similarly to the control group, displaying similar learning accuracies, and discrimination times. Additionally, in more complex odour discrimination tasks at supra-threshold concentrations, NR1^{GAD65(+/-)}, and GluA2^{GAD65(+/-)} animals demonstrated similar odour discrimination abilities as control animals. However, when animals were trained using sub-threshold odour concentrations, NR1^{GAD65(+/-)}, and GluA2^{GAD65(+/-)} animals showed altered threshold concentration for odour detection and discrimination. This hints at the role of iGluRs in finer odour discrimination paradigms. Previous studies have highlighted the relation between odour concentration and the number of active glomeruli upon odour exposure during associative learning. At sub-threshold concentrations, a lesser number of glomeruli are activated compared to supra-threshold concentrations (Abraham et al., 2014). Thus, the local inhibitory network might be helpful in learning odour discrimination at sub-threshold concentrations. However, in NR1^{GAD65(+/-)}, and GluA2^{GAD65(+/-)} animals, the inhibitory circuit function is disturbed leading to impairment in odour detection and discrimination at sub-threshold concentration.

In nature, odorant molecules are carried with an air plume, associated with varying airflow rates. Previous research has shown activity in the olfactory system upon airflow stimulation (Connelly et al., 2015; Grosmaître et al., 2007; R. Wu et al., 2017). Our lab has demonstrated the involvement of the OB in airflow discrimination (Mahajan et al., in communication). We aimed to investigate the role of iGluRs in the airflow discrimination abilities of the animals. We trained NR1^{GAD65(+/-)}, and GluA2^{GAD65(+/-)} animals on different airflow discrimination tasks. We observed unimpaired airflow discrimination in NR1^{GAD65(+/-)} animals similar to controls. However, GluA2^{GAD65(+/-)} animals displayed impaired airflow discrimination abilities. The plausible explanation for the compromised airflow discrimination learning in GluA2^{GAD65(+/-)} animals can be the altered optimal inhibition required for this compared to odour discrimination. It has been shown that the removal of the GluA2 subunit from AMPA receptors makes them calcium permeable (Seeburg et al., 2001). The increased calcium influx, leads to more GABA release, enhancing the inhibition of M/TCs (Abraham et al., 2010).

The optogenetic experiments done by Mahajan et al., demonstrate opposite results for odour discrimination and airflow discrimination learning pace. Optogenetic activation of GAD65-expressing interneurons during odour discrimination enhances the learning abilities of animals while photoactivation of GAD65-expressing neurons during airflow discrimination impairs the airflow discrimination abilities (Mahajan et al., under review). Thus, the optogenetic and genetic modulations of GAD65-expressing interneurons, demonstrate differential optimal inhibition needed to refine odour and airflow information.

CHAPTER 1

Introduction

1.1. The sense of smell

The ability of organisms to efficiently detect and discriminate the odorants in the environment is essential for their reproduction and survival. Most vertebrates rely extensively on their sense of smell, i.e., olfaction, to navigate and localize food sources, evade potential predators, choice of mates, and inter- or intra-social communication. Olfaction also plays a major role in mate choice, intra-social communication, and mother-offspring recognition (Stoddart 1980, Malakoff 1999). It is the structural diversity that exists in the olfactory system that enables the efficient execution of a plethora of functions. This structural diversity exists in the form of different subsystems with various sensory receptors, a specialized and layered pre-cortical region (olfactory bulb) having a variety of neurons that aid in information processing, that is fed into several cortical areas (reviewed in Le Bon et al., 2016; Leon & Johnson, 2006). The mammalian olfactory system possesses immense power in odour discrimination, with estimates suggesting that humans can discriminate a trillion odours (Bushdid et al., 2014; Firestein, 2001). Given the structural and functional diversity of the olfactory system, it is fundamentally important to elucidate how they both contribute to olfactory perception and decision-making. Since mice's primary sensory modality is smell, and since the genetic and anatomical makeup of rodent olfactory systems are well understood (R. Doty, 2012; R. L. Doty, 2019), they serve as an excellent model system to study olfactory information processing and decision-making.

1.2. Mouse Olfactory System

The olfactory world consists of a vast repertoire of natural odours containing a mixture of various components that differ in their physio-chemical properties. The complex structure of the olfactory system enables the mammalian brain to make sense of multidimensional odorant stimuli (Su et al., 2009). The mouse olfactory system consists of the Olfactory Epithelium (OE), Olfactory Bulb (OB), and Olfactory Cortex (OC). Odorant molecules enter the nasal cavity through sniffing (an active sensing behaviour) and travel to the OE that has Olfactory Sensory Neurons (OSNs). OE hosts three types of cells: OSNs, supporting cells, and basal cells (Morrison & Costanzo, 1990). At the ciliary ends of OSNs, Olfactory Receptors (ORs) are expressed. The binding of odour molecules with the receptors initiates a series of

biochemical reactions that convert the chemical energy into electrical energy. This information is then carried to the OB. Within OB, projection neurons known as Mitral and Tufted Cells (M/TCs) collect the odour information and transmit the signal to higher olfactory cortical areas. In the OB, the activity of M/TCs is heavily modulated by excitatory and inhibitory interneurons, that are present across various layers of OB. In addition, OB receives feedback projections from the OC (Davis & Macrides, 1981; Pinching & Powell, 1972; D. A. Wilson et al., 2006) and inputs from different neuro-modulatory brain centres such as noradrenergic, cholinergic, and serotonergic areas (Devore & Linster, 2012; Doucette et al., 2007; Linster & Cleland, 2002; McLean et al., 1989; Rothermel et al., 2014). The following sections comprehensively cover the anatomical and physiological components of the mouse olfactory system.

1.2.1. Olfactory Epithelium

The mouse nasal cavity possesses four subsystems known for sensing odorants with different physiochemical properties, as well as mechanical and temperature variations. These systems include the Vomeronasal Organ (VNO), Septal Organ (SO), Gruneberg Ganglion (GG) and the Main Olfactory Epithelium (MOE) (Broman, 1921; Graziadei & Metcalf, 1971; Gruneberg, 1973; Harrison, 1987). Once the odorant molecules reach the posterior end of the nasal cavity, they bind to the olfactory receptors (ORs) located at the terminal ends of OSNs in the turbinates of the MOE (L. Buck & Axel, 1991; L. B. Buck, 1996). The sensory neurons in MOE are known to be distributed among four zones along an antero-dorsomedial-caudoventral-lateral axis (Ressler et al., 1993; Vassar et al., 1993). However, whether this categorization is based on the type of OSN is still debatable (Ma, 2009; Mori & Sakano, 2011; Munger et al., 2009; Murthy, 2011). The OSNs send their projection to the Main Olfactory Bulb (MOB), which also displays the four zones as observed in the OE. In addition to the MOE, the VNO is also known to be involved in sensing non-volatile odorants and pheromones (Halpern & Martínez-Marcos, 2003; Luo et al., 2003; Wysocki et al., 1980). The other two subsystems, namely the Septal Organ and Gruneberg Ganglion, can sense odorants, alarm pheromones (Enjin & Suh, 2013) and temperature (Mamasuew et al., 2011). While the OSNs of the epithelium are known to send their projections to the MOB, OSNs from the VNO project to the Accessory Olfactory Bulb (AOB). Specifically, sensory neurons in the apical and basal regions of VNO project, respectively, to the anterior and posterior AOB (C. Jia & Halpern, 1996; Salazar & Sánchez Quinteiro, 2003). The plausible overlapping functions of MOE and

VNO in olfactory perception and pheromonal information processing have been understudied (Boehm et al., 2005; Mandiyan et al., 2005; Yoon et al., 2005).

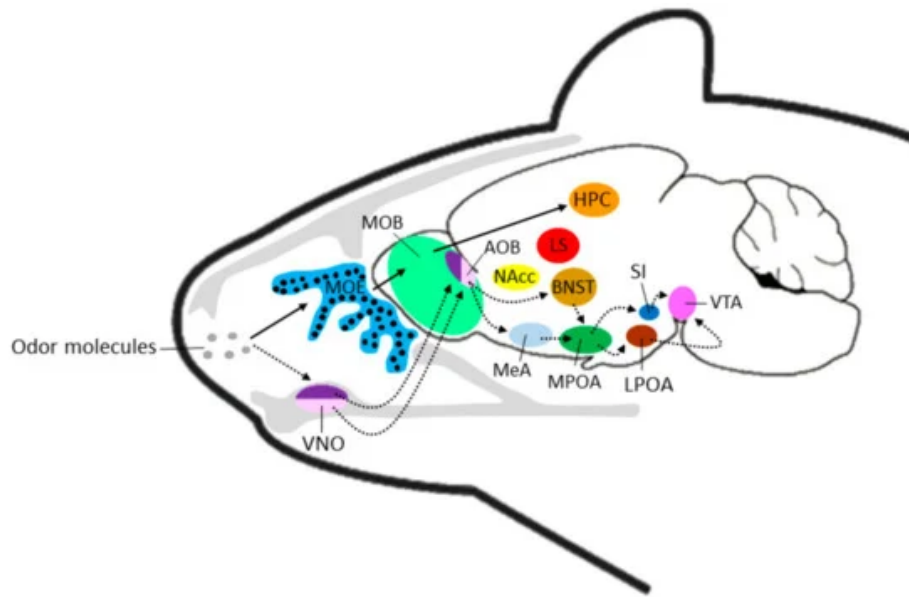


Figure 1.1: Mouse olfactory system

Odour information transmission via the main olfactory system (shown in solid black arrows) and accessory olfactory system (shown in dotted black arrows). VNO: Vomerolateral Organ, MOE: Main Olfactory Epithelium, MOB: Main Olfactory Bulb, AOB: Accessory Olfactory bulb, NAcc: Nucleus Accumbens, MeA: Medial Amygdala, LS: Lateral Septum, BNST: Bed Nucleus of the Stria Terminalis, MPOA: Medial Preoptic Area, SI: Substantia Innominata, LPOA: Lateral Preoptic Area, VTA: Ventral Tegmental Area [taken from (Rymer,2020)]

1.2.2. Olfactory bulb

The mouse olfactory bulb (OB) is a laminar brain structure consisting of the outermost Glomerular Layer (GL), External Plexiform Layer (EPL), Mitral Cell Layer (MCL), Internal Plexiform Layer (IPL), and the innermost Granule Cell Layer (GCL).

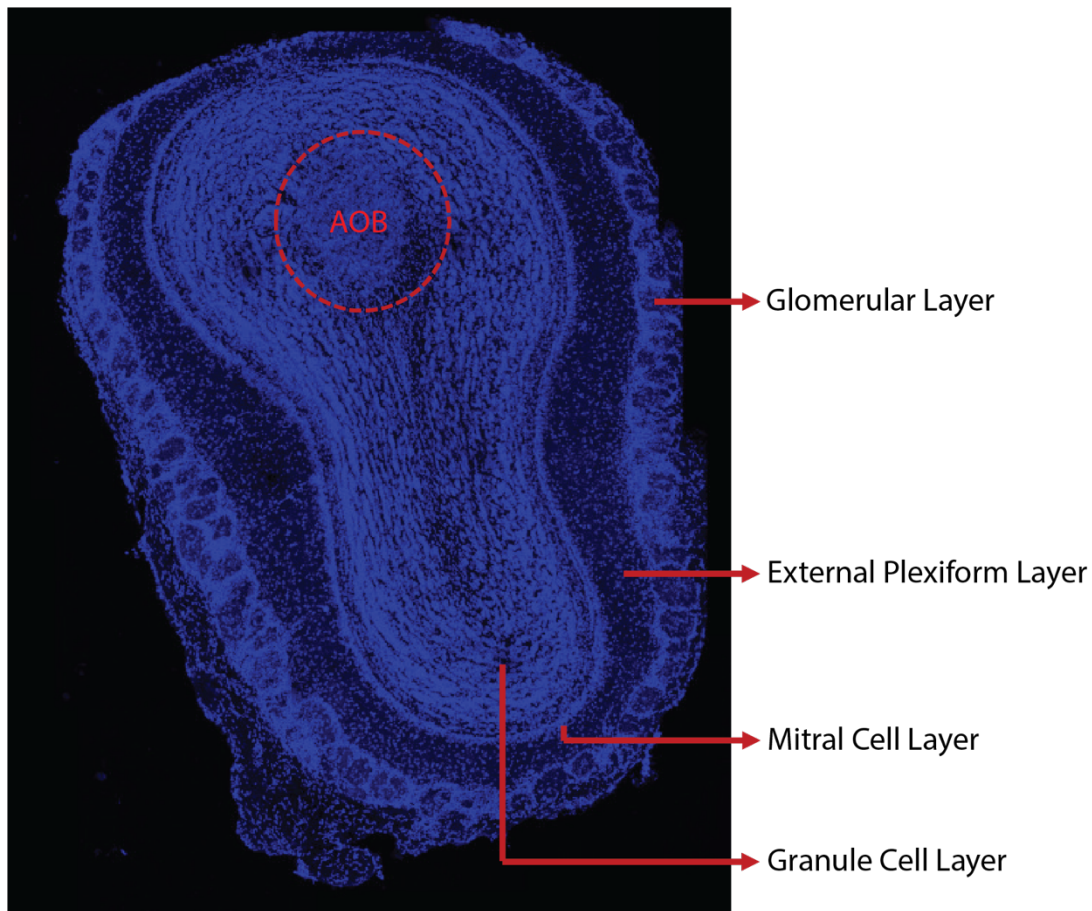


Figure 1.2: Coronal section of Mouse olfactory bulb

The coronal section of the mouse olfactory bulb stained in DAPI displaying various layers of OB. From outermost to innermost, Glomerular Layer: GL, External Plexiform Layer: EPL, Mitral Cell Layer: MCL, Granule Cell Layer: GCL, Accessory Olfactory Bulb: AOB

1.2.2.1. Glomerular Layer

The glomerular Layer (GL) contains neuropil-like structures called glomeruli (singular: glomerulus). OSNs relay information from OE to the GL in a combinatorial fashion, where one OR can recognize multiple odorant molecules and conversely, one odorant molecule can be recognized by multiple ORs (Malnic et al., 1999). The glomerulus is where sensory neurons make synapses with projection neurons, specifically the Mitral/Tufted cells (M/TCs) of the OB (Wachowiak & Shipley, 2006; R. I. Wilson & Mainen, 2006). The GL contains morphologically heterogeneous cell populations, including Periglomerular Cells (PGCs), Superficial Short Axon Cells (sSACs), and External Tufted Cells (ETCs) (Pinching & Powell, 1971a). These cells are collectively referred to as Juxta Glomerular Cells (JGCs). Most of which are interneurons. Among them, PGCs are the most abundant interneurons present in the GL

(Parrish-Aungst et al., 2007). The cell body of PGCs ranges from 5 to 10 μm . While the majority of PGCs mainly project their dendrites to a single glomerulus, some may project to multiple glomeruli. Although most PGCs have axons (Pinching & Powell, 1971a) there are also axon-less PGCs (Kosaka & Kosaka, 2011). The axon of PGCs can extend up to 600 μm , reaching 5 to 6 glomeruli (Pinching & Powell, 1971a). Molecularly, PGCs have subpopulations based on their identity; for instance, GABAergic PGCs are uniglomerular, while Dopamine-expressing PGCs are ‘oligoglomerular’, extending to 5 to 12 glomeruli (Kiyokage et al., 2010). sSACs are fewer in number compared to PGCs and have larger cell bodies, typically ranging from 8 to 12 μm in diameter. Their dendrites occupy the interglomerular space, while their axons extend to 1-2 glomeruli (Pinching & Powell, 1971a, 1971b). ETCs, on the other hand, have a cell body with a diameter of 10 to 15 μm (Pinching & Powell, 1971b). The primary dendrites of ETCs span a single or double glomerulus (Ennis & Hayar, 2008).

ETCs can be classified into two main types: one group lacks secondary dendrites, and their axons are restricted to the OB, while the other group has secondary dendrites extending to the EPL. The cell bodies of the first group are located in the GL, while those of the second group are found in the deeper layer of the GL. The axons of the second group extend either to IPL of the same OB or to Anterior Olfactory Nucleus (AON) pars externa (Macrides & Schneider, 1982; Schoenfeld et al., 1985). These axons can also project to either IPL or the anterior AON.

All of these cell types play a role in modulating the activity of the projection neurons in the OB.

1.2.2.2. External Plexiform Layer and Mitral Cell Layer

Once the M/TCs receive information from the OSNs, their primary dendrites propagate odour signals into the External Plexiform Layer (EPL). The signal reaches the soma of M/TCs and then backpropagates horizontally through the secondary dendrites in the EPL (Chen et al., 1997). The backpropagation signal is inhibited by local inhibition from the granule cells (GCs) (Xiong & Chen, 2002). The horizontal signal propagation inhibits the activity of other M/TCs via dendrodendritic synapses with GCs and other interneurons (Egger & Urban, 2006; Schoppa & Urban, 2003). The EPL is mostly populated by the secondary dendrites of M/TCs and apical dendrites of GCs.

Mitral and tufted cells are the two types of projection neurons in the OB. They share many morphological characteristics such as extending a single primary dendrite to one of the

many thousands of glomeruli in the OB. The sister cells (neurons associated with a single glomerulus) receive homogeneous input from the same OSN and thus have similar odour response properties (Dhawale et al., 2010). However, they have variable odour-tuning properties which are mainly dependent on odour concentration and spatial location (Kikuta et al., 2013; Tan et al., 2010). Mitral cells and tufted cells have similar biophysical properties as well, for example, the dendrodendritic reciprocal synapse between M/TCs and GCs is mediated by ionotropic glutamate receptors, NMDA and AMPA. NMDA receptors are known to be important for driving the dendrodendritic inhibition (Christie et al., 2001).

In mammals, M/TCs can be morphologically distinguished. The secondary dendrites of tufted cells extend to the superficial layer of the EPL while mitral cells have their secondary dendrites in the deep EPL (Mori et al., 1983; Orona et al., 1984). The cell bodies of tufted cells are sparsely distributed across EPL, while the cell bodies of mitral cells are adjacent to each other. This is speculated to be the reason for a stronger interaction between GCs and mitral cells compared to GCs and tufted cells (Kikuta et al., 2013; reviewed in Nagayama et al., 2014). The axonal targets of mitral cells and tufted cells also differ. Tufted cells mainly project to the anterior olfactory cortex, comprising the olfactory peduncle, the OT and the ventro-rostral subdivision of the PCx. The axons of mitral cells extend to the entire OC (Haberly & Price, 1977; Scott et al., 1980; Skeen & Hall, 1977).

Functionally, tufted cells have a lower threshold of spike discharges, as shown by electrical stimulations or odour stimulation of OSNs (Ezeh et al., 1993). Mitral cells have a lower firing frequency than tufted cells (Kikuta et al., 2013; Nagayama et al., 2004). Tufted cells respond to a broader array of odorants than mitral cells. During the earlier respiratory phase, tufted cells are activated while, at the later respiratory stage, mitral cells are activated (Fukunaga et al., 2012; Igarashi et al., 2012).

1.2.2.3. Granule Cell Layer

The Granule cell Layer (GCL) is the innermost layer of the OB. The OB circuitry is majorly modulated by dendrodendritic synapses formed between the lateral dendrites of M/TC and Granule Cells (GCs), which are the most abundant type of inhibitory interneurons in the OB (Shepherd et al., 2007). These axon-less cells possess cell bodies with a diameter of 6 to 8 μm (Price & Powell, 1970c). Most of the GCs have their soma situated in GCL; however, a few GCs may have it in the MCL as well. The apical dendrites of GCs form reciprocal synapses with the secondary dendrites of M/TCs in the EPL. Based on the dendritic extension patterns

in the EPL, GCs are categorized into three groups: Type-I GCs have their dendrites at any depth of the EPL, Type-II GCs' dendrites extend to the deep EPL, and Type-III GCs' dendrites reach superficial GCs. Type-II and type-III GCs mainly regulate the activities of mitral cells and tufted cells, respectively. In 2014, Merkle et al. proposed two additional types of GCs, Type-IV and Type-V. Type-IV GCs do not project their dendrites beyond the IPL and show dendritic branching within the GCL. Type-V GCs are devoid of basal dendrites and are limited to the MCL (Merkle et al., 2014). The majority of GC subpopulations possess calretinin (CR) as a molecular marker and release GABA as the primary neurotransmitter. In addition, a leucine-rich repeat protein, 5T4, also labels a subset of GCs in the superficial GCL (Imamura et al., 2006).

The GCL also contains Deep-short axon cells (dSACs), which are further classified based on soma location and morphology (Schneider & Macrides, 1978; Shepherd et al., 2004). These cells have a cell body diameter of 10 to 20 μm and typically do not have their dendrites extended beyond the MCL. In 1978, Schneider and Macrides classified dSACs into four cell types: Blanes cells, Golgi cells, horizontal cells, and Cajal cells. Blanes cells have a cell body diameter of 16 to 23 μm and are present in the GCL. Golgi cells have cell body diameters of 12 to 22 μm and are situated in GCL. Horizontal and Cajal cells have cell body diameters of 15 to 18 μm and are restricted to MCL (Schneider & Macrides, 1978). In 2008, Eyre et al. re-categorized dSACs into three groups depending on their axonal projections in the OB. GL-dSAC axons extend in EPL up to the GCL. They form synapses with PGCs in the GL. The axons EPL-dSAC predominantly ramify within the EPL and never enter the GL. They form synapses with the dendritic shafts and spines of GCs in the EPL. The axons of GCL-dSA are mostly restricted to GCL and have connections with the proximal dendrites and somata of GCs (Eyre et al., 2008). dSACs express some molecular markers such as Vasoactive Intestinal Peptide-expressing (VIP) (Gracia-Llanes et al., 2003), somatostatin (Burton, 2017), neuropeptide Y (Gall et al., 1986), calbindin (CB) (Brinon et al., 1992) and neuronal nitric oxide synthase (nNOS) (Kosaka & Kosaka, 2007).

1.2.2.4. Synaptic organization of the Olfactory Bulb

Olfactory perception begins with the activation of unmyelinated OSNs located in the OE. The activated OSNs send axons to the OB, where they bundle up to form the olfactory nerve. The olfactory nerve carries electrical signals to the OB, where the axons of OSNs establish glutamatergic excitatory synapses at glomeruli (Shepherd et al., 2004). Along with

relaying odour information to the OC, the OB processes and refines the information through a neural circuit involving two types of interneurons: PGCs and GCs. They form dendro-dendritic connections with M/TCs reciprocally (Shepherd et al., 2004). In the GL, the GABAergic PGCs inhibit M/TCs via GABA_A receptors. Additionally, the synaptic transmission from the axons of OSNs to the OB is regulated by GABAergic and dopaminergic PGCs via presynaptic GABA_B and D2 receptors (Jackowski et al., 1978; Rall et al., 1966). M/TCs extend their secondary dendrites to the EPL, up to 1000 µm, where the GCs form reciprocal dendro-dendritic synapses with M/TCs (Mori et al., 1983; Orona et al., 1984). GCs depolarize in response to the glutamate released by activated M/TCs. Once activated, GCs release GABA onto the same M/TCs (recurrent inhibition) or onto other M/TCs (lateral inhibition). This ensures a tight balance between inhibitory and excitatory signalling. These local interneurons in the OB also integrate information from various centrifugal systems, including the Piriform Cortex (PCx), telencephalic basal ganglion, magnocellular basal forebrain nuclei, and brain stem locus coeruleus (Price & Powell, 1970b; Shepherd et al., 2004). The high degree of plasticity in dendritic reciprocal synapses might result from central feedback coming from higher centres (Hendin et al., 1997; D. A. Wilson & Sullivan, 1994). Thus, during learning, the level of synchronisation in subsets of projection neurons might alter, leading to changes in the strength of temporal binding of signals coming from different ORs (reviewed in Lledo et al., 2004). The activity of M/TCs within a particular glomerulus is influenced by the M/TCs connected with neighbouring glomeruli. Along with recurrent inhibition, M/TCs also experience lateral inhibition (Isaacson & Strowbridge, 1998; Margrie et al., 2001; Rall et al., 1966). Lateral inhibition is highly sensitive to NMDAR blocking (Isaacson, 1999). The effect of lateral inhibition on M/TC depends on the strength and location of the inhibitory input (reviewed in Lledo et al., 2004). Lateral inhibition is necessary to sharpen the receptive fields of individual M/TCs and dampen the activity of neighbouring projection neurons. It enhances both the quality and quantity of information reaching the OC (Aungst et al., 2003). Hence, the functions performed by the GABAergic interneurons in the OB exhibit complexity, underscoring the central role of the OB circuit in various aspects of odour information processing.

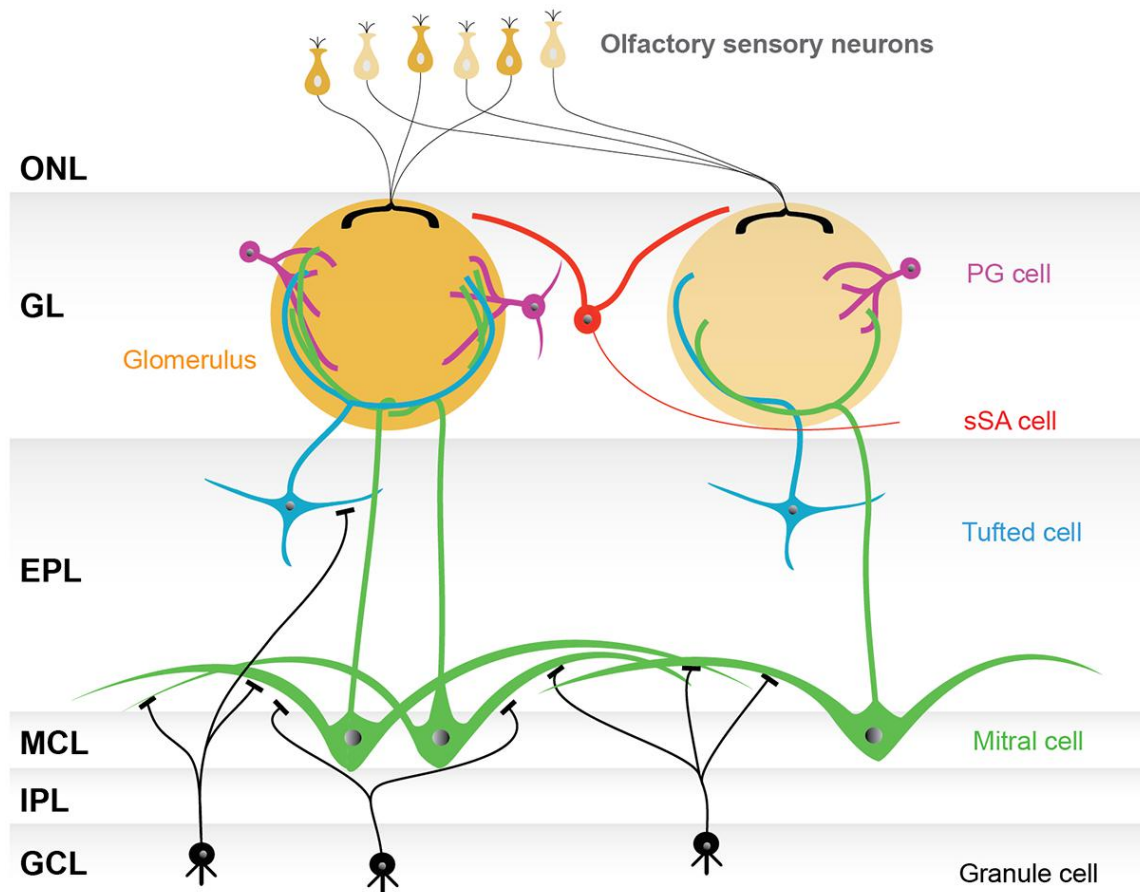


Figure 1.3: Basic model of Olfactory Bulb network

The OSNs form synapses at the GL. The neurons surrounding glomeruli are termed Juxtglomerular cells (JGCs). The main two types of projection neurons are Mitral cells and Tufted cells. The cell bodies of mitral cells are at the Mitral Cell Layer (MCL) and those of tufted cells are scattered in the External Plexiform Layer (EPL). Granule Cell Layer (GCL) contains cell bodies of granule cells (GCs). ONL: Olfactory Nerve Layer, IPL: Internal Plexiform Layer (Taken from Nagayama et al., 2014)

1.2.2.5. Interneurons in the Olfactory Bulb

In the OB, the local inhibitory circuit modulates the odour representations by altering the activity of M/TCs. It has been shown that the interaction between M/TCs and GCs drives the major source of modulation (reviewed in Lledo et al., 2004; Shepherd et al., 2004). However, the contributions from other inhibitory interneurons remain elusive. Nerve cell communication is dominated by two modes: excitation and inhibition. Traditionally it has been understood that excitation has a main role in conveying information, while inhibition serves to eliminate unwanted activity. There has been a continuous emergence of new functional,

molecular, and anatomical characteristics of inhibitory local interneurons (Lledo et al., 2004). Interneurons manage neurotransmission by modulating information processing (Bartolini et al., 2013; Paredes et al., 2016). At the cellular level, they are responsible for controlling synaptic strength and plasticity, as well as for the generation and pacing of large-scale, synchronous oscillatory activity (Bonzano et al., 2016; Bragin et al., 1995; G. Liu et al., 2019). Interneurons constitute one of the most anatomically diverse cell populations of the central nervous system (CNS) (McBain & Fisahn, 2001). The vast heterogeneity in the morphology and connectivity of interneurons makes them challenging to study (Lledo et al., 2004). One way to characterize interneurons is based on their neurochemical content, such as calcium-binding proteins such as calretinin, calbindin or parvalbumin, as well as neuromodulators such as somatostatin, and neuropeptide-Y (reviewed Lledo et al., 2004). However, it is been shown that neurochemically identical cells can exhibit surprisingly different functional properties (Freund, 2003). Characterization based on function also presents another challenge due to pleiotropic actions displayed by inhibitory interneurons (Fricker & Miles, 2001). Hence, the OB appears to be the best system for studying the functions of inhibitory interneurons, because the biochemical, morphological, and physiological characteristics of interneurons are thoroughly investigated in the olfactory system (Lledo et al., 2004).

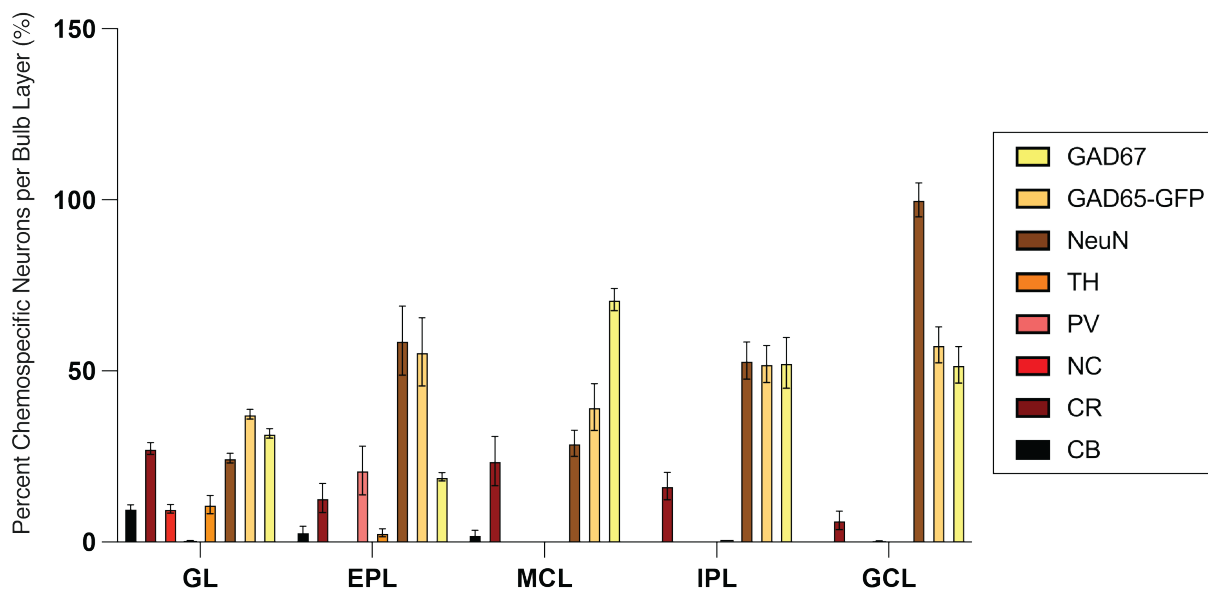


Figure 1.4: Molecular diversity of interneurons in mouse olfactory bulb

Each layer of the olfactory bulb hosts various interneuron populations. GAD: Glutamic Acid Decarboxylase, TH: Tyrosine Hydroxylase, PV: Parvalbumin, NC: Neurocalcin, CR: Calretinin, CB: Calbindin [Adapted from (Parrish-Aungst et al., 2007)]

1.2.3. Accessory olfactory system (AOS)

The Vomeronasal Organ (VNO), the Accessory Olfactory Bulb (AOB), and higher cortical areas such as the vomeronasal amygdala and hypothalamus comprise the accessory olfactory system. Previously, it was thought that the AOS only responds to pheromones, but recently it has been shown that the AOS also responds to odorants (Mucignat-Caretta et al., 2012; Xu et al., 2005). The VNO and AOB will be comprehensively discussed in upcoming sections.

1.2.3.1. Structure of vomeronasal organ

A Danish anatomist, Ludvig Jacobson, first described the VNO in 1813, hence VNO is also termed Jacobson's organ (Trotier et al., 1998). In mammals, it is situated at the base of the septum of the vomer bone. The sensory epithelium of the VNO is not directly exposed to airflow; it has a pumping mechanism to flush mucus to the VNO, which helps the sensory epithelium access stimuli (Meredith et al., 1980a; Meredith, 1994; Moon et al., 2014). The VNO sensory epithelium consists of bipolar glutamatergic neurons that project their axons to the AOB (Dudley & Moss, 1995). Additionally, the sensory epithelium contains sustentacular cells and stem cells, which differentiate into new neurons over the lifetime of the animal (Barber & Raisman, 1978a). Upon degeneration of receptor neurons, stem cells migrate to their final position, differentiate, and send their axons to the AOB (Barber, 1981; Barber & Raisman, 1978b; De La Rosa-Prieto et al., 2009).

According to their location on the epithelium, the expression of G protein and their bulbar target, VNO receptor cells are classified into two groups. Receptors with cell bodies present at the superficial part of the epithelium express the G-alpha subunit G₁₂-alpha. They project their axons to anterior AOB. Receptors situated deep in the epithelium express the G₀ - alpha subunit and project to the posterior AOB (C. Jia et al., 1997; Wekesa & Anholt, 1999).

1.2.3.2. Accessory olfactory bulb

The AOB serves as the first integration centre for VNO inputs and is located dorsocaudally to the MOB. Axons of the VNO synapses with M/TCs in glomeruli of AOB, with a convergence ratio of 1000:1. Compared to the MOB, the glomeruli and EPL of the AOB are comparatively smaller, while MCL is larger in width (Mohrhardt et al., 2018; Mucignat-Caretta, 2010). Axons from AOB M/TCs are connected to interneurons, which, in turn, contact adjacent M/TCs, suggesting lateral inhibition of inputs (Larriva-Sahd, 2008). The anterior and

posterior parts of the AOB receive dendrites from different populations of M/TCs (C. Jia & Halpern, 1997; Sugai et al., 1997). Unlike the MOE, VNO receptor neurons expressing a specific receptor protein project to multiple glomeruli situated within spatially restricted domains (Belluscio et al., 1999).

In contrast to the MOB, the AOB has projections running through the bed nucleus of the stria terminalis, accessory olfactory tract, and medial and posteromedial cortical nuclei of the amygdala (Scalia & Winans, 1975; Shipley & Adamek, 1984; Winans & Scalia, 1970). Both subregions of the AOB project to medial and posteromedial cortical nuclei of the amygdala (Salazar & Brennan, 2001). The amygdala provides feedback projections to AOB Through stria terminalis (Raisman, 1972). The bed nucleus of the stria terminalis projects GABAergic axons to the MCL of the AOB, while amygdala nuclei send glutamatergic axons to the GCs of the AOB. In the amygdala, inputs from both MOB and AOB converge onto single neurons, integrating the information processed by the main olfactory system (MOS) and accessory olfactory system (AOS) (Fan & Luo, 2009). This cross-talk between the two systems is vital, as the amygdala is involved in modulating many social behaviours (reviewed Murray, 2007; Siever, 2008).

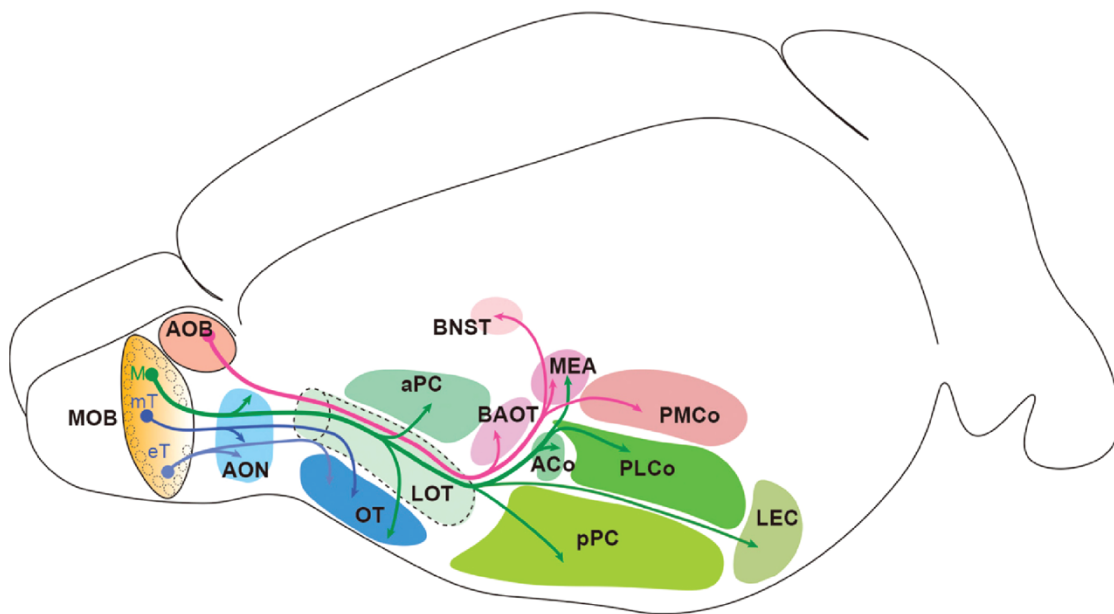


Figure 1.5: Innervations of olfactory bulb projections neurons

Mitral cells of the MOB project to the Anterior Olfactory Nucleus (AON), the anterior and posterior Piriform Cortex (aPC and pPC), Olfactory Tubercle (OT), Lateral Entorhinal Cortex (LOC), Medial Amygdaloid Nucleus (MEA), and anterior and posterolateral cortical Amygdaloid Nucleus (ACo and PLCo). Mitral cells of AOB send their projections to the Bed Nucleus of the Accessory

Olfactory Tract (BAOT), Bed Nucleus of Stria Terminalis (BNST), MEA, and Posteromedial Cortical Amygdaloid Nucleus (PMCo) [Taken from (Imamura et al., 2020)]

Together with the MOS, the VNO is involved in mate recognition and sexual behaviour (Keller et al., 2009). In male mice, same-sex interactions elicit responses in the caudal AOB, while interactions with females stimulate the rostral AOB (Kumar et al., 1999). In the AOB, responses sustain for a longer time compared to the MOB, with a gradual increase in firing rate over many seconds after the exposure to stimuli. AOB inhibitory responses start slowly and maintain a peak for about 1 minute (Luo & Katz, 2004). The stimuli activate VNO, so the inhibition seen at the level of AOB comes from the inhibitory local circuit. In the amygdala, pheromone exposure-induced activity is seen at a delayed time point, 120-140 minutes after pheromone exposure. This suggests the involvement of the AOS in the long-term modulation of the hypothalamus-pituitary axis (HPA) (Mucignat-Caretta et al., 2006).

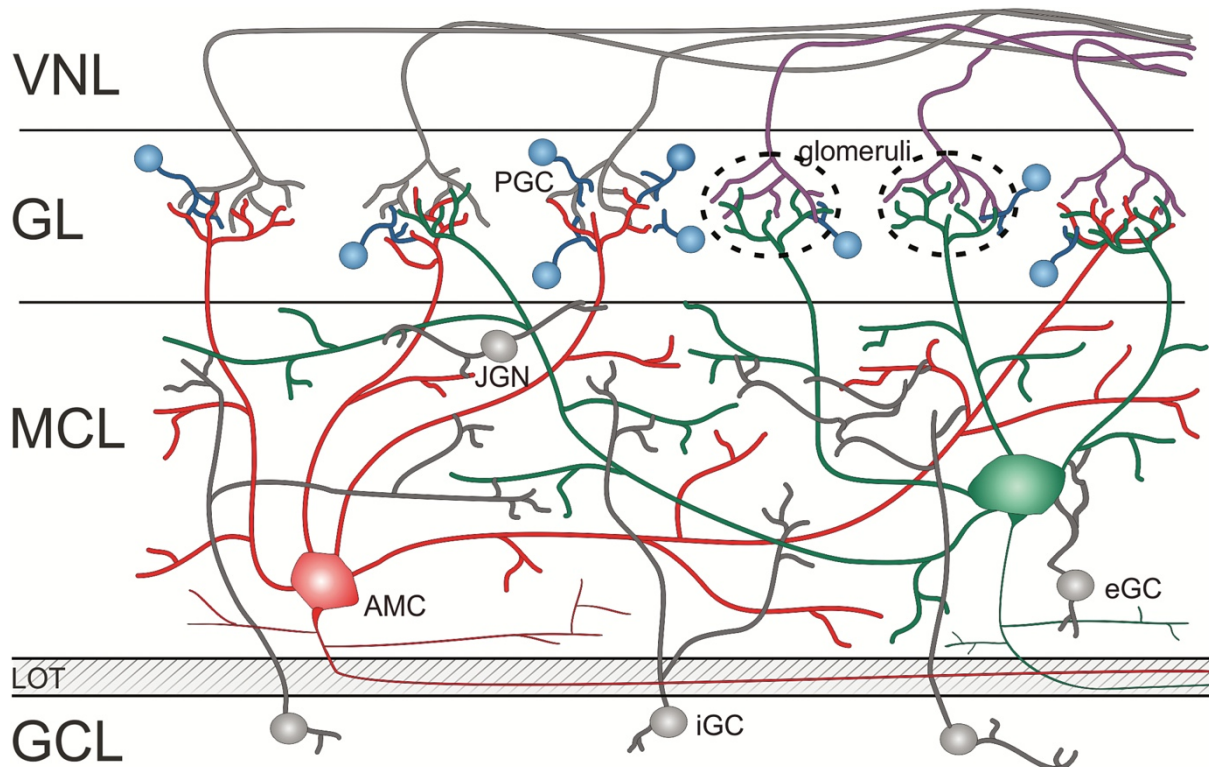


Figure 1.6: Basic model of Accessory Olfactory Bulb network [Taken from (Mohrhardt et al., 2018)]

Vomerolnasal Sensory Neurons (VSNs) innervate loosely defined glomeruli in the GL. The MCL contains juxtglomerular neurons (JGNs) and AOB mitral cells (AMCs) that each innervate

several glomeruli. In the MCL, some external granule cells (eGCs) are present. The Lateral Olfactory Tract (LOT) receives efferent axons from both main bulb projection neurons and AMCs. The internal cellular layer mainly harbours axon-less GABAergic internal granule cells (iGCs) and is thus designated as the granule cell layer (GCL).

1.2.3.2. Interneurons of the Accessory Olfactory Bulb

Morphologically, AOB interneurons can be classified into six categories (Larriva-Sahd, 2008). The most abundant among them are the spiny interneurons called External Granule Cells (EGCs). EGCs are located in the External Cellular Layer (ECL), adjacent to mitral cells (MCs). MCs are the sole projection neurons present in the AOB. EGCs do not have axons and form reciprocal dendrodendritic synapses with MCs. They possess extensive radial dendritic arbours with long-necked spines, facilitating extensive lateral connections within the circuit.

Juxtglomerular cells (JGCs) and Internal Granule cells (IGCs) also are axon-less. EGCs, IGCs and JGCs exhibit characteristic physiological features that remain consistent across differently labelled interneurons such as Calb2, GAD65, and Cort. This molecularly diverse population of interneurons demonstrates strong expression in the AOB (Taniguchi et al., 2011). The expression levels of GAD65 and Calb2 are high, as confirmed by fluorophore expression and antibody staining observations, with a predominant presence in the Internal Cellular Layer (ICL) (Maksimova et al., 2019). GAD65-expressing interneurons are primarily populated in the anterior AOB, whereas Calb2 interneurons are prevalent in the posterior AOB. Cort-positive interneurons are mostly found in the External Cellular Layer (ECL), albeit in lower percentages compared to GAD65- and Calb2- positive interneurons.

ICGs play a crucial role in AOB plasticity, exhibiting moderate rates of spontaneous EPSPs (sEPSPs) mediated by NMDAR and AMPARs. Due to their high intrinsic variability, IGCs are believed to comprise diverse subpopulations. Similarly, EGCs also display NMDAR and AMPAR-dependent sEPSPs, albeit with significantly higher frequency and amplitude, indicating denser connectivity with MCs on a per-cell basis. JGCs in the AOB consist of Periglomerular Cells (PGCs) and Short-axon Cells (SAs). Some JGCs exhibit low capacitance and moderate depolarization with sustained action potential generation, while others have higher capacitance and lower input resistance, suggesting a larger and/or more conductive dendritic arbour. Based on their morphology, AOB EGCs are believed to support broad innervation and gain-scaling, while IGCs are hypothesized to perform broad lateral inhibition among spatially distributed MCs. GAD65-expressing interneurons, which are prevalent in the

AOB, can fire sustained action potential upon moderate depolarization. They also exhibit low capacitance and high input resistance (Maksimova et al., 2019). Thus, the mouse AOB plays a pivotal role in processing social chemosensory information, and the intricate inhibitory circuitry within the AOB helps in refining this information.

1.2.4. Olfactory Cortex

The olfactory cortex (OC) is defined as the area in cortical regions that receive direct inputs from the OB, including the AON, Olfactory Tubercle (OT), cortical nucleus of the amygdala, PCx, and lateral regions of the entorhinal cortex. A few regions of the neocortex, such as the lateral entorhinal cortex and Orbitofrontal Cortex (OFC), receive input from the OB via the primary OC. Most OB projections project to PCx (Haberly, 1985; Price, 1973). PCx has three distinct layers. Layer I receives inputs from the OB. At layer Ia, they form synapses with dendrites from deeper layers of PCx. Layer II contains pyramidal neurons and semilunar cells. The innermost layer of PCx is Layer III, which has pyramidal neurons in superficial areas. Although M/TCs project to a limited number of pyramidal cells in PCx, the excitatory dendrodendritic synapses among pyramidal neurons ensure wide odour-evoked responses (Franks et al., 2011; Poo & Isaacson, 2011; Suzuki & Bekkers, 2011). While semilunar cells have a robust input from OB, PCx also receives input from AON, OT, and many regions of the amygdala (Haberly, 2001; Luskin & Price, 1983). PCx also receives inputs from the brainstem, thalamus, hypothalamus, and basal forebrain. AON is responsible for maintaining inter-bulbar connections. It receives input from M/TCs and provides feedback to contralateral OB GCs (Imai & Sakano, 2008; Yan et al., 2008). AON stores memory engrams (Aqrabawi & Kim, 2020). OT is also part of the OC because it receives direct input from the OB. OT forms associations with reward centres, indicating its active role in the odour-reward associations (Ikemoto, 2007; Wesson & Wilson, 2011). OT represents reward value independent of odour identity (Millman & Murthy, 2020). OFC receives odour information via PCx and is activated during the trial initiation, odour sampling, and reward consumption (Ramus & Eichenbaum, 2000). OB at the GL and GCL also receives dense feedback projections, mainly from PCx and AON (Boyd et al., 2012a; Luskin & Price, 1983; Markopoulos et al., 2012; Matsutani, 2010). This feedback is primarily inhibitory, resulting in the suppression of M/TCs activity upon odor stimulation. This inhibition occurs via the activation of inhibitory interneurons in the OB. Furthermore, these excited interneurons inhibit the activity of M/TCs (Boyd et al., 2012a; Markopoulos et al., 2012). Short-Axon cells (SAs) in the GL receive excitatory feedback

projections from PCx, which also lead to the disinhibition of M/TCs through the inhibition of GCs and PGCs (Boyd et al., 2012a). OB also receives noradrenergic input from the Locus Coeruleus (LC) (Devore & Linster, 2012; Doucette et al., 2007), cholinergic input from the horizontal limb of the diagonal band of Broca (HDB) (Shepherd et al., 2004), and serotonergic input from dorsal and medial raphe nuclei (McLean et al., 1989). The noradrenergic inputs are received at IPL and GCL, while moderate projections are seen at the EPL and MCL (McLean et al., 1989). The noradrenergic system helps hyperpolarize GCs, thus inhibiting M/TCs activity induced due to weak OSN input (Devore & Linster, 2012; Hayar et al., 2001; Linster et al., 2011; Nai et al., 2009). Blocking of noradrenergic receptors in the OB results in impairment of odour discrimination of complex odours (Doucette et al., 2007). The cholinergic system helps in odour habituation, short-term olfactory memory, and complex odour discrimination (Linster & Cleland, 2002; Ma & Luo, 2012).

The spatially segregated chemotropic map of odours seen in GL is no longer represented at the level of PCx. M/TCs from a glomerulus project across PCx without any apparent spatial preference (Apicella et al., 2010; Isaacson, 2010), and a single pyramidal neuron receive inputs from M/TCs from different glomeruli. Approximately 3 to 15% of the pyramidal neurons in PCx are activated in response to an odorant (Rennaker et al., 2007; Stettler & Axel, 2009). Neurons activated in response to dissimilar odours often fail to respond to similar odours, indicating discontinuous receptive fields in PCx, i.e., the specificity of odorant molecules is represented by distributed and unique ensembles of neurons that do not display the coarse chemotopy or spatially segregated patterns of neural activity observed in the OB (Stettler & Axel, 2009). Early-firing M/TCs are reliably represented in layers II and III of the PCx while late-firing M/TCs activity is reduced by global inhibition in the PCx (Bolding & Franks, 2018; Rennaker et al., 2007; Stern et al., 2018). PCx also sends odour information to the OFC, which is activated during the trial initiation, odour sampling, and reward consumption (Ramus & Eichenbaum, 2000). Apart from the PCx, the AON is responsible for maintaining inter-bulbar connections. It receives input from M/TCs and provides feedback to GCs of the contralateral OB (Imai & Sakano, 2008; Yan et al., 2008). AON is mainly known for storing olfactory memory engrams (Aqgrabawi & Kim, 2020). OT, another part of the cortex, receives direct input from the OB primarily by the tufted cells (Scott et al., 1980). OT is known to form associations with reward centres, indicating its active role in odour-reward associations (Ikemoto, 2007; Wesson & Wilson, 2011). OT represents the value of reward irrespective of odour identity (Millman & Murthy, 2020).

The neurons of the olfactory bulb, especially those in the GL and GCL, also receive dense feedback projections from the cortical areas, primarily from the PCx and AON (Boyd et al., 2012a; Luskin & Price, 1983; Markopoulos et al., 2012; Matsutani, 2010). This feedback is predominantly inhibitory, resulting in the suppression of M/TCs activity upon odor stimulation (Boyd et al., 2012a; Markopoulos et al., 2012). Short-Axon cells (SAs) in GL receive excitatory feedback projections from the PCx, which also lead to the disinhibition of M/TCs through the inhibition of GCs and PGCs (Boyd et al., 2012a). The OB also receives noradrenergic input from the Locus Coeruleus (LC) (Devore & Linster, 2012; Doucette et al., 2007), cholinergic input from the horizontal limb of the diagonal band of Broca (HDB) (Shepherd et al., 2004), and serotonergic input from the dorsal and medial raphe nuclei (McLean et al., 1989). Noradrenergic inputs are received at IPL and GCL, while moderate projections are seen at the EPL and MCL (McLean et al., 1989). The noradrenergic system helps hyperpolarize GCs, thus inhibiting M/TCs activity induced by weak OSN input (Devore & Linster, 2012; Hayar et al., 2001; Linster et al., 2011; Nai et al., 2009). Blocking noradrenergic receptors in the OB results in the impairment of odour discrimination of complex odours (Doucette et al., 2007). The cholinergic system aids in odour habituation, short-term olfactory memory, and complex odour discrimination (Linster & Cleland, 2002; Ma & Luo, 2012). Firing latencies of early-firing M/TCs encode odour intensity and late-firing M/TCs are needed for fine discrimination (Hopfield, 1995). Olfactory cortical neurons from the AON and PCx respond to many odour components present in the odour mixture (Lei et al., 2006; D. A. Wilson, 2000). In rodents and humans, PCx also plays a role in adapting to stable odour inputs (Kadohisa & Wilson, 2006; Sobel et al., 2000; D. A. Wilson, 1998). The OC has a role in odour discrimination and odour generalization (Barnes et al., 2008; Chapuis & Wilson, 2012). In summary, cortical feedback on M/TCs improves odour-evoked inhibition, maximizing the signal-to-noise ratio to produce effective perception (Boyd et al., 2012b).

1.3. Role of Ionotropic glutamate receptors in olfaction

The reciprocal synapses between projection neurons and GCs are located in the EPL. They consist of asymmetric synaptic junctions between projection neurons and GCs adjacent to symmetric GCs and projection neuron synapses (Price & Powell, 1970a, 1970c). This dendrodendritic connection results in recurrent inhibition of the projection neurons and provides the mechanism for lateral inhibition, which ultimately helps in odour detection, discrimination and olfactory learning (P. Brennan & Keverne, 2015; Yokoi et al., 1995). The

excited projection neuron releases glutamate onto the GC, which is received by NMDA and AMPA receptors. This results in the release of GABA from the GC. The GABA, in turn, inhibits the same or different projection neurons, which is termed recurrent or lateral inhibition respectively. Few electrophysiological studies have shown that the activation of GCs is dependent on NMDARs (Isaacson & Strowbridge, 1998; Schoppa & Westbrook, 1999). Similarly, the self-excitation of M/TCs is dependent on NMDARs (Aroniadou-Anderjaska et al., 1999; Isaacson, 1999).

1.3.1. Ionotropic Glutamate Receptors

1.3.1.1. NMDA receptors

NMDA receptors (NMDARs) are a subtype of ionotropic glutamate receptors that play an important role in mediating many changes during synaptic plasticity. NMDARs are heteromeric proteins composed of seven different subunits. NR1(GluN1) codes for the NR1 subunit, and undergoes extensive splicing to produce eight variants: NR1A to GluN4A and NR1B to GluN4B. GluN2 codes for the NR2 subunit and has four variants (GluN2A - GluN2D), while GluA3 codes for the GluA3 subunit, consisting of two variants, GluN3A and GluN3B (Iacobucci & Popescu, 2017). All these variants are encoded in three separate genes: GRIN1, GRIN2 and GRIN3 respectively. To form a functional NMDAR, the presence of the NR1 subunit is necessary. The major subtypes of NMDARs pair with NR1 and various variants of NR2 subunits to form NR1/NR2A, NR1/NR2B, NR1/NR2C and NR1/NR2D, displaying distinct developmental expression patterns (Van Dongen, 2009). Sometimes, a combination of NR1 with NR2A and NR2B or NR2C, as well as NR1 with NR2B and NR2D, is also observed within the same receptor (Bhattacharya et al., 2018; Stephenson, 2001). These various combinations regulate different aspects of neural circuit development and the expression of plasticity (Sheng et al., 1994). NR3 subunits pair with NR1 subunits and not with NR2 subunits and are expressed very early during the development for a brief amount of time (Pachernegg et al., 2012; Pérez-Otaño et al., 2016). NMDARs are highly permeable to Ca^{2+} and Na^{+} (Garaschuk et al., 1996; Jahr & Stevens, 1993; McBain & Mayer, 1994; Müller & Connor, 1991; Schneggenburger et al., 1993; Yu & Salter, 1998). This property makes NMDARs a good candidate in the processing of plasticity.

NMDARs exhibit a Mg^{2+} -dependent blockade of receptors, termed Mg^{2+} block. In conditions where the extracellular Mg^{2+} was reduced, the response of NMDAR is facilitated. In the presence of Mg^{2+} , the probability of NMDA channel opening is reduced (Nowak et al.,

1984). Studies have shown that blocking postsynaptic inhibition using GABA inhibitors facilitates LTP (Wigström & Gustafsson, 1985) and hyperpolarization of postsynaptic neurons- reversibly blocks LTP (Malinow & Miller, 1986). This suggests that LTP is regulated by changes in membrane voltage during depolarization. Based on this, NMDARs are potential candidates to induce LTP and compute the Hebbian conditions required for associative memory. In 1986, it was demonstrated that NMDAR activation is directly linked to memory formation during a spatial learning task (Morris et al., 1986). Other experiments in spatial memory tasks have also discussed the role of NMDAR in memory formation and LTP expression (Tsien et al., 1996). This highlights the role of NMDARs in long-term potentiation and memory.

1.3.1.2. AMPA receptors

Many glutamatergic synapses contain a large population of AMPARs which are further categorized based on their subunit composition. AMPARs are assembled from four subunits, forming a dimer of two identical homo or heterodimers to create a tetramer. The different subunits are GluA1, GluA2, GluA3, and GluA4. Each subunit contains a ligand-binding site that, upon binding with the ligand, undergoes a conformational change, allowing the channel to open (Mayer, 2005). Each subunit consists of an amino-terminal domain (ATD), a ligand-binding domain (LBD), a transmembrane domain (TMD) and a cytoplasmic carboxy-terminal domain (CTD). The CTD helps subunits interact with specific cytoplasmic and transmembrane proteins. This is facilitated through the presence of phosphorylation, palmitoylation, ubiquitination, and nitrosylation sites along the length of the CTD.

GluA1

GluA1, also known as GluR1 or GluRA, is expressed in various structures of the CNS such as the cortex, amygdala, cerebellum, thalamus, brainstem, CA1-CA3 and DG, making it a subject of study in the context of synaptic plasticity, learning and memory. GluA1 mRNA has been found not only in excitatory principal neurons but also in inhibitory interneurons, oligodendrocytes, and glial cells (J. R. P. Geiger et al., 1995; Matthias et al., 2003; Patneau et al., 1994). GluA1 mainly forms heterodimers with GluA2, giving rise to GluA1/2 AMPAR. GluA1 is synthesized via the de novo pathway as well as through activity-dependent local translation (Cajigas et al., 2012; Ju et al., 2004). In the hippocampal CA1 region, GluA1 mainly forms heterodimers with GluA2, comprising nearly 80% of the total AMPAR population (W.

Lu et al., 2009), while GluA1 homomers make up about 8 % of the total AMPAR population (Wenthold et al., 1996a).

Each subunit has a similar core structure; however, differences in their CTDs determine their trafficking dynamics in response to activity. The long CTD of GluA1 allows phosphorylation by various activity-driven proteins such as Ca^{2+} /Calmodulin kinase (CaMKII), protein kinase A (PKA), protein kinase C (PKC) and protein kinase G (PKG), which regulates trafficking and stabilization of GluA1-containing AMPARs (Hayashi et al., 2000). The stabilization of receptors is facilitated by chaperons and scaffold proteins such as PSD-95 (Anggono & Huganir, 2012; Shi et al., 2001a), synapse-associated proteins 97 (SAP97) (Leonard et al., 1998), A-kinase anchoring protein 79 (AKAP79) (Colledge et al., 2000), cornichon Family AMPA Receptor Auxiliary Proteins 2/3 (CNIH2/3) (Herring et al., 2013) and transmembrane AMPA receptor regulatory proteins (TARPs) (Diering & Huganir, 2018).

Calcium-permeable AMPA receptors

Calcium-permeable AMPA receptors (CP-AMPA receptors) are distributed throughout the CNS, including regions such as the hippocampus (Katsumaru et al., 1988; Koh et al., 1995), motor and auditory cortices (Angulo et al., 1999; Otis et al., 1995), amygdala (Mahanty & Sah, 1998), dorsal horn (Albuquerque et al., 1999), cochlear (Gardner et al., 1999, 2001), basal ganglia (Götz et al., 1997) and retina (Mørkve et al., 2002). CP-AMPA receptors exhibit transient expression both developmentally (Gray et al., 2007; Ho et al., 2007; J. Lu et al., 2007) and in response to activity (Morise et al., 2019; Park et al., 2016; Plant et al., 2006; Shi et al., 2001b). CP-AMPA receptors are primarily composed of GluA1 and GluA2 subunits, which render them permeable to Ca^{2+} . AMPAR subunit genes, *GluR1*-*GluR4* have a conserved codon coding for the glutamine site at the second transmembrane of the channel. The GluA2 mRNA undergoes a post-translational modification by the ADAR2 enzyme, converting glutamine to arginine (Q/R modification) at the 607 position (Horsch et al., 2011). This editing prevents calcium permeability when GluA2 complexes with GluA1 and GluA3 (Wright & Vissel, 2012). Approximately 90 to 95% of GluA2 mRNAs undergo Q/R editing (Isaac et al., 2007; Pachernegg et al., 2015). CP-AMPA receptors can also consist solely of GluA1 subunits, forming homomeric GluA1 receptors (Rozov et al., 2012). CP-AMPA receptors exhibit fast kinetics and high conductance (J. R. P. Geiger et al., 1995; Grosskreutz et al., 2003; Verdoorn et al., 1991).

GluA1 homomers are highly expressed during early development (Pickard et al., 2000). In adulthood, they are predominantly found in extra-synaptic locations or intracellular

stores under basal conditions (Jaafari et al., 2012; W. Lu et al., 2009; Y. Yang et al., 2010). These homomers have been observed to significantly contribute to AMPAR transmission during synaptic activity (Rozov et al., 2012) and play a role in the induction of LTP (Cepeda-Prado et al., 2019; Guire et al., 2008; J. Lu et al., 2007; Park et al., 2016; Plant et al., 2006; Y. Yang et al., 2010). However, there are opposing views from some groups suggesting that GluA1 homomers do not contribute to LTP induction (Adesnik & Nicoll, 2007; Gray et al., 2007).

Recent studies have highlighted the crucial role of CP-AMPA receptors in synaptic plasticity and memory formation (Man, 2011). Genetic knockout of GluA2 in the mouse hippocampus has been shown to enhance LTP and improve hippocampus-dependent learning tasks (Asrar et al., 2009; Wiltgen et al., 2010). However, it is important to note that CP-AMPA receptors do not govern all forms of LTP, as evidenced by numerous studies (Adesnik & Nicoll, 2007; Gray et al., 2007; Guire et al., 2008; Purkey & Dell'Acqua, 2020). Additionally, their regulation is highly influenced by factors such as animal species and developmental age (J. Lu et al., 2007).

At Post-synaptic density (PSD), CP-AMPA receptors accumulate and are rapidly internalized, being replaced by GluA2-containing AMPARs in an activity-dependent manner (Mattison et al., 2014; Plant et al., 2006; Y. Yang et al., 2010). Disrupting the interaction between GluA2 and trafficking proteins leads to subsequent LTP mediated by CP-AMPA receptors. Knockouts of GluA1 and GluA2 subunits disrupt the initiation and completion of this synaptic exchange, respectively, highlighting the distinct roles of these subunits in this process (McCormack et al., 2006).

The rapid delivery of GluA1-containing AMPARs to the PSD is believed to initially occur through lateral diffusion, and later, exocytosis of AMPARs replenishes these surface pools. However, studies have also shown that an increase in GluA1 (but not GluA2) followed by in vitro LTP induction, induction is subsequently accompanied by an increase in GluA2-containing AMPARs. This indicates a dynamic process involving both GluA1 and GluA2 subunits during synaptic plasticity.

GluA2, GluA3, GluA4

The GluA1 subunit is known as a plasticity-promoting AMPAR subunit, whereas GluA2 and GluA3 subunits are involved in consolidating changes (Wenthold et al., 1996a). Knockout of the GluA2 gene (*Gria2*) leads to increased LTP, intact LTD, depotentiation, and basal synaptic transmission. However, a double knockout of *Gria2* and *Gria3* results in

impaired LTD and depotentiation, while LTP remains enhanced (Meng et al., 2003). Despite the enhanced LTP in GluA2 knockout animals, studies have shown impaired spatial working memory and spatial reference learning tasks (Shimshek et al., 2005). In 2000, Liu and Cull-Candy demonstrated that activity in CP-AMPA promotes the replacement of these AMPARs by GluA2-containing AMPARs (S.-Q. J. Liu & Cull-Candy, 2000).

GluA2/3 containing AMPARs are the second most abundant heteromeric AMPARs in cortical principal cells (J. R. P. Geiger et al., 1995; Tsuzuki et al., 2001; Wenthold et al., 1996b). The expression of GluA3-containing AMPAR increases throughout the hippocampus during development (Ritter et al., 2002). GluA3-containing AMPARs have been shown to play a crucial role in synapse maturity and stabilization. Under basal conditions, they contribute to stabilizing changes in AMPAR surface expression by replacing synaptic GluA1/2-containing AMPARs (Malinow & Malenka, 2002; Passafaro et al., 2001; Shi et al., 2001a).

GluA4-containing AMPARs exhibit high expression during early development (Henley & Wilkinson, 2016; Ritter et al., 2002). They are predominantly found in the cerebellum (Pelkey et al., 2015; Schwenk et al., 2014). GluA4-containing AMPARs are later replaced with the GluA2-containing AMPARs (Zhu et al., 2000). Knockout of GluA4 impairs the acquisition of spatial reference memory while maintaining intact LTP (Sagata et al., 2010).

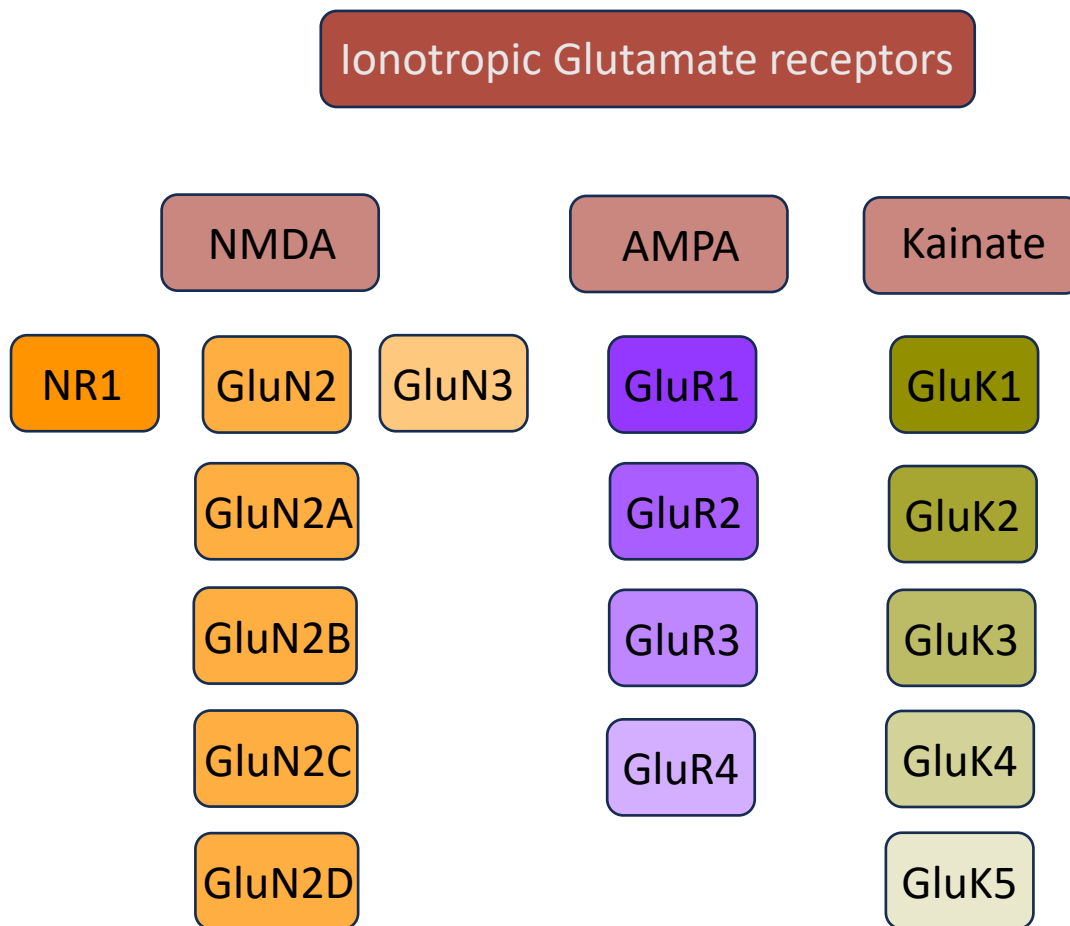


Figure 1.7: Subunit composition of Ionotropic glutamate receptors

NMDA receptors are made from two NR1 subunits and two GluN2 or GluN3 subunits. AMPA receptors are composed of four types of subunits encoded by GluR1, GluR2, GluR3 and GluR4, forming tetramers. Kainate receptors are multimeric proteins with the assembly of GluK1-GluK3 and GluK4 or GluK5.

1.4. Social Odors

Rodents encounter various odours emanating from food sources, predators, and potential mates (A. Liu et al., 2020). The detection of such odours leads to many events, such as active sniffing of the odorants and avoiding or approaching the source of the odorant. Ultimately, the processing of these odorants leads to appropriate innate or learned behaviour. Encountering such odorants can also be stored in long-term memory and used when needed in the future. One example of such a social odour is a pheromone. The term “pheromone” was coined more than 50 years ago and has been described as airborne communication molecules that help individual animals evoke specific and robust physiological/behavioural effects in the

recipient individual of the same species (Karlson & Lüscher, 1959). The events taking place during the detection and processing of these pheromones in rodent brains have been studied in-depth. In this section, the studies conducted on pheromonal signalling through olfactory subsystems will be discussed.

1.4.1 Scent marking

In nature, a variety of innate as well as adaptive behaviours are displayed by animals, which help them communicate with conspecifics and defend themselves from predators (Brown & Macdonald, 1985; Kats & Dill, 1998). Odorants are used to recognize and distinguish individuals of the same or different species. The social relationships with conspecifics are shaped by chemo-signals. Scent-marking behaviour is exhibited by rodents to establish the territories (Anderson & Hill, 1965; Crowcroft & Rowe, 1963) even when the owner is not present at the location (Hurst, 1987b, 1989).

As early mammals were nocturnal, major roles were played by senses like olfaction, audition and tactile senses in intra-species communication and predator avoidance (Brown, 1979; Eisenberg, 1981). Although olfactory signals show a delay between signal emission and reception compared to the audition, a “rapid on-rapid off” signal (Eisenberg & Kleiman, 1972), olfactory cues stay in the environment longer. This provides a benefit in providing information to a wider range of recipients about the location of the animals that are no longer present there (Blanchard et al., 2003; Brown & Macdonald, 1985; Hurst & Beynon, 2004).

Scent marks deposited by rodents provide information about the social status (Hurst, 1993; R. B. Jones & Nowell, 1973, 1974), health status, and reproductive health of the individual (Barnard & Fitzsimons, 1988; Brown & Macdonald, 1985; Kavaliers et al., 2005; Mossman & Drickamer, 1996). Information via scent marks may be maintained for over 24 hours even in the owner’s absence (Hurst & Beynon, 2004) which is absent in auditory or tactile communication (Hurst et al., 1998). Scent marking and counter-marking of scents by other males are a means to display dominance in male house mice (Hurst et al., 2005; Mugford & Nowell, 1970). Dominant males tend to make more urinary scent marks than subordinate males, indicating the social status in the hierarchy (Desjardins et al., 1973; Hurst, 1990). Non-volatile components in the urine help males recognize urine from other animals in the territory (Humphries et al., 1999; Hurst et al., 2005).

It has been observed previously that male mice release more scent marks in the presence of an adult female compared to a juvenile female (Arakawa et al., 2007) and the occurrence of scent marks is not dependent on the female’s estrous cycle (Brown, 1988; Coquelin, 1992).

Female mice show an interruption in the establishment of pregnancy in the presence of unfamiliar male odours; this is called the Bruce effect (Bruce, 1959; Kumar & Dominic, 1993; Parkes & Bruce, 1961). The AOB is a region where female mice form a memory for the stud male shortly after mating (P. Brennan et al., 1990; P. A. Brennan & Peele, 2003). This process is hormonal-dependent. Prolactin and LH stimulate neuronal production in the OB and hippocampus, respectively (Mak et al., 2007). Prolactin is released if the female is exposed to an unfamiliar male odour within five days of mating, leading to a failure in embryo implantation (Peele et al., 2003). Major Urinary proteins (MUPs) aid in releasing MHC-associated odours produced through a molecular mixture of volatile metabolites (Singer et al., 1993). Both males as well as females express MUPs (Beynon et al., 2002; Payne et al., 2001). However, males invest more in MUP production and scent marking (Beynon et al., 2002; Stopka et al., 2007). Different MUP patterns help mice distinguish self-urinary marks from others (Hurst et al., 2001; Nevison et al., 2003).

Odorants are primarily received in the nose by two receiving regions: the main olfactory epithelium (MOE) and the Vomeronasal organ (VNO). The MOE is located in the dorsal posterior region of the nasal cavity, while the VNO is located in the inferior region of the nasal cavity (Greer & Bartolomei, 1994; Matsunami & Buck, 1997; Rodriguez et al., 2002). The MOE sends signals to the MOB, while the VNO sends its axons to the AOB, which is at the posterior part of the OB. Previously, it has been shown that the MOB is responsible for processing general odours that provide information regarding the surroundings, while the AOB responds to pheromones (Dulac & Torello, 2003; Halpern & Martínez-Marcos, 2003; Wysocki et al., 1982) and kairomones (Staples et al., 2008). However, the role of these two subsystems in the detection of biologically relevant odours seems to be partially overlapping (Restrepo et al., 2004). Removal of the VNO in males results in reduced scent marking and aggression towards other males (Maruniak et al., 1986; Pankevich et al., 2004). VNO removal does not cause changes in mating behaviour towards females however, it results in a loss of preference for estrous females (Pankevich et al., 2004). These data indicate male aggression and scent-

marking behaviour are VNO-dependent, while normal male-female sexual interactions require an intact main olfactory system (reviewed in Arakawa et al., 2008).



Figure 1.8: Diagrammatic representation of scent-marking behaviour in nature

In nature, male mice deposit urine (shown in yellow) at different locations. The female mice encounter the volatiles and non-volatiles from male urine on stones, foliage etc. The olfactory and whisker systems aid in associating the spatial location of pheromones and thus facilitate multimodal learning and memory formation (Image made in BioRender).

1.4.2 Pheromone processing through olfactory subsystems

Rodents have two olfactory systems: the vomeronasal and main olfactory systems (P. A. Brennan & Kendrick, 2006). Originally, they were considered to be independent of each other, with the vomeronasal system responsible for the processing of social cues such as pheromones. However, it has been seen that the two systems have overlapping and possibly complementary functions in the processing of pheromonal cues (Dulac & Torello, 2003; Kang et al., 2011).

The VNO is situated at the rostral end of the nasal cavity, where it functions as a vasomotor pump-like structure, primarily responsible for collecting non-volatile odorants from the source (Meredith et al., 1980b; Meredith & O'Connell, 1979). The VNO contains three types of vomeronasal receptors (Holy, 2018), belonging to the GPCR family, which are categorized based on the location in the VNO (Dulac & Torello, 2003). V1Rs and V3Rs are present at the apical end, while V2Rs are present in the basal region of the VNO (Boschat et al., 2002; Dulac & Axel, 1995; Pantages & Dulac, 2000; Zhang et al., 2010). There is another

type of receptor that binds to formyl peptides (Liberles et al., 2009; Rivière et al., 2009). All these types of receptors (V1R, V2R and V3R) are expressed in different Vomeronasal Sensory Neurons (VSNs) that specifically bind to the pheromone molecules. Unlike OSNs, VSNs send their projections to distributed glomeruli in the AOB (C. Jia & Halpern, 1996). V1Rs show a broader activation pattern to many social odours, while V2Rs have a specific activation pattern towards social odours, such as those emitted by a specific sex in particular environmental conditions (Isogai et al., 2011). The VSNs carry information to the AOB, which further conveys the information to the Medial Amygdala (MeA), Postero-medial cortical amygdala (PMcO), bed nucleus of stria terminalis (BNST), and hypothalamus (Dulac & Wagner, 2006). It has been shown that the Ventrolateral hypothalamus (VMHvl) regulates the sexual behaviour of both sexes (Lee et al., 2014; D. Lin et al., 2011; C. F. Yang et al., 2013).

The view that the VNE is solely responsible for collecting the pheromonal cues while the MOE only receives information related to non-pheromonal volatiles is no longer accepted (Holy, 2018). Social cues are also processed through the MOB. Immediate Early Genes (IEGs) have been expressed due to urine exposure in the glomeruli of the MOB (Bepari et al., 2012). A study combining extracellular electrophysiology and solid-phase microextraction gas chromatography showed the presence of specific M/TCs responding to volatiles present in the urine in both males and females (D. Y. Lin et al., 2005). Connectivity from the MeA to the MOB has been shown by Kang et al. through retrograde tracing, and these neurons have been demonstrated to respond to male urine (Kang et al., 2011). This indicates a direct connection between the MOB and MeA in pheromonal odour processing, suggesting that MOB and AOB have partially overlapping functions during odour information processing.

1.5. Adeno-associated viruses (AAV): A tool used in viral gene transfer method

Adeno-associated virus (AAV) is a single-stranded DNA virus belonging to the parvovirus family, with a broad range of hosts. Its ability of AAV to perform as a provirus as well as a lytic virus has made it a suitable tool as a mammalian transduction vector. In 1984, Hermonat and Muzyczka isolated recombinant viruses containing AAV capsid genes in replacement of the neomycin gene. They showed that these recombinant viruses can be used to introduce foreign genes into the host genome (Hermonat & Muzyczka, 1984). This strategy is widely used in neuroscience to both target and manipulate specific neuronal types based on location, gene expression or connectivity and non-neuronal cell types within the nervous

system (Haery et al., 2019). One of the major advantages of using AAVs to target the nervous system is a lack of toxicity (During et al., 2002; Tenenbaum et al., 2004). The infectivity of the virus to the cell type depends on the serotype of the virus e.g., serotype 1 viruses target neurons and glial cells while serotype 2 cells mainly target neurons. Another advantage of using AAVs is to induce long-term gene expression without the T-cell responsivity (Hernandez et al., 1999; Jooss et al., 1998).

AAVs are widely used to efficiently transfer the 38-kDa protein, Cre recombinase enzyme to mammalian cells in vitro as well as to various regions in the rodent brain in vivo (Ahmed et al., 2004). AAV1 and AAV2 can be packaged into the same virion due to the high level of similarity between them (Hauck et al., 2003). We have used pAAV.CMV.HI. eGFP-Cre.WPRE.SV40 (AAV5) viral particles selectively modulate neuronal populations in OB and AOB regions of the mouse olfactory system. The manipulation of microcircuitry in OB and AOB subsystems through Cre-mediated deletion of NR1 or GluA2 made it possible to carry out long behavioural experiments to deduce the role of ionotropic glutamate receptors in odour information processing through olfactory subsystems. AAV viral particles were specifically injected in the OB or AOB region of the olfactory subsystem to delete NR1 and GluA2 subunits, thereby altering the calcium influx into these neurons. Previously in 2005, Shimshek et al, showed that knocking-out GluA2 subunit specifically from the forebrain region led to faster olfactory learning while causing compromised olfactory memory (Shimshek et al., 2005). In 2010, researchers knocked-out NR1 subunit of NMDA receptor and GluA2 subunit of AMPA receptors specifically from the granule cell layer of olfactory bulb, by stereotactically injecting Cre-viral particles (Abraham et al., 2010). This resulted in bidirectional changes of synaptic inhibition in the OB. At behavioural level, NR1 knockout animals took longer time to discriminate the difficult (60:40) odour mixtures while GluA2 knockout animals were faster at discriminating difficult odour mixtures. However, the olfactory memory in both groups was unaltered. In one part of our study, we have used a similar approach, to knockout NR1 and GluA2 subunits. We trained the animals to pheromone place preference assay and tested their memory on day 7 and day 15. We found that the animals displayed no preference towards male urine zone indicating lack of learning of pheromone locations. In the previous study, the focus mainly was on Go/No-go olfactory learning while the current study investigates the involvement of OB circuitry in multimodal learning of pheromone locations.

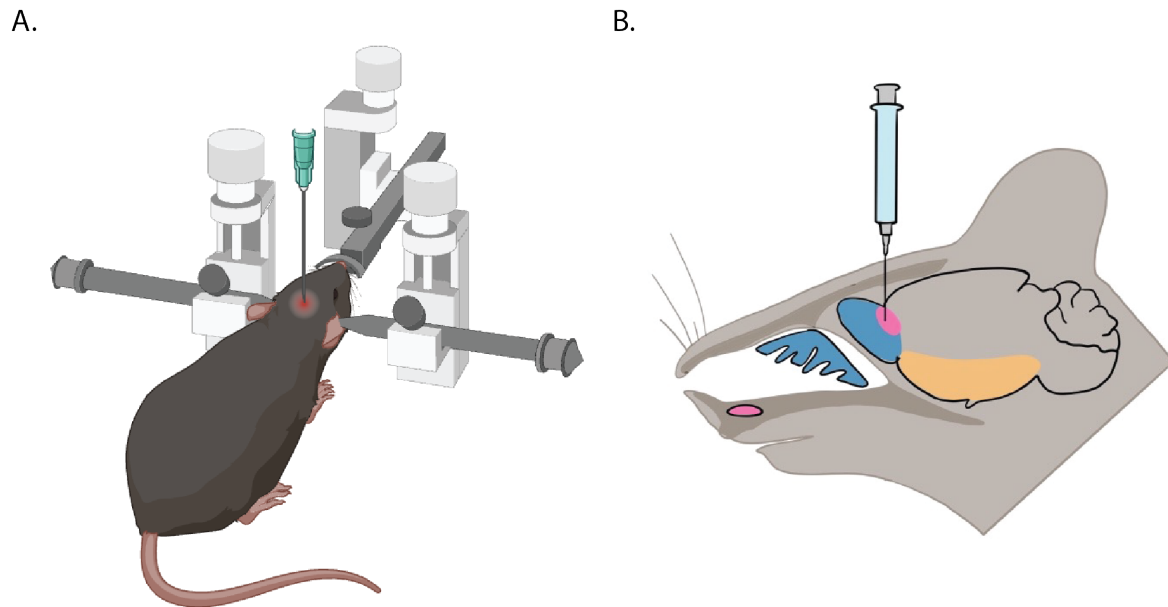


Figure 1.9: Diagrammatic representation of Stereotaxic viral injection strategy

- A. The stereotaxic setup is used to deliver AAV-viral particles. An animal in an anaesthetized condition is placed on the setup using the ear bars and mouthpiece. A glass capillary is used to deliver the viral particles at the specific brain coordinates. (Image is partially made in BioRender)
- B. A diagrammatic representation of the injection site (AOB).

1.6. Quantification of Immediate Early genes as a tool to observe neuronal activity during learning and memory

To access the activity-dependent changes in neurons, the mRNA or protein levels of Immediate Early genes (IEGs) are quantified. Quantification of IEGs during learning or memory is a powerful tool to understand the ensemble of cells getting activated during the activity. The expression of IEG proteins is not hampered in the presence of protein synthesis inhibitors and they do not need to undergo de novo protein synthesis (Matthies, 1989; Robertson, 1992; Sheng & Greenberg, 1990). The protein products of IEGs show faster decay times and their expression is dependent on the duration and the nature of the task (Tischmeyer & Grimm, 1999). Induction of IEG can be initiated by various stimulating factors such as avoidance learning in rodents, sensory discrimination tasks, and exposure to social stimuli such as pheromones (Freeman et al., 1995; Nikolaev et al., 1992).

The IEGs are distributed in various gene classes such as inducible transcription factors, cell-surface receptors, growth factors and protein kinases. One of the most studied IEGs is, c-Fos which helps in the activation of downstream processes upon neuronal activation. IEGs act as transcription factors which produce neurotransmitters and cytoskeletal proteins (Chiu et al.,

1988; Herrera & Robertson, 1996; Heurteaux et al., 1993). A study involving temporal profiling of cfos-eGFP in the retrosplenial cortex using two-photon microscopy showed different neuronal populations activated upon dual context exposure (Meenakshi et al., 2021). In another report, the researchers combined photo-acoustic imaging with Fos-LacZ expressing rats to gain high spatial resolution based on ex vivo imaging of mPFC following cocaine induction (Matchynski et al., 2021).

The expression of these IEGs can be graded and seems to be highly correlated at the single neuron level (Gonzales et al., 2020). One study showed that after the spatial learning task, the activation levels of c-fos, Arc and zif 268 were similar. However, Arc m-RNA expression was higher in the rats proficient in doing the task than in those who learned an unfamiliar trial (Guzowski et al., 2001). Another study where researchers exposed the female mice to clean bedding vs the soiled bedding from a male mouse, showed elevated expression of Egr-1 compared to Arc, c-fos, Fos-B and c-Jun, in the VNO (Isogai et al., 2011). These studies indicate that the expression of IEGs is highly dependent on the context of the task as well as on the nature of the stimulus.

Activity-regulated cytoskeleton (Arc) IEG is one of the effector-IEGs which is more efficient in tasks requiring long-term experience-dependent plasticity, as Arc remains stable over time. Arc acts as a reliable marker for learning and memory because of its ability to remain at steady-state levels in specific neuronal networks. During a continuous exploration of the same spatial location, Arc has been seen to be expressed consistently. It plays a major role in memory consolidation. Arc knock-out animals have been shown to have intact short-term memory, however they displayed impairment in forming long-term hippocampal-dependent memory and learning. This was also accompanied by a lack of consolidation of potentiation at the CA1 and a reduction in long-term depression (LTD) (Plath et al., 2006). Thus, Arc might be partly responsible for maintaining a balance between LTP and LTD. Arc regulates the dynamics between actin and cytoskeletal proteins which helps maintain LTP and mediate receptor endocytosis needed for LTD (Bramham et al., 2010).

Thus, the use of IEGs to locate the activity of neuronal ensembles is widely accepted. However, selecting a specific IEG depends mainly on the nature of the task and the stimulus.

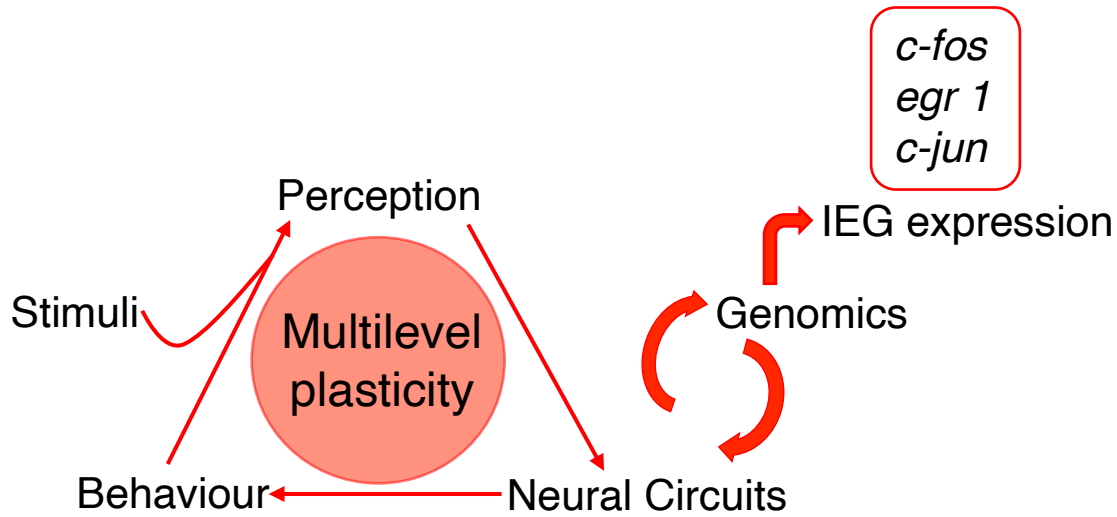


Figure 1.10: Expression pathway of Immediate Early Genes

Stimuli from the environment are integrated and processed within neurocircuits. Sensory exposure and learning activate a genomic response cascade in neurons that leads to changes in the neurocircuits. The first transcriptional step after neuronal activation includes the expression of immediate early genes (IEG), which orchestrate plasticity at the neuronal, behavioural, and perceptual levels. Their central role in controlling mechanisms of plasticity and the transient nature of their translation-independent expression makes IEGs promising markers for activated neuronal circuits [Adapted from (Sommerlandt et al., 2019)]

1.7. Aim of the thesis

This thesis mainly aimed to investigate the role of ionotropic glutamate receptors in odour information processing through olfactory subsystems with a focus on pheromonal cues. Specific molecular perturbations in the OB and AOB were achieved by altering the ionotropic glutamate receptor composition of the most abundant interneurons of OB, namely GAD65-expressing interneurons (Maksimova et al., 2019; Parrish-Aungst et al., 2007). GABAergic interneurons provide the synaptic inhibition onto projection neurons of OB (Isaacson & Strowbridge, 1998; Jahr & Nicoll, 1980; Nicoll, 1969; Urban, 2002). We modified the GAD65-expressing interneuron functions by knocking out the NR1 subunit of NMDA receptors and the GluA2 subunit of AMPA receptors from GAD65-expressing interneurons by crossing GAD65-Cre animals with NR1^{2Lox} or GluA2^{2Lox} animals. Through protein quantification, we have shown that there is a reduction of NR1 and GluA2 protein levels in the olfactory bulb of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals respectively. To observe if this brain-wide modification of GAD65-expressing neurons causes any deficits in mood disorder-related behaviours, we conducted a battery of tests.

Firstly, we subjected the animals to pheromone place preference assay (explained in Chapter 2) to investigate the role of ionotropic glutamate receptors of GAD65-expressing interneurons in multimodal learning of pheromone locations. Next, we modified the neuronal circuitry specifically in OB and AOB to investigate the involvement in multimodal learning of pheromone locations. Using a viral gene transfer method, we knocked out NR1 and GluA2 subunits of NMDAR and AMPAR, respectively, from OB and AOB. This allowed us to modify neuronal functions in a spatially restricted manner and carry out long-term behavioural experiments (Chapter 3).

Secondly, we trained the control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals on a Go/No-go behavioural training paradigm to understand the involvement of ionotropic glutamate receptors of GAD65-expressing interneurons in odour discrimination of both simple and complex odour mixtures. We also demonstrated changes in odour concentration threshold for detection and discrimination in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals. Lastly, previous research in our lab has shown that the olfactory bulb is also involved in processing airflow information (Mahajan et al., under review). Therefore, we sought to identify the molecular players in this phenomenon by subjecting NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals to airflow discrimination tasks.

CHAPTER 2

Materials and Methods

2.1. Ethics Statement

All experimental procedures used in this thesis work are presented in this chapter. The Institutional Animal Ethics Committee (IAEC) at IISER Pune, and the Committee for the Control and Supervision of Experiments on Animals (CCSEA), Government of India (Animal facility CCSEA registration number 1496/GO/ReBi/S/11/CCSEA), have approved the experimental protocols. The protocol number licensed for this project is IISER_Pune/IAEC/2016_01/001. The experimental procedures are discussed in the following sections:

2.2. Maintenance of mice used in the study

Adult male or female mice aged 8 to 12 weeks were used for the various experiments. The mice were maintained on a 12-hour day-light cycle (Light phase: 8.45 A.M. to 8.45 P.M.; Dark phase: 8.45 P.M. to 8.45 A.M.). Three to five mice were housed together in each Individually Ventilated Cage (IVC), with each cage providing a floor area of 800 cm². The cages were equipped with standard rodent bedding and nesting material. The temperature of the mouse holding room was maintained between 22⁰C and 25⁰C, with relative humidity ranging from 45% to 55%. All behavioural experiments were conducted during the light phase (between 8.45 A.M. and 8.45 P.M.). Except during the discrimination training paradigms, mice were given food ad libitum but were placed on a water-deprivation schedule. This schedule was designed to maintain the mice at $\geq 80\%$ of their initial body weight, and water restriction did not exceed 12 hours in a single day.

2.3. Generation of heterozygous knockout animals

The following lines of animals were crossed to generate a heterozygous F1 population of animals:

- 1) GAD65 Cre mouse line – B6N.Cg-GAD-65^{tm2(Cre)Zjh} /J was obtained from the Jackson Laboratory, USA.
- 2) NR1^{2Lox} mouse line – NR1^{2Lox} (Grin1^{tm2Rsp}) was obtained from Heidelberg University, Germany.
- 3) GluA2^{2Lox} mouse line – GluA2^{2Lox} (Gria2) was obtained from Heidelberg University, Germany.

To obtain heterozygous knockout animals for NR1, we crossed the GAD65-Cre line with the NR1^{2Lox} line. The F1 progeny was named NR1^{GAD65(+/-)} animals. Similarly, GluA2^{2Lox} animals were crossed with GAD-65 Cre animals to obtain GluA2^{GAD65(+/-)} animals. The males and females from the F1 generation were used for experiments after they were genotyped for confirmation of heterozygous knockout for NR1 and GluA2. We confirmed the presence of Cre through the expression of EYFP and the success of knockout was confirmed by western blotting (Figure 2.1).

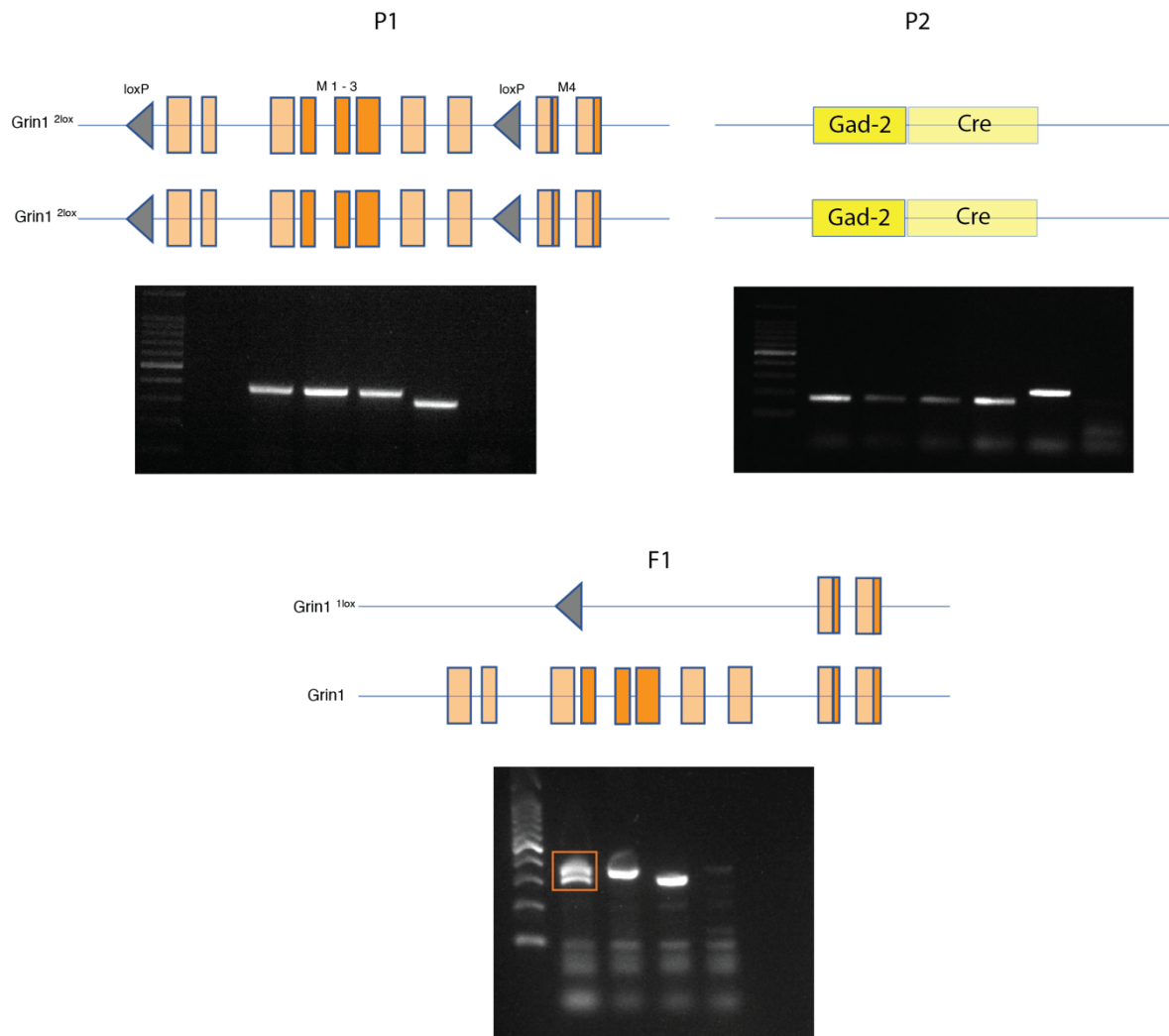


Figure 2.1: Genetic scheme used for NR1^{GAD65(+/-)} heterozygous knockout generation

NR1^{2Lox} animals were crossed with GAD65-Cre (GAD2-Cre) animals to obtain heterozygous knockouts of NR1 in GAD65-expressing interneurons in the F1 generation. The agarose gel images of the respective line are shown along with controls. The animals with heterozygous band patterns (shown in the orange box) were selected for further study.

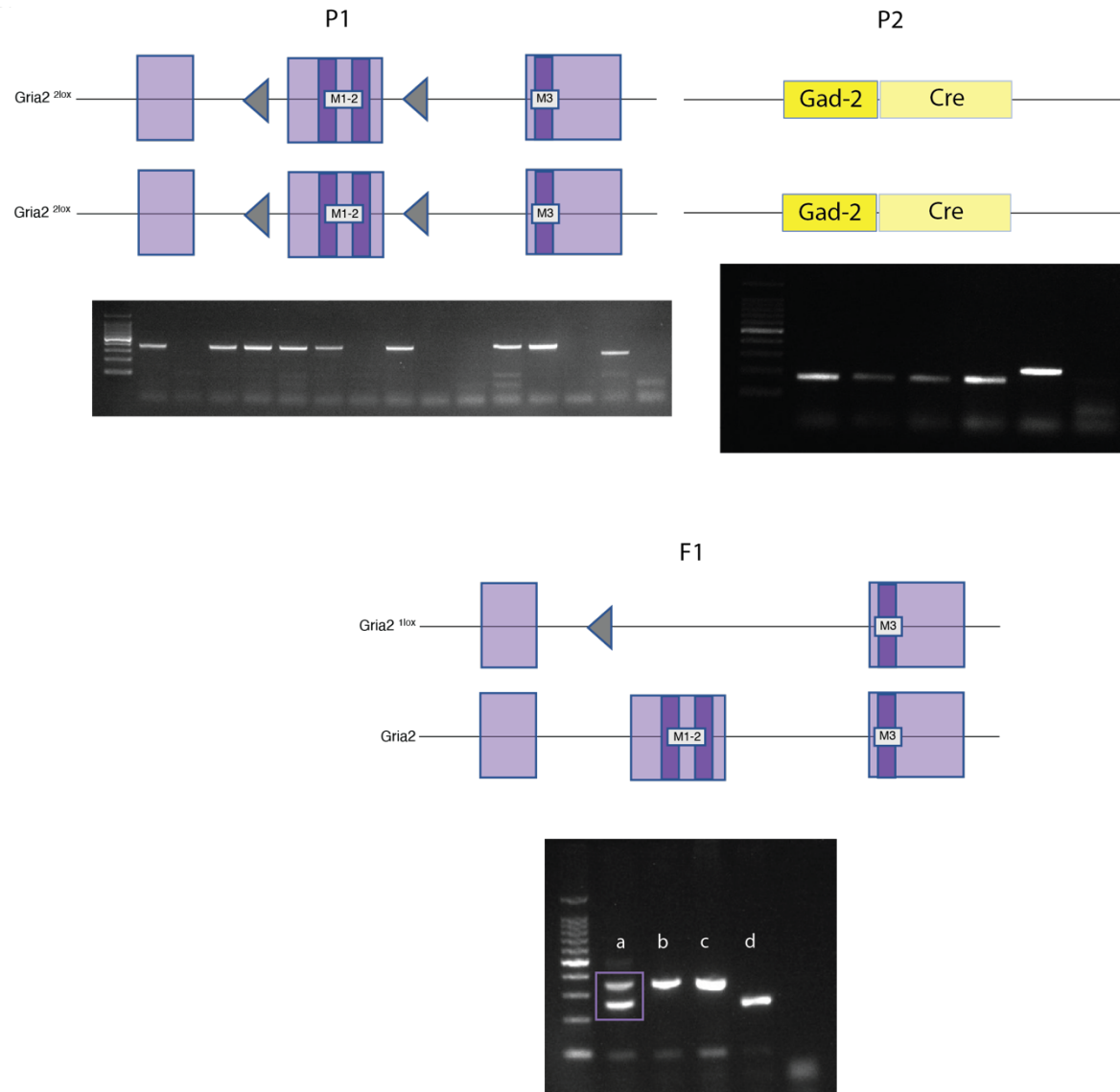


Figure 2.2: Genetic scheme used for $GluA2^{GAD65(+/-)}$ heterozygous knockout generation

$GluA2^{2Lox}$ animals were crossed with GAD65-Cre (GAD2-Cre) animals to obtain heterozygous knockouts of GluA2 in GAD65-expressing interneurons in the F1 generation. The agarose gel images of the respective line are shown along with controls. The animals with heterozygous band patterns (shown in the purple box) were selected for further study.

2.3.1. Genotyping

Tail samples from the F1 males and females were collected. DNA isolation was performed using a KAPA DNA extraction kit (Cat # KK5510) according to the manufacturer's instructions. The quality of the DNA and concentration were confirmed using NanoDrop (Thermo Fisher). Approximately, 50 to 70 μ g of DNA was used for the Polymerase Chain

Reaction (PCR). The primer sequences and details of the thermocycling protocol are described in Table 2.1. The amplified DNA was loaded onto 2 % agarose gel, and the DNA band pattern was observed as shown in Figure 2.1.

Name	Primers	PCR Cycling conditions
GAD65-Cre	5'-CACTGCATTCTAGTTGTGGTTTG- 3' (Mutant forward) 5' TCGTTGCACTGACGTGTTCT- 3' (Wild type forward) 5' AACAGTTTGATGAGTGAGGTGA -3' (Common)	Initial denaturation: 94°C, 2 minutes Denaturation: 94°C 20 seconds Annealing temp: 65 °C (-0.5°C per cycle), 15 seconds, Extension: 68°C, 10 seconds 10x Denaturation: 94°C, 15 seconds Annealing temp: 60°C 15 seconds, Extension: 72°C, 10 seconds 28x Final extension: 72°C, 2 minutes
NR1 ^{2Lox}	P1: N1Ex18do1: 5' - CTGGGACTCAGCTGTGCTGG- 3' P1b: NR1.2: 5'- CTCAAGTGAGTCTGCCCCATGCTG- 3' P2: N1In18up1: 5' - AGGGGAGGCAACACTGTGGAC- 3' P2b: NR1.3as: 5' - CACAGGGGAGGCAACACTGTGGAC- 3'	Initial denaturation: 94°C, 2 minutes Denaturation: 94°C, 30 seconds Annealing temp: 62.4°C, 30 seconds, Extension: 72°C, 50 seconds 35x Final extension: 72°C, 2 minutes
GluA2 ^{2Lox}	VM10: 5' - GTTGTCTAACAAGTTGTTGACC- 3' VM12: 5'- GCGTAAGCCTGTGAAATACCTG- 3' VM17: 5'- GAATCATTGTTGACAGATTGCCAC-	Initial denaturation: 94°C, 2 minutes Denaturation: 94°C, 30 seconds Annealing temp: 54.4°C, 30 seconds, Extension: 72°C, 50 seconds

	3'	35x Final extension: 72 ⁰ C, 2 minutes
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Table 2.1: Details of Genotyping protocol and DNA band pattern

2.3.2. Protein quantification

To quantify the drop in the protein levels of NR1 and GluA2, western blotting was performed. The following groups of animals were used for western blotting: control (NR1^{2Lox}, GluA2^{2Lox}), NR1^{GAD65(+/-)}, and GluA2^{GAD65(+/-)} animals (n = 3 each, N = 2). Animals aged 6 to 10 weeks were utilized. The animals were anaesthetized in a CO₂ chamber and then decapitated. Both OBs were dissected and immediately flash-frozen. The samples were kept at -80⁰C until further use. OB lysates were prepared in RIPA buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM Naf), supplemented with a complete protease inhibitor (Roche Cat # 04693116001). The BCA assay was performed to quantify the total protein concentration using a Pierce BCA protein assay kit (Thermofisher Cat # 23225). Approximately, 20 µg of protein from each sample was loaded onto a 12% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). After separating the proteins based on size, the proteins were transferred to the Immobilon-P PVDF membranes (Millipore Cat # IPVH00010) through methanol buffer transfer. Blocking was carried out with 5 % milk/Tris Buffer Saline (TBS)-Tween 20 for 1 hour at room temperature. The membranes were probed with primary antibodies: anti-NR1 antibody (Millipore Cat # AB9864R), anti-GluA2 antibody (Millipore Cat # AB1786-l), and anti-GAPDH (Sigma Cat # G9545) at 1:1000, 1:250 and 1:5000 dilutions, respectively, for approximately 20 hours at 4⁰C. The blots were washed with 0.1% TBST three times for 15 minutes each. The appropriate secondary antibody used was peroxidase-conjugated AffiniPure Goat anti-rabbit IgG (Jackson ImmunoResearch Cat # 111-035-003), diluted at 1:5000 for 1 hour at room temperature. The bound antibodies were detected using Clarity ECL Western Blotting Substrate (BioRad Cat # 1705061), with the image digitally captured using an ImageQuant LAS 4000.

2.4. Operant conditioning paradigms

The Go/No-Go (GNG) operant conditioning paradigm was used to study the odour and airflow discrimination abilities of mice, as described previously (Abraham et al., 2004; Bodyak, 1999). Briefly, mice were trained to discriminate between two odours (olfactory stimuli) or two airflows (mechanosensory stimuli). One of the stimuli was associated with a

water reward (S+), and the other stimulus was not associated with any reward or punishment (S-). The water reward given per correct response to the S+ stimuli was between 3 and 5 μ l. The water reward was given only when the animal fulfilled the reward criteria as specified in section 2.4.1.3. Animals were not supposed to lick for an S- stimuli, however, a few licks were permissible (discussed in section 2.4.1.3) to consider it as a correct response. There was neither reward nor punishment associated with an S- stimuli. In the paradigm, the response window overlapped with the stimulus presentation time. Animals received stimuli in a block of 20 trials containing an equal number of both S+ and S- stimuli presented in a pseudorandomized fashion. Animals underwent training until they reached the asymptotic phase of learning. Usually, animals took 3 to 4 tasks (wherein each task consists of 300 trials) to reach this phase. The GNG paradigm was employed for different experiments: odour discrimination under freely moving (FM) conditions, airflow discriminations and optogenetics experiments under head-restrained conditions.

2.4.1. GNG paradigm under freely moving conditions

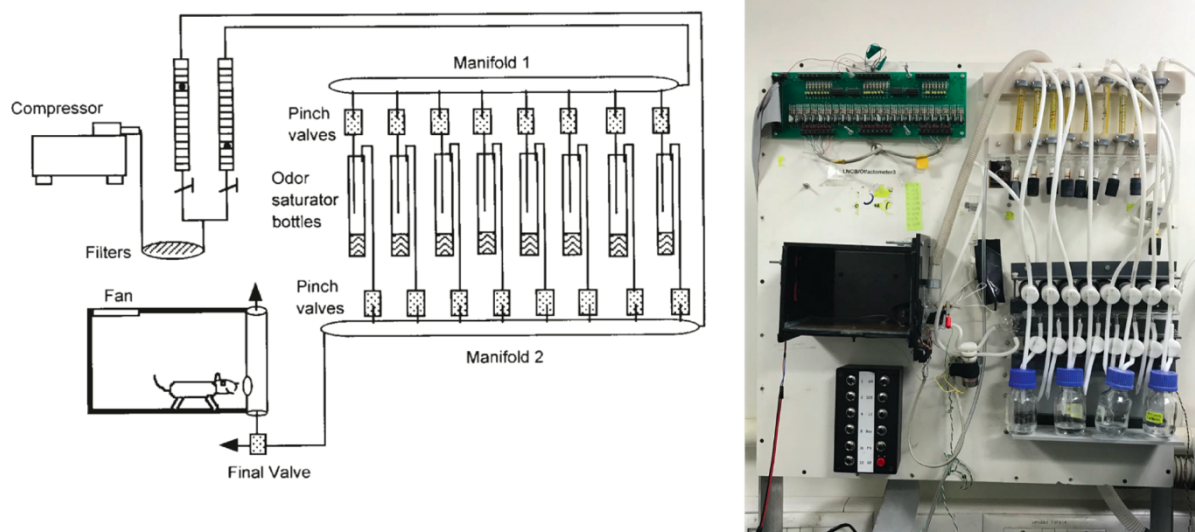
2.4.1.1. Apparatus

The animals were trained to discriminate odours using a custom-modified 8-channel olfactometer (Knosys, Washington, USA) (Bodyak, 1999), which was controlled by a custom-written program in IgorPro (Wavemetrics, OR). The olfactometer operated on the GNG operant conditioning paradigm, wherein one stimulus was associated with the reward and the other was not. The olfactometer comprises the following components: 1) Operant chamber, 2) Flowmeters, 3) Solenoid valves, 4) Sampling port and reward port, 5) Final Valve (FV), 6) Water valve, 7) Manual control box, and 8) Digital I/O board (Figure 2.3). The operant chamber is made of plexiglass and has a metallic floor. One side of the chamber consists of a sampling port, and the opposite side is mounted with an exhaust. The sampling port is guarded by an Infrared (IR) beam, which aids in initiating trials and recording the sampling behaviour of the animal. In this apparatus, the reward port and sampling port are coupled to ensure a firm association between reward and stimulus. The delivery of the stimulus is controlled by a set of solenoid valves, whereas the flow rate of clean and odorized air is controlled by two separate flowmeters.

Once the animal inserts its head into the sampling port, the IR beam breaks, and the trial is initiated. Upon trial initiation, the odour valve opens followed by the opening of the FV after 500 milliseconds. The FV helps divert odorized air away from the animal for an initial

500 milliseconds and then upon its opening the stimulus is delivered to the animal. The FV ensures fast and precise odour delivery to the animal. Once the animal samples the odour, it needs to either lick or not lick (depending on the type of stimulus) on the lick tube connected to a circuit for measuring lick responses. The lick responses are recorded once the animal's tongue is in contact with the lick tube, allowing the conductance through the animal. The sampling, as well as licking behaviour data, are stored in 20-millisecond time bins, which can be customized according to the experimenters' needs.

Figure 2.3: Olfactometer to study the behaviour under freely-moving conditions



Diagrammatic representation of the olfactometer (Bodyak, 1999) used in the study. A compressor pumps air into the olfactometer, and the airflow is controlled by the two flowmeters. The manifold distributes the air across different channels. A mouse is placed in an operant chamber under freely moving conditions to sample the odour coming from an odour tube. The animal responds based on the reward conditions.

2.4.1.2. Pre-training paradigm

Mice underwent a habituation phase during which they acclimatized to the setup and learned the procedural aspects of the paradigm. Two days after starting the water restriction schedule, animals underwent pre-training for the next 4 to 6 days. The pre-training paradigm consists of 8 phases. During the first phase, the water reward was given to the animal for poking its nose into the sampling port. After 15 such pokes, training progressed to the next phase where the animal needed to register a lick to receive the reward. After 20 trials of this phase, the animal moved to the next phase where a beam break triggered the valve to open for 2 seconds. If the animal licked during this time window, it received a water reward. Mineral oil was used

as the stimulus during this habituation phase, as it is a solvent used for odour dilution. Starting from the next phase, the lick criteria became more stringent. Initially, the animal had to lick for 1 bin, and then subsequently for 2 bins out of four bins to receive the water reward. In the last two phases, the lick criteria matched with those during the training paradigm, requiring the animal to lick for 3 out of 4 bins to get the water reward. Animals typically completed the pre-training paradigm within 4 to 6 days, involving 2 to 3 sessions of 30 minutes each.

2.4.1.3. Odour discrimination training

During the odour discrimination paradigm, the trial was initiated when the animal broke the IR beam by poking its head into the sampling port (Figure 2.4). After a delay of 500 milliseconds, an odour was represented to the animal for a duration of 2 seconds. The stimulus was presented in a block of 20 trials with an equal number of S+ and S- stimuli. These stimuli were presented in a pseudorandomized fashion, ensuring that not more than two successive deliveries of the same odour type were presented to the animal. For an S+ odour, if the animal licked correctly and satisfied the reward criteria, it received a water reward (3 to 5 μ l). The stimulus duration coincided with the response window, within which animals had to lick. In this case, the response window of 2 seconds was virtually divided into four windows of 500 milliseconds. For an S+ trial to be correct, animals had to register a lick in at least 3 out of those 4 bins. In contrast, for an S- trial to be correct, animals were allowed to lick at a maximum in 2 bins. Two consecutive stimuli were separated with a mandatory Inter-Trial Interval (ITI) of 5 seconds i.e., the next trial could not be initiated during that time. During the discrimination training, there was no control set for the pre-stimulus licking done by the animals. Each animal was trained for two sessions each day with 60 to 80 trials per session. The motivation level during the training was monitored using the ITI and licking frequency.

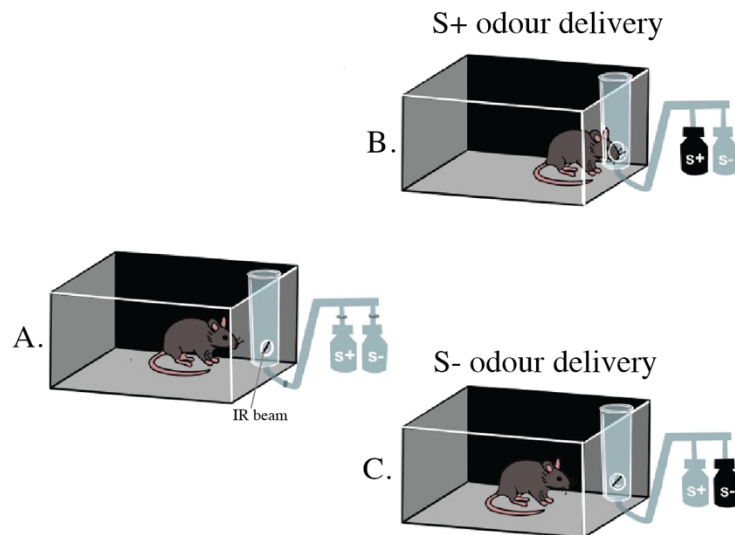


Figure 2.4: Schematic representation of the training paradigm under freely-moving conditions

- A. A trial begins when the animal breaks the IR beam (shown as the black line) and the stimulus is presented to the animal.
- B. If the odour is rewarded (S+), the animal continues to sample the odour and licks the lick tube to receive the water reward.
- C. If the odour is not rewarded (S-), the animal retracts its head, and the IR beam is sealed again.

2.4.1.4. Behavioral readouts

Learning accuracy

The progression of accurate performance was quantified using learning curves. A learning curve depicts the progression of average accuracies of 100 trials (50 S+ and 50 S-, averaged across all animals) with the number of trials. Initially, at the start of the discrimination task, the average accuracy of the animals remains near the chance level (50%), which improves with the number of trials and reaches an asymptotic phase of learning (> 80%) during the final phase (Figure 2.5). The discrimination task is considered concluded when the animals reach this advanced stage of learning.

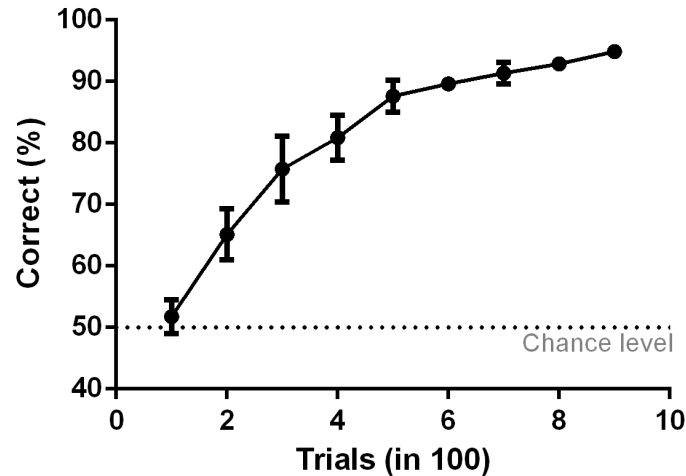


Figure 2.5: Learning curve

A typical learning curve representing the progression of accuracies with a number of trials when animals were trained to discriminate an odour pair under freely moving or head-restrained conditions. Learning accuracy is averaged for every 100 trials represented as each dot in the graph. Animals start with a chance level performance at 50%. Once the animal reaches 80% accuracy, it is considered to have learnt to discriminate the odours.

Discrimination Time (DT)

The time point after the onset of the odour stimulus, where the responses (licking or sampling – refer to Figure 2.6) by the animal for S+ and S-odours significantly diverges, is considered to be an Odour Discrimination Time (ODT). The sampling behaviour of individual animals is measured using the IR beam. Once the animals have achieved learning with > 80 % accuracy, they continue licking for S+ odour while keeping their head inside the sampling port, which is detected by the continuous breaking of the IR beam. For S- odours, as the animal retracts its head, the IR beam is sealed again. The mean difference in sampling behaviour for S+ and S- odours follows a sigmoid curve and provides a sensitive measure to determine ODT. The ODT is calculated task-wise using data from 300 trials (i.e., one task consisting of 150 S+ and 150 S- trials), where the learning accuracy has reached > 80%. Using a one-tailed paired t-test, each time bin of the 150 S+ and 150 S- trials are statistically compared. This statistical comparison yields a p-curve as a function of time. The last point in that p-curve showing $p \leq 0.05$ is considered to be the ODT.

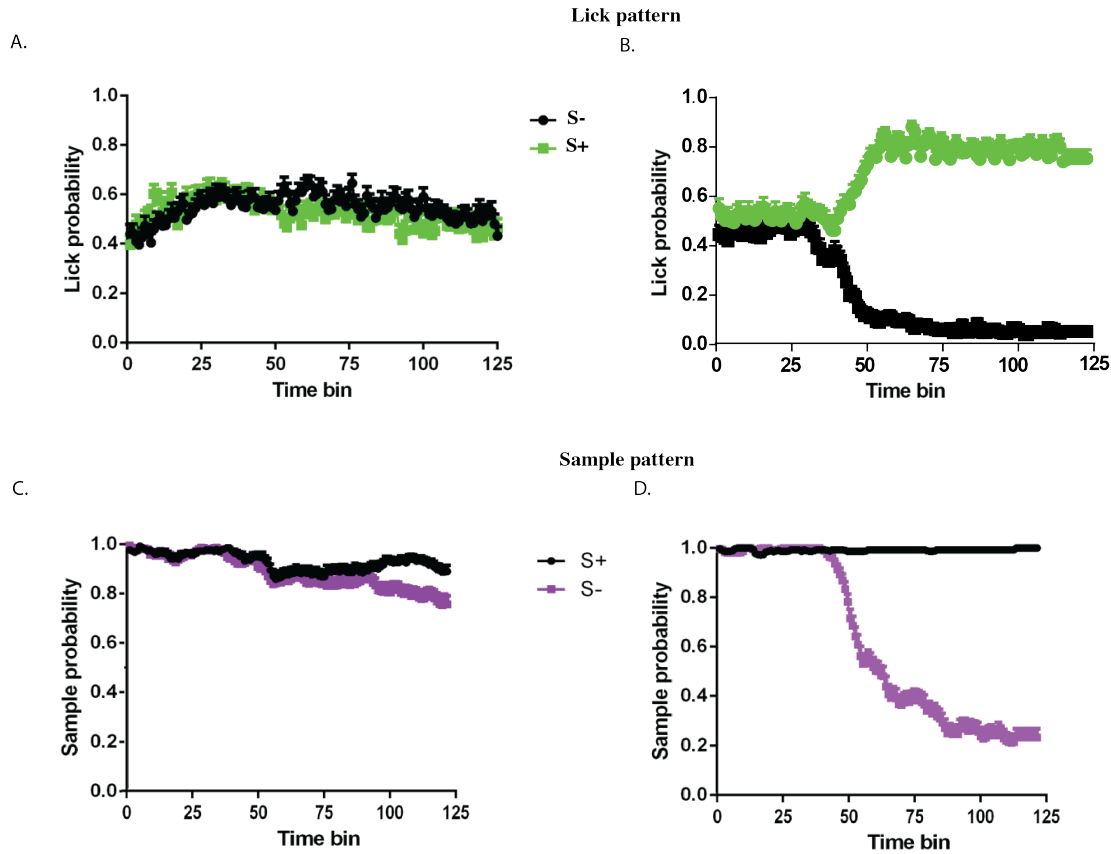


Figure 2.6: Representative graphs showing sampling and licking behaviour during training

A,B. The licking behaviour of a single animal during the first and last task of training is depicted.

The licking patterns for S+ (shown in green) and S- (shown in black) trials diverge as training progresses, as illustrated in the data from the last task.

C,D. The sampling behaviour of a single animal during the first and last task of training is illustrated.

The sampling patterns for S+ (shown in purple) and S- (shown in black) trials show divergence with the progression of training as demonstrated for the last task.

2.4.2. GNG paradigm under head-restrained conditions

2.4.2.1. Surgical implantation of head-post

Animals also underwent discrimination under head-restrained conditions. The head-restrained experiments provide an advantage over freely moving conditions if experimental conditions require the recording of certain physiological parameters, such as sniffing are required. To carry out optogenetic studies, a head-restrained setup is beneficial. Under head-restrained conditions, animals are mounted on a polyvinyl chloride (PVC) tube with a custom-made stainless-steel bracket. The animals undergo surgery during which a head post is attached to their skull, allowing for mounting. The surgical procedure is as follows:

- 1) Mice were first given an intraperitoneal (IP) injection of anaesthesia containing ketamine (50 µg/g body weight) and xylazine (10 µg/g body weight).
- 2) After the animal reached a state of anaesthesia, it was mounted on the stereotaxic setup. Before mounting, the animal's mouth was kept open by positioning its tongue outside to prevent fluid accumulation in the pharynx. The ear bars were fixed at the external auditory meatus, and the incisor teeth were placed on the incisor bars. Additionally, the nose clamp was fixed over the animals' noses.
- 3) The fur over the skull of the animal was removed. During the surgical procedure, the animal's eyes were continuously rehydrated with artificial tears (Refresh liquigel, Allergen India).
- 4) The skin over the skull was cut circularly with scissors. The area was cleaned with artificial cerebrospinal fluid (aCSF) with the following composition: 125 mM NaCl, 5 mM KCl, 10 mM Glucose, 10 mM HEPES, 2 mM CaCl₂, 2 mM MgSO₄, in 1L sterile Distilled water, pH = 7.4).
- 5) The periosteum was removed using a scalpel blade. Subsequently, the area was cleaned and dried using cotton buds.
- 6) Phosphoric acid (Etching agent: Ivoclar Vivadent) was applied over the skull for 10 to 15 seconds to increase the skull surface roughness, which provides a good landscape for adhering to the white dental cement. The skull was then cleaned with cotton buds.
- 7) After air drying, the skull was coated with dental primer and polymerized using UV light for 10 to 20 seconds to provide a better surface to adhere the white dental cement.
- 8) A thin and even layer of white dental cement (UV-cured-Ivoclar Vivadent) was spread on the skull and polymerized using UV light for 20 to 30 seconds.
- 9) White dental cement was applied to the base and sides of the headpost, which was then placed upright on the previous cement layer. The white dental cement on the head post was fused with the layer of cement on the skull, and the position of the head post was adjusted accordingly. The cement then was polymerized by exposing it to UV light for 50 to 60 seconds.
- 10) The exposed area of the skull was covered with dental acrylic cement (DPI RR Cold Cure), which was mixed with a polymerizing agent and two drops of cyanoacrylate gum to provide stability to the preparation.
- 11) Once the cement dried, the animal was kept in a home cage and monitored for the next few hours. The mice were given a recovery period of two days during which they had ad libitum access to food and water.

2.4.2.2. Apparatus

Optogenetic modulations during airflow discrimination under HR conditions were conducted using a setup consisting of a custom-built 10-channel olfactometer and lickometer. The lickometer served as an interface between all individual components, which were used to record and digitize the licks done by the animals towards various airflows. The olfactometer consisted of 11 mass flow controllers (MFCs; one main MFC and 10 mini MFCs, Pneuculus Technologies LLC, Hollis, NH) and electromagnetic solenoid valves (SMC Pneumatik AG, Switzerland), this helped to achieve temporal accuracy during the airflow delivery. A manifold was utilized diverting the clean air to different MFCs. Air through mini MFCs entered empty bottles and air from the main MFC was controlled using solenoid valves. A customized glass nozzle was employed for stimulus delivery. A pre-loading time of 3.2 seconds was set during which the air was directed to exhaust to establish a steady-state stimulus presentation. After a 3.2-second pre-loading period, the exhaust was shut down, and the stimulus (airflow) was represented to the animal. The stimulus nozzle was positioned near the nostrils of the animal to minimize the stimulus travel time. The lick tube, where the water reward was dispensed, was placed near the animal's mouth. The lick tube was connected to a lick circuit which allowed for the recording of lick responses during the training period. This comprehensive setup facilitated precise control and monitoring of airflow stimuli and the animals' behavioural responses, essential for studying airflow discrimination under head-restrained conditions and performing optogenetic modulations.

2.4.2.3. Pre-training

During the pre-training paradigm, which spanned 5 to 7 days, the animals were acclimated to the procedural aspects of the task. On the first day, the animal was placed inside the PVC tube and given water to habituate it with the setup. On the second day, phase 1 pretraining commenced with 20 trials, each involving of 200-millisecond tone at the onset of trial. Following the tone, water (3 to 5 μ l) was presented to the animal. Phases 2 (15 trials) and 3 (20 trials), gradually increased the delay between the tone and the water reward to 1 second and 2 seconds, respectively. The number of trials varied among animals based on their motivation levels. In the subsequent phase, a 2-second airflow pulse was introduced, and the animal received water upon exhibiting a lick response. Following that, baseline licking was recorded for 1 second after the tone. If the animal refrained from licking during the baseline duration, water reward criteria were adjusted based on the licking time during stimulus

presentation. Throughout the stimulus presentation, the required total licking time for the animal to receive the reward increased gradually from 40 milliseconds to 240 milliseconds (Bhattacharjee et al., 2019). A lick penalty was imposed, increasing the licking time from 100 % to 200%, if the animal licked during the baseline period. These progressive training phases and adjustments in reward criteria aimed to shape the animals' responses and acclimate them to the task's requirements.

2.4.2.4. Discrimination training

An ITI of 13.2 seconds, which includes 3.2 seconds of pre-loading time, was implemented during the airflow discrimination task. During the FM discrimination paradigm, it had been seen that the average ITI lies between 7 and 13 seconds. Hence, a constant ITI (3.2 seconds + 10 seconds) was implemented during HR conditions. The stimulus presentation and response window overlapped, with a duration of 2 seconds. The total lick duration needed to receive the water reward after S+ airflow was determined by the licking duration during the baseline period. If the animal licked during the baseline period, it had to lick twice the amount of time during the response window to receive the water reward. If the animal did not lick during the baseline period, it had to lick 80 milliseconds in total in any three out of four bins to get a water reward. A correct response was recorded if the animal licked properly for the S+ stimulus. For an S- stimulus, if the animal did not lick during the baseline period, a total of 80 milliseconds in two out of four bins was allowed to be considered as a correct response. If the animal licked during the baseline period, the S- response was registered as a correct response if the animal did not lick more than 25% of the baseline licking. There was no punishment involved for an incorrect response.

The stimulus sequence was pseudorandomized blocks of 20 trials. Counterbalancing of stimuli was performed to eliminate any bias towards one of the stimuli. Motivated animals completed 160 to 200 trials each day in two sessions. The licking frequency was monitored to assess the motivation level of the animal. If the animal showed a decrease in motivation level, visible by the licking frequency, the training for the session was stopped.

2.4.2.5. Quantification of discrimination time

The licking response was averaged into time bins with a resolution of 2 milliseconds. As the animals learned the discrimination paradigm, they consistently licked during the presentation of the S+ stimulus and refrained from licking during the presentation of an S-

stimulus within the response window. Blocks with $\geq 80\%$ learning accuracy were selected to calculate the discrimination time. The first point of divergence that exhibited a significant difference between S+ and S- trials was used to measure of discrimination time.

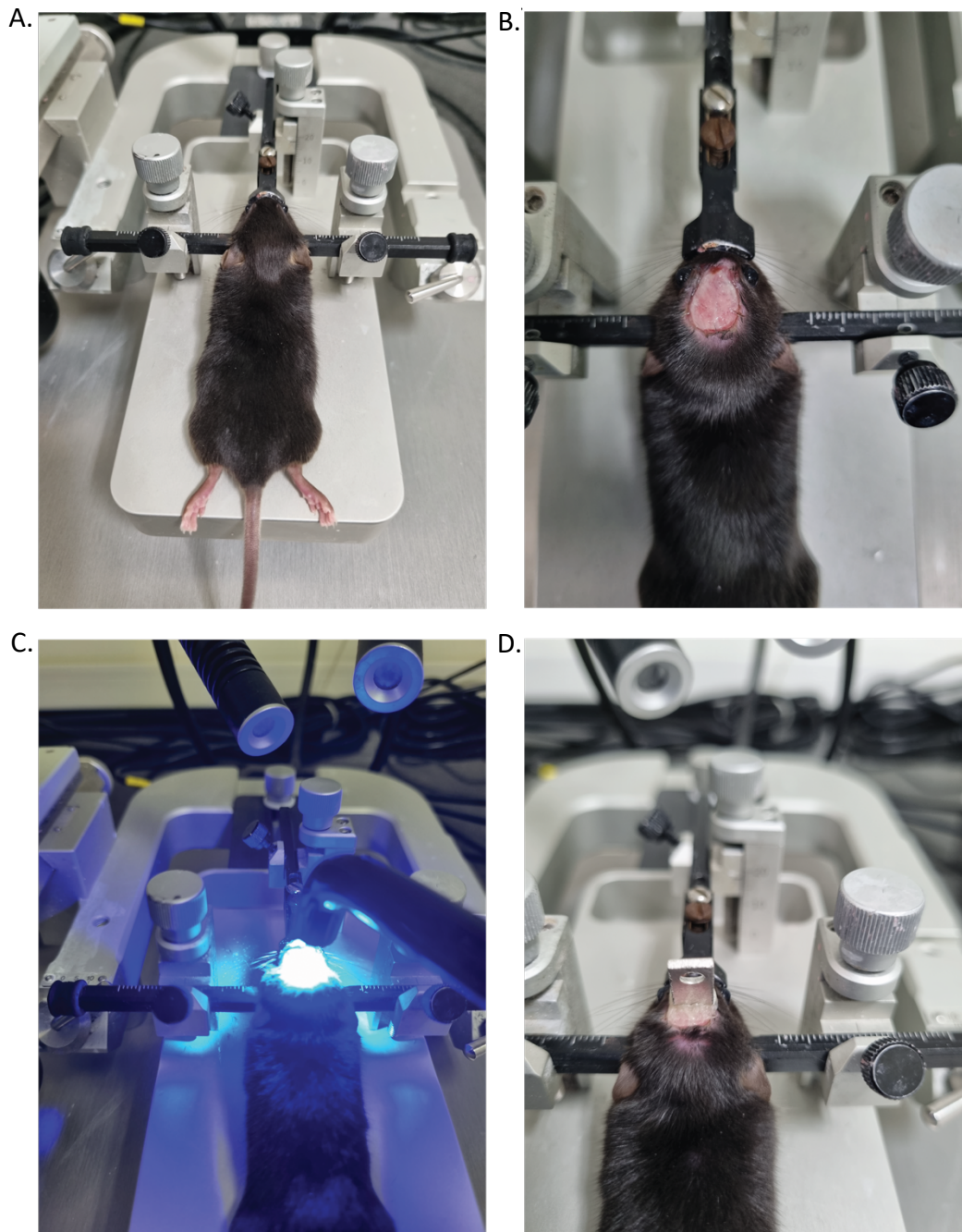


Figure 2.7: Procedure for head post-implantation

- A. The mouse was anaesthetized and secured on the stereotaxic setup.
- B. The skin over the skull was cleaned, and a thin base layer of UV-polymerizing white dental cement was applied.
- C. The applied thin layer of white cement was polymerized using UV light exposure.

D. The head-post was attached to the skull, and the surrounding exposed area was covered with acrylic pink dental cement.

2.4.3. Optogenetic modulation of GAD65-expressing interneurons

2.4.3.1. Cranial window implantation

LED implantation was performed on top of the OB to optogenetically modulate the GAD65-expressing interneurons in the GCL during airflow discrimination tasks. To specifically express ChR2 in GAD65-expressing interneurons in the GCL, GAD65-EYFP males were stereotactically injected with ChR2 viral particles (pAAV-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA). A cranial window was then implanted over the top of the OB to avoid direct contact with the LED and minimize the risk of infection. The surgical procedure for cranial window implantation is detailed as follows:

- 1) Mice were anaesthetized and mounted on the stereotaxic instrument as previously described. The skin over the OB was removed, and the skull was cleaned with artificial cerebrospinal fluid (aCSF).
- 2) A circular cranial window was created over the OB using a 2.5 mm biopsy punch.
- 3) A circular slit was made around the circumference of the cranial window using a blunt dental drill. This slit helps the glass window to fit properly on the top of the OB. Continuous application of aCSF was done to prevent tissue heating during drilling.
- 4) Once the proper slit depth was achieved, the 2.5 mm biopsy punch was used to remove the circular portion of the cranium.
- 5) The exposed dura was then covered with an optical window (glass coverslip) measuring 3 mm in diameter (Thomas Scientific, #1 thickness). aCSF was used to keep the dura moist during the process.
- 6) The optical window was temporarily held in place by supporting it with a Hamilton needle pushing at the centre of the window.
- 7) Acrylic dental cement, along with a drop of cyanoacrylate gum, was used to seal the optical window along the edges. The cement was allowed to dry for the next 10 minutes.
- 8) Following the window implantation, head-post implantation was performed to the posterior side of the window as previously described.
- 9) Animals were kept in a cage supplemented with a heating pad after the surgery until they started moving freely in the cage.
- 10) Animals were kept in their home cages for 3 weeks to allow the expression of the Cre enzyme. Before beginning the pre-training, LED implantation was performed.

- 11) For LED implantation, animals were anaesthetized and mounted on the stereotaxic setup as previously explained.
- 12) A ~ 2 mm LED (Blue LED (473 nm), NFSB036BT) with a connector (ED 11100— ND), connector socket .0207 100POS 0.050SMT, Digikey, and ED8250-ND Connector strip header 50POS, 0.050) was fixed on top of the optical window with the acrylic dental cement.
- 13) The animals were allowed to recover before starting with LED standardization. During this period, animals had ad libitum access to food and water.

2.4.3.2. LED standardization

The purpose of the optogenetic experiment was to determine if modulation of GAD65-expressing interneurons in the GCL during the representation of airflow stimulus could influence airflow discrimination learning abilities. Due to the surgical variabilities, the transparency of the optical cranial window may vary across the animals. Hence, it is necessary to perform a step of LED standardization for each animal to optimize the intensity of light illumination over the optical window. LED standardization involves finding the optimal light intensity where the behaviour of animals remains normal and the light source temperature does not exceed the physiological range. In addition to variations in window transparency, the distribution and quantity of Cre-viral particles in the GCL may vary, leading to differences in the light power required to activate GAD65-expressing interneurons in the GCL. Therefore, LED standardization is an essential step. To adjust the LED intensity, the LED driver (LEDD1B, T-Cube LED driver, ThorLabs) was used and the surface temperature was measured for every light power setting. We selected power settings near the ambient body temperature of the animal.

Before the LED optimization protocol, animals underwent pre-training on the head-restrained setup. During the LED (473 nm) standardization protocol, blocks of 20 trials with light stimulation (40 Hz) were interleaved with trials without light stimulation. Light stimulation occurred during the stimulus presentation for 2 seconds. Previous studies have shown that overstimulation of inhibitory activity leads to an inhibition of M/TCs activity, causing the cessation of licking behaviour (Gschwend et al., 2015). Therefore, during the LED standardization protocol, the licking response to trials with light stimulation versus without light stimulation was quantified. Different licking behaviour was observed with changes in

light power. For the airflow discrimination tasks, to modulate GAD65-expressing interneurons in the GCL, we selected the highest light power where licking behaviour remained intact.

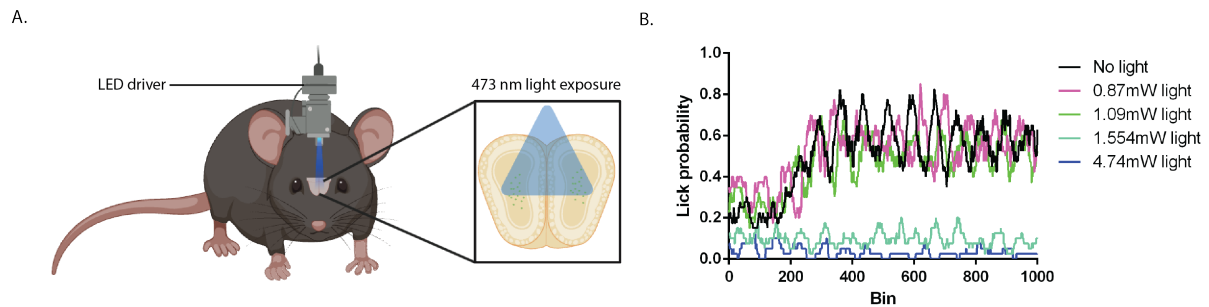


Figure 2.8: LED implantation and standardization for optogenetic experiments

- A. A diagrammatic representation of the implantation of a 473 nm LED implantation over the OB in ChR2-expressing GAD65-expressing interneurons of the GCL.
- B. Representation of licking behaviour for GAD65^{GCL}-ChR2 expressing animals. Licking patterns during airflow presentation with varying light intensities are depicted. An increase in light intensities resulted in a suppression of the licking response compared to licking during no-light conditions (as shown in black). For each animal, the highest light intensity showing licking behaviour was selected during the airflow discrimination tasks.

2.4.4. Assays to quantify anxiety and behavioural despair

2.4.4.1. Open Field Test (OFT)

OFT was conducted as a proxy to measure the anxiety-like behaviour in mice (Gould et al., 2009; Kraeuter et al., 2019). The arena, measuring 60 cm x 45 cm, was utilized for the assay. The setup was coated with black acrylic paint on all sides. Fresh bedding was used during the experiment. Initially, animals were placed in a habituation chamber measuring 42 cm x 26 cm x 18 cm for 10 minutes to acclimate. Subsequently, animals were individually allowed to explore the arena for 10 minutes each, while their activity was video recorded. The videos were later analyzed using Ethovision XT 8.5 software to determine the time spent by the animals in the centre and at the corners of the arena.

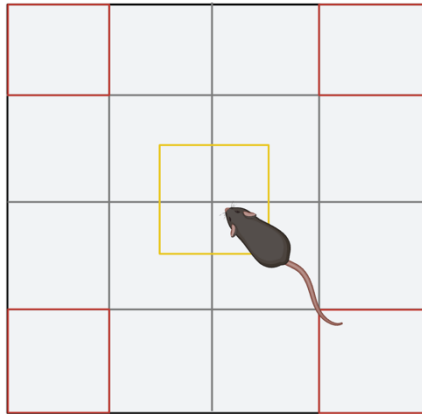


Figure 2.9: Setup for Open Field Test (OFT)

A diagrammatic representation of the open field test setup was created. The animal was allowed to explore the arena, and the time spent in the corners (shown in red) and the centre (shown in yellow) was calculated.

2.4.4.2. Elevated Plus Maze Test (EPMT)

The EPMT was also conducted to assess anxiety-like behaviour in mice (Komada et al., 2008; Walf & Frye, 2007). The maze consisted of two open arms and two closed arms on an elevated platform (50 cm above the ground). Each arm measured 55 cm in length and 5 cm in width. The closed arms were enclosed with walls of 15 cm in height. During the test, the animal was allowed to explore the setup for 10 minutes, and its activity was recorded via video. Subsequently, the videos were analyzed using Ethovision XT 8.5 software to determine the time spent by the animal in both the closed arms and open arms of the maze.

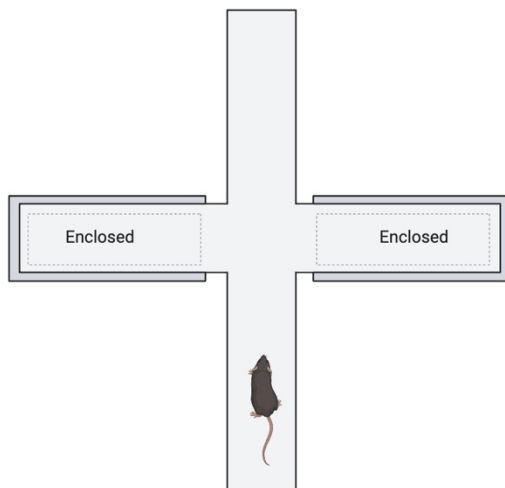


Figure 2.10: Elevated Plus Maze Test (EPMT)

A diagrammatic representation of the elevated plus test setup was created. The animal was introduced at the centre of the arena, and the number of entries and time spent in the closed versus open arms were calculated.

2.4.4.3. Tail Suspension Test (TST)

The TST was performed to assess despair-like behaviour in mice (Can, Dao, Terrillion, et al., 2012; Castagné et al., 2011). The animal was suspended upside-down by its tail using a 15 cm piece of tape attached to a stick at a height of 40 cm from the ground. The activity was

recorded for 5 minutes, and later the time spent by the animal in moving and stationary positions was manually calculated.



Figure 2.11: Tail Suspension Test (TST)

A diagrammatic representation of the tail suspension test setup was created. The animal was suspended upside down, and the time spent stationary versus mobile states was quantified.

2.4.4.4. Forced Swim Test (FST)

The FST was also performed to assess despair-like behaviour in mice (Can, Dao, Arad, et al., 2012; Castagné et al., 2011). A transparent jar measuring 15 cm in diameter and 20 cm in height was filled with distilled water up to a height of 12 cm. The animal was placed in the water for 10 minutes, and its activity was recorded. The time spent by the animal in moving and non-moving phases was calculated manually.



Figure 2.12: Setup of Forced Swim Test (FST)

A diagrammatic representation of the forced swim test setup was created. The animal was introduced into the water, and the time spent in stationary versus mobile states was quantified.

2.4.5. Measurements of Sniffing Frequency

We investigated the sniffing strategies of 10 to 12-week-old female mice across three groups (Control, NR1^{GAD65(+/-)}, GluA2^{GAD65(+/-)}) during exposure to male urine. This investigation utilized an air-pressure sensor (AWM2300V, Honeywell) in a head-restrained

setup. The head-restrained setup was employed for the precise delivery of volatiles to the animal.

The females were implanted with a custom-built head-post on the skull, as previously described. Upon recovery, the animals were mounted on a head-restrained setup. The stimulus was presented to the animals, with the odour emanating from one of two bottles containing either 100 μ l of male urine or 100 μ l of water in a pseudorandom order. Sniffing was detected using an airflow pressure sensor (AWM2300V, Honeywell) placed on one of the nostrils of the animal. The odour stimulus delivery lasted for 2 seconds. The stimulus was presented in blocks containing 20 trials (10 for male urine and 10 for water). Data was collected over four consecutive days, ensuring a total of 40 trials for each stimulus type.

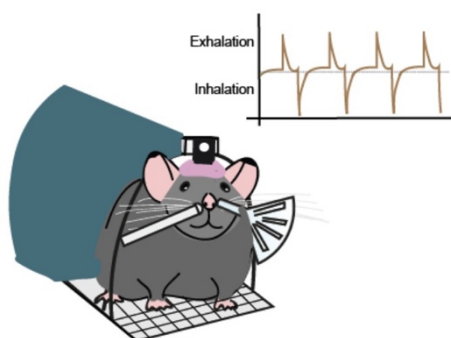


Figure 2.13: Sniffing Measurements

Diagrammatic representation of the head-restrained animal on the apparatus used to measure sniffing frequency. Odour molecules were introduced near one nostril, and sniffing was measured through another nostril using a pressure-based sensor. A trace of an inhalation-exhalation cycle is shown [taken from (Pardasani et al., 2021)].

2.4.6. Pheromone detection assay

A total of 72 female C57Bl6/J mice aged 8 to 12 weeks were used for the experiment. Pheromone detection was performed using a setup made of plexiglass coated with black acrylic paint. The dimension of the setup was 40 cm x 60 cm x 45 cm (L x W x H). For pheromone detection, 100 μ l of male urine and male soiled bedding collected from the male cage was kept in a 6 mm petri dish at the centre of the arena. The female mouse was allowed to explore the arena for 10 minutes, and the activity was recorded using an overhead recording setup. After 10 minutes, the recording was stopped, and the animal was returned to its home cage. The videos were analyzed using EthoVision (8.5 XT version) tracking software to determine the amount of time spent by the animal near the petri dish.

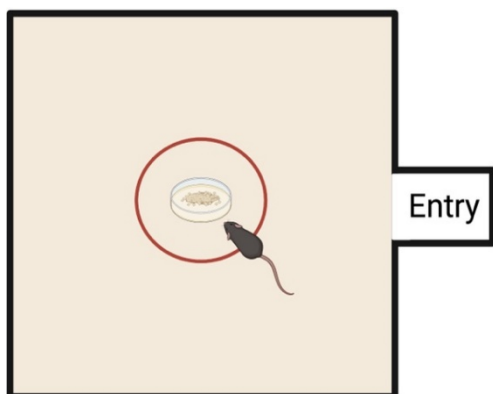


Figure 2.14: Pheromone Detection Test (PDT)

A diagrammatic representation of the pheromone detection test setup was created. The animal was introduced into the setup through the entry zone, and the time spent by the animal near the petri dish containing male urine (shown in red) was quantified.

2.4.7. Multimodal Pheromonal Location Learning

2.4.7.1. Apparatus

The three-chambered box used for the multimodal pheromone location learning paradigm had the following dimensions: 60 cm x 30 cm x 15 cm (L x W x H). It was made from plexiglass and coated with non-reflective black paint. The setup was divided into three equally sized zones. Zone 1 and Zone 2 (as shown in Figure 2.14) each had one chamber measuring 10 cm x 10 cm x 15 cm (L x W x H). Zone 1 and Zone 2 were positioned opposite to each other. Each chamber had a removable plate with either a 5 mm or 10 mm hole size. One pair, consisting of one 10 mm hole-sized plate and one 5 mm hole-sized plate, was dedicated to the male urine chamber (marked on top), while the other set was used only for the water chamber. The other three sides of these chambers were without any holes and were fixed. This was to prevent animals from sniffing odours from other sides of the chamber and ensure they could only access odours from the front end of the chamber. Each chamber contained a 55 mm petri dish. The Petri dish kept in the urine chamber contained fresh 100 μ l male urine (opposite sex pheromone in chamber 1), while the water chamber had 100 μ l distilled water (neutral stimulus in chamber 2). Two identical setups were used for training sessions and memory sessions, respectively.

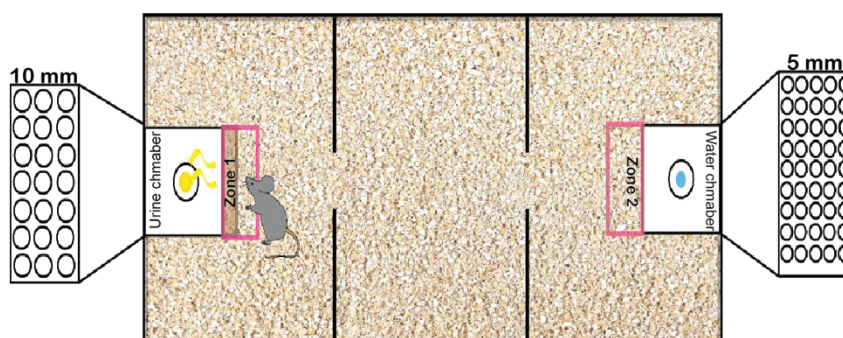


Figure 2.15: Diagrammatic representation of multimodal pheromone learning setup

The setup comprises two oppositely placed chambers containing male urine and water. Zone 1 and zone 2 represent the areas in front of the chambers used to quantify ‘time spent’ parameters. The number of active attempts made on the 10 mm and 5 mm plates was also quantified.

2.4.7.2. Training paradigm

The assay consists of three phases: 1) the Testing Phase (TP), 2) the Training Phase (TrP) and 3) the Memory Testing Phase (MP). The TP is performed for an initial four days. During the TP, the female mouse was allowed to roam freely in the setup for 10 minutes, and it's the activity was video recorded. The TP was conducted to determine if the female mouse exhibited any spatial preference towards the male urine chamber over the water chamber in the absence of any stimuli. The setup was rotated by 180° every day to avoid bias towards spatial location throughout the TP and TrP. During the TP, male urine (MU) and water (NS) were kept in their respective chambers. Soiled bedding (SB) from the male cage, from which urine had been collected, was also spread in front of the male urine chamber. This ensured the detection of both volatile and non-volatile molecules from the male pheromones.

During each experiment, female mice were counterbalanced toward a particular hole size on the plate (e.g., half of the female mice had a 10 mm hole-sized plate in the urine chamber, and a 5 mm hole-sized plate in the water chamber. For the other half, the urine chamber had a 5 mm hole-sized plate, and the water chamber had a 10 mm hole-sized plate). This counterbalancing was done to avoid any predisposition of female mice towards a particular hole size.

The TrP was conducted for the next 15 days. During TrP, the female animal was confined to both zones for 15 minutes each in three cycles; i.e., after the first 5 minutes in Zone 1, the animal was allowed to go to Zone 3 for 5 minutes. This was continued for 30 minutes every day for 15 days. Every day, the setup was rotated by 180° to avoid any possible bias toward a specific direction. Fresh MU, MB, and water were used on each training day. Memory was tested on a different setup that was identical to the training setup. This was done to prevent animals from sensing any remaining odours in the setup.

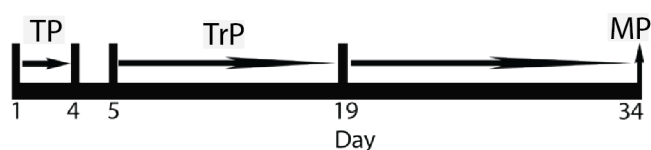


Figure 2.16: Timeline of pheromonal place preference assay

The training paradigm involved an initial four days of testing followed by 15 days of training. The memory for the pheromonal location was assessed on post-training day 15.

2.4.7.3. Behavioural parameters

Two parameters were quantified during the pheromone place assay: 1) 'Time spent' near Zone 1 versus Zone 2, and 2) 'number of active attempts' towards the respective plates. EthoVision software (Noldus Information Technology, version 8.5 XT) was used for quantifying 'time spent'. The nose point feature was utilized for the quantification. The number of active attempts referred to nose pokes made by the animal, which were counted manually.

2.4.7.4. Immunohistochemistry

After memory days 7 and 15, animals were sacrificed within 30 minutes of the experiment. This was done to quantify the expression of the Activity-regulated cytoskeletal (Arc) protein in the OB (MOB and AOB), dentate gyrus (DG) and somatosensory cortex (SSC). Perfusion was performed using the trans-cardial method with 4% paraformaldehyde (PFA) in 1X phosphate buffer saline (PBS). Brains were kept in 4% PFA overnight at 4°C. After a brief wash with 1X PBS, the brains were transferred to 30% sucrose, which acted as a cryoprotectant. Once the brains sank in the sucrose solution, a block of the brain with tissue freezing media (Leica Tissue freezing media cat # 14020108926) was prepared. 50 µm thick coronal sections were cut using a cryotome. We selected every 6th section of the OB, hippocampus and SSC for staining purposes. The sections were washed with 1% triton in 1X PBS for 10 minutes, followed by incubation in a blocking buffer (5% BSA in 0.5% triton and 1X PBS) for 1.5 hours at room temperature. Primary antibody incubation (1:750 ul of anti-Rabbit Arc antibody in 0.5% BSA in 0.1% triton and 1X PBS) was performed overnight at 4°C. Three washes with 1X PBS were carried out for 15 minutes each. During the last wash, 4,6-diamidino-2-phenylindole (DAPI) was added to visualize nuclei under the microscope. Secondary antibody incubation (anti-Rabbit Alexa-fluor 488 1:1000) was performed for 2 hours at room temperature. Three washes with 1X PBS were conducted for 10 minutes each. The sections were mounted on slides and protected from light with a VectaShield light protectant. The sections were imaged using a Leica SP8 confocal fluorescent upright microscope.

In addition, c-fos staining was conducted using female mice after training days 1, 7 and 15. The staining procedure was similar to Arc staining, with the exception of the blocking buffer, which contained 10% normal goat serum (NGS) in 1X PBS, and primary antibody

dilution (1:500). The sections were imaged under the 40X objective. Quantification of Arc and c-fos positive cells was performed using Imaris software.

2.4.7.5. Confocal imaging and cell count quantification

c-Fos and Arc expressing cells across OB, AOB, hippocampus and SSC were imaged using a Leica SP8 confocal microscope. We utilized 4 to 6 sections per brain area per mouse for quantification. The cell quantification process was conducted using Imarisx64 software (Bitplane, Oxford Instruments). For quantification, each section with a Z-stack of 1 μm step size was loaded into the software. A constant Gaussian filter was applied to every stack to reduce the background noise. We applied a constant value XY diameter (7.54 μm) for c-Fos and a value XY diameter (13.1 μm) for Arc. The threshold used to count the c-Fos and Arc-expressing cells was automatically set by the Imaris software, and false positive counts were manually removed. The total cells counted for each brain region equaled the sum of the cells counted per stack. The total volume was obtained by multiplying the thickness of the stack with the XY diameter of the image. The final number of c-Fos and Arc positive cells counted was measured per 1 mm^3 .

2.4.8. Stereotaxic delivery of Cre-viral particles

Stereotaxic surgery is a method used to manipulate the neural circuitry of the brain, providing spatial specificity (B. M. Geiger et al., 2008). This technique allowed us to accurately target different subsystems in the OB such as the MOB and AOB, using a stereotaxic atlas. For injections of Cre-viral particles, we used NR1^{2Lox} and GluA^{2Lox} female mice aged 6 to 8 weeks. The BENCHMARKTM stereotaxic instrument (myNeuroLab, St. Louis, USA) was used along with the LEICA MZ6 stereo microscope (Leica Microsystems, Germany). Henceforth, the anterior-posterior axis is referred to as the Y-axis, the mediolateral as the X-axis and the dorsoventral direction as the Z-axis.

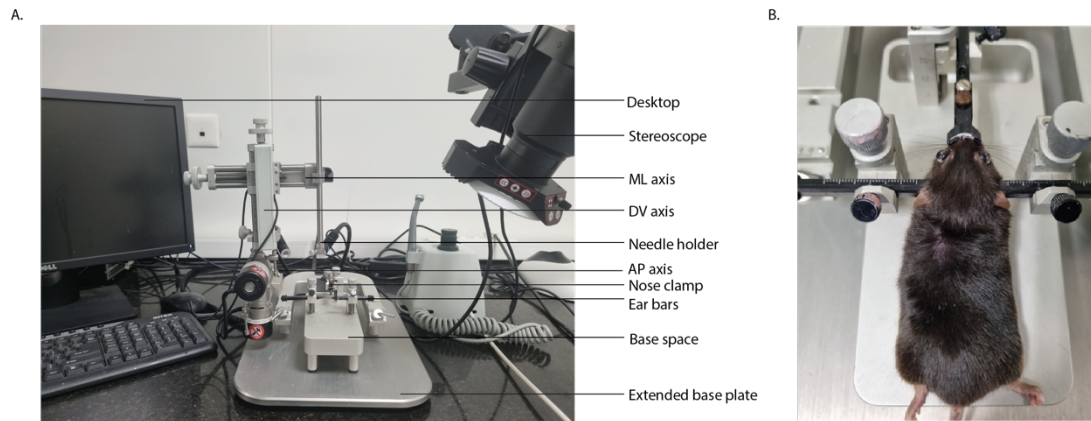


Figure 2.17: Stereotaxic setup used for viral injections

- A. The stereotaxic setup.
- B. An anaesthetized animal was fixed on the stereotaxic setup with the help of ear bars and mouthpiece to proceed with the surgery.

2.4.8.1. Preparation of capillaries

The injection capillaries were pulled using a needle puller (Narishige, Japan, model # PN-31, Serial # 12017), employing marked micropipettes (intraMARK, BLAU BRAND). The needles were pulled using specific settings (Heat = 660, Vel = 54). The elongated tips of the needles were cut using scissors to obtain a sharp-edged tip with a diameter of approximately 50 μm . The 1 μl calibration mark on the micropipette was used to estimate the volume of the injecting solution. One injection resulted in approximately 100 nl of the solution being injected.

2.4.8.2. Surgical procedure

- 1) The animal was anaesthetized and mounted on the stereotaxic instrument as described in section 2.4.2.1.
- 2) The fur over the skull of the animal was removed. During the surgery, the eyes of the animal were continuously rehydrated with artificial tears (Refresh liquigel, Allergan India).
- 3) Using a scalpel blade mounted on a holder, a midline incision of the skin on the dorsal surface of the head was made. The skin was laterally kept apart with the help of clips for the experimenter to access the skull. The incision was made in one stroke using enough pressure to facilitate suturing once the procedure was completed.
- 4) The skull was cleaned with a scalpel blade and then with a cotton bud dipped in aCSF and air dried.

- 5) Craniotomies were performed over each lobe of the OB using a dental drill. Gel foam was utilized to stop any bleeding that occurred during the surgical procedure.
- 6) The injection capillary was mounted along the dorsoventral axis of the stereotaxic setup. Negative pressure was manually applied through the capillary to fill it with the injecting solution.
- 7) The tip of the injection capillary was positioned at bregma, where the coordinates (X, Y, Z) were set to 0.
- 8) After establishing bregma as a reference point, the capillary was moved further anterior, near the intersection of coronal and sagittal sutures, where the Z coordinate was adjusted according to the Z coordinate at bregma. All the coordinates at this point were set to the origin (0,0,0). This was considered a local reference point.
- 9) Injection coordinates were based on the local reference point. Four injections were performed, and at each injection site, 100 to 150 μ l of solution was injected.
- 10) The injections were performed manually with the help of a syringe.
- 11) After each injection, the injection capillary was kept stationary for 3 to 5 minutes to prevent backflow of the injected solution.
- 12) Finally, the skin over the skull was sutured after completing the surgical procedure, and the animal was kept on a heating pad for further monitoring. Three weeks after the injections, the animals were used for experiments.

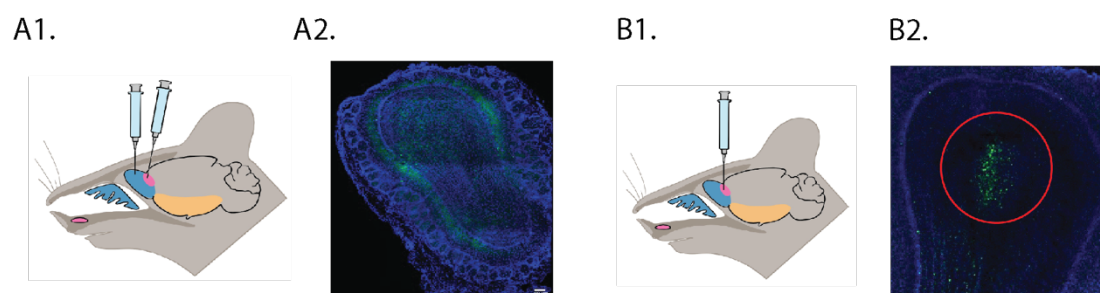


Figure 2.18: Stereotaxic injections for modulating the pheromonal information processing pathway

A1. Viral injections using a stereotaxic approach were performed in both the main and accessory olfactory bulb.

A2. Expression of EGFP after AAV-mediated Cre delivery in OB.

B1. Viral injections were targeted specifically at the accessory olfactory bulb using a stereotaxic approach.

A2. Expression of EGFP after AAV-mediated Cre delivery in AOB.

CHAPTER 3

Role of ionotropic glutamate receptors in multimodal learning of pheromone locations

3.1 Introduction

The sense of smell is vital for rodents' survival, allowing efficient detection, discrimination, and long-lasting memory of pheromones from their mates and predators. Male mice are known to mark their scent through urine depositions at various places in the environment (Arakawa et al., 2008). Urine scent marks are the display of their position in the hierarchy (Humphries et al., 1999; Hurst, 1993). Female mice encounter these pheromonal deposits and remember the location (Roberts et al., 2010). We have previously shown that this learning of pheromonal locations is multimodal. It involves both the olfactory as well as whisker systems (Pardasani et al., 2021). While for olfactory memory formation and storage, the PCx (Barkai & Hasselmo, 1997; Haberly & Bower, 1989; Quinlan et al., 2004), hippocampus (Fortin et al., 2002; Wood et al., 1999) and OB (P. Brennan et al., 1990; D. A. Wilson & Sullivan, 1994; Yuan et al., 2003) are involved, the cellular correlates of this multimodal memory formation for pheromonal locations remain unknown.

In the glomerular layer (GL) of the OB, odours are first represented as a spatiotemporal pattern of activity (Kauer & White, 2001; Mori et al., 1999; Schaefer & Margrie, 2007). Projection neurons, which are Mitral/Tufted cells (M/TCs), act as receivers of input from OSNs as well as output neurons that carry the information to the PCx (Shepherd et al., 2004). Glomeruli are spherical-like structures in the GL where synapses are formed between terminals of OSNs and dendrites of M/TCs. At the GL and GCL, M/TCs have synaptic connections with inhibitory interneurons (Aungst et al., 2003). The dendro-dendritic synapses formed between M/TCs and granule cells (GCs) are predominantly present in the OB. GCs are axon-less interneurons (Shepherd et al., 2007). Activated M/TCs excite GCs, which in turn inhibit the same or different M/TCs, a process called recurrent and lateral inhibition, respectively (Isaacson & Strowbridge, 1998; Jahr & Nicoll, 1980, 1982a, 1982b; Mori et al., 1999; Nicoll, 1969; Nowycky et al., 1981; C. G. Phillips et al., 1963; Urban, 2002; Wellis & Kauer, 1993, 1994). This interplay between inhibitory interneurons and M/TCs modulates olfactory sensory processing (Cleland & Linstner, 2005). Many studies have focused on the inhibitory interactions between M/TCs and GCs; however, the role of ionotropic glutamate receptors in multimodal

learning of pheromone locations has not yet been probed.

GABA is the most abundant inhibitory neurotransmitter present in OB interneurons, which is derived from glutamic acid decarboxylase (GAD). The adult brain contains two isoforms of GAD; 65-kDa (GAD65 or GAD2) and 67-kDa (GAD 67 or GAD1). In the OB, the ratio of GABAergic interneurons to excitatory neurons is 100:1 (Shepherd et al., 2004). In mouse OB, GAD65 accounts for approximately 82 % of the GAD protein (Parrish-Aungst et al., 2007; Sheikh et al., 1999). The GCL contains 58% of GAD65 (Parrish-Aungst et al., 2007). The balance between inhibitory and excitatory neurons has been maintained across mammalian species, indicating their vital role in olfactory information processing (Lledo et al., 2005; Tsai et al., 2018).

It has been discussed that volatiles in nature are sensed by the ORs in the MOE, and the information is then sent to glomeruli in the MOB while the non-volatile chemical cues are sensed by ORs in the vomeronasal organ (VNO), which project to the accessory OB (AOB). Many groups have shown that the MOB further projects to the “olfactory” amygdala i.e., the anterior cortical amygdala and posterior lateral cortical amygdala, while the AOB projects to the “vomeronasal” amygdala, which is the medial amygdala (MeA) (Broadwell, 1975; Carmichael et al., 1994; Davis et al., 1978; Jansen et al., 1998; Martinez-Garcia et al., 1991; Pro-Sistiaga et al., 2007; Scalia & Winans, 1975; Shipley & Adamek, 1984). However, recent studies have shown that a subpopulation of M/TCs coming from the MOB directly projects to the MeA, and these cells are preferentially excited by volatiles present in the urine of the opposite sex (Kang et al., 2009). Any impairment in VNO or MOE-based signalling leads to drastic deficits in avoiding predators and social communication in rodents (Bean & Wysocki, 1989; Isogai et al., 2011; Kimchi et al., 2007; Kobayakawa et al., 2007; Mandiyan et al., 2005; Stowers et al., 2002; Yoon et al., 2005). This indicates that chemical cues play a key role in social information processing in rodents and that the MOB and AOB pathways work together in responding to chemical cues (Dulac & Kimchi, 2007; Dulac & Wagner, 2006). How the changes in the MOB and AOB micro-circuitry affect the multimodal learning of pheromone locations is yet to be elucidated.

At dendro-dendritic synapses between M/TCs and GCs, Ca^{2+} influx through ionotropic glutamate receptors (iGluRs) causes the release of GABA from GCs onto M/TCs, resulting in the inhibition of M/TCs (Chen, Xiong, and Shepherd 2000; Halabisky et al. 2000; Isaacson 2001). GCs contain iGluRs, which are fast AMPARs and slow NMDARs (Montague & Greer, 1999; Sassoè-Pognetto & Ottersen, 2000). NMDARs containing an essential subunit, NR1, are calcium-permeable, while AMPARs containing a GluA2 subunit are calcium-impermeable

(Seeburg et al., 2001). AMPARs containing GluA2 are highly expressed in mouse OB (Montague & Greer, 1999; Sassoè-Pognetto & Ottersen, 2000). These receptors are also present at the dendro-dendritic synapses in GCs (Isaacson, 2001; Jardemark et al., 1997). Hence, we specifically manipulated iGluRs in GAD65-expressing interneurons to assess the role of these receptors in multimodal learning of pheromone locations. We also specifically modulated the iGluRs in mouse OB and AOB to deduce the involvement of these micro-circuitries in multimodal learning of pheromone locations.

3.2 Materials and Methods

3.2.1 Animals used

A total of 280 C57BL/6J animals aged 8 to 12 weeks were used for the experiments conducted in this chapter. The utilization of the animals for each experiment is outlined below. The transgenic mice were generated by crossing the following genotypes obtained from either Heidelberg University or Jackson Laboratory. The details are as follows:

- 1) GluA2^{2Lox}: GluA2^{2Lox} (Gria2) was obtained from the Heidelberg University, Germany
- 2) NR1^{2Lox}: NR1^{2Lox} (GluN1) was obtained from the Heidelberg University, Germany
- 3) GAD65-Cre: B6N.Cg-Gad2^{tm2(cre)Zjh}/J was obtained from the Jackson's Laboratory
- 4) NR1^{GAD65(+/-)} knockout: NR1^{2Lox} with B6N.Cg-Gad2^{tm2(cre)Zjh}/J for the knockout of GluA2 in GAD65-expressing interneurons
- 5) GluA2^{GAD65(+/-)} knockout: GluA2^{2Lox} with B6N.Cg-Gad2^{tm2(cre)Zjh}/J for the knockout of GluA2 in GAD65-expressing interneurons

Genotyping and protein quantification

Tail samples were frequently collected from the F1 progeny. The PCR amplification and agarose gel electrophoresis were performed as described in Chapter 2. To quantify the protein levels of NR1 and GluA2 in the F1 generation, OBs from six adult animals aged 8 to 12 weeks were taken. Western blotting was then performed following the procedures outlined in Chapter 2.

Anxiety and despair-like behaviour tests

The anxiety and despair-like behavioural experiments were performed on adult females aged 6 to 12 weeks, with varying numbers of animals across the tests.

EPMT: control (n = 5), NR1^{GAD65(+/-)} (n = 5) and GluA2^{GAD65(+/-)} (n = 6)

FST: control (n = 6), NR1^{GAD65(+/-)} (n = 6) and GluA2^{GAD65(+/-)} (n = 6)

TST: control (n = 5), NR1^{GAD65(+/-)} (n = 6) and GluA2^{GAD65(+/-)} (n = 6)

OFT: control (n = 8), NR1^{GAD65(+/-)} (n = 10) and GluA2^{GAD65(+/-)} (n = 11)

EPMT, FST, TST, and OFT were performed as explained in Chapter 2.

Pheromone Detection Test (PDT)

A total of 72 female mice were used for the pheromone detection test.

Sniffing frequency

To observe the sniffing frequency of female mice towards male urine pheromone, 8 to 12-week-old females per group were used (N = 14). Sniffing quantification was carried out following the procedures outlined in Chapter 2.

Pheromone place preference assay

A total of 129 female mice were used to conduct the pheromone place preference assay, following the procedures outlined in Chapter 2. The experimental conditions were as follows:

As previously demonstrated by Pardasani et al.(2021), memory related to pheromone locations is multimodal involving olfactory and whisker systems (Pardasani et al., 2021). For this study, we obtained a new set of 12 WT (C57Bl6/J) females aged 8 to 12 weeks. Male urine was collected from age-matched C57BL6/J animals. Following initial testing days, the animals were divided into whisker-intact (WI) and whisker-deprived (WD) groups. The whiskers of the animals in the WD group were trimmed after the last testing day. To eliminate any input through the whiskers, the snout of the animals was applied with an anaesthetic gel, Lignocaine, 15 minutes before the start of training. Whiskers were trimmed every fourth day of training to ensure no regrowth. WI and WD animals were trained for the multimodal pheromone place preference assay and memory was accessed on post-training day 7 and day 15.

In the next set of experiments, we investigated the role of iGluRs (NMDA and AMPA) in multimodal learning of pheromone locations. We modified the GAD65 interneurons by knocking out the NR1 subunit of NMDARs and the GluA2 subunit of AMPAR. The details are discussed in Chapter 2. They are named NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} throughout. 8 to 10 weeks-old female animals are exclusively used for the pheromonal place preference assay. Male urine was collected from age-matched non-littermate males from the control group (NR1^{2Lox} or GluA2^{2Lox}). The initial testing phase (TP) was conducted to observe any innate preference towards one of the zones (male urine zone or water zone, control: n = 12,

NR1^{GAD65(+/-)}: n = 10, GluA2^{GAD65(+/-)}: n = 12). The post-training day 15 memory (MD 15) was tested in a completely new setup that was never exposed to odour stimuli. The time duration spent by these females toward the male urine zone and water zone was quantified using Ethovision software 8.5 XT (control: n = 15, NR1^{GAD65(+/-)}: n = 19, GluA2^{GAD65(+/-)}: n = 13). The number of active attempts was quantified manually (control: n = 13, NR1^{GAD65(+/-)}: n = 13, GluA2^{GAD65(+/-)}: n = 8). We repeated the experiments in three batches, and the results were pooled. The last set of experiments was done to sacrifice the animals after memory day 15 to quantify Arc protein expression. The protocol is described in Chapter 2. Three animals from each group were used for immunohistochemistry. From each brain, we collected 8 to 12 OB sections, and 6 to 8 SSC and DG sections for quantification of Arc using Imaris software.

To test the pheromonal location memory on early recall periods, a new set of 8 to 10-week-old females was used (n = 6 for each group). The memory on post-training 7 was quantified. After memory day 7, Arc expression was quantified in the OB, SSC and DG regions (n = 3 for each group).

We also investigated the memory for earlier recall periods (MD 1 and MD5). We used a new set of age-matched females (control: n = 5, NR1^{GAD65(+/-)}: n = 5, GluA2^{GAD65(+/-)}: n = 6). After the training phase was completed, the memory was assessed the next day. Using the same set of animals, we quantified the memory of post-training day 5.

We observed the behaviour of animals during the training phase (TP) by quantifying the time spent and number of active attempts on TrD 1, 7, and 15 (control: n = 4, NR1^{GAD65(+/-)}: n = 4, GluA2^{GAD65(+/-)}: n = 5). Immediate early gene (IEG) expression was quantified using c-fos in the OB, SSC and DG (n = 3 for each group).

The mouse olfactory system is organized into functional systems involving different sensory regions, such as MOE, VNO, SO, and GG. The MOE sends projections to the MOB, while receptors in the VNO project to the AOB. To investigate the role of the OB and AOB, we modified the microcircuitry using stereotaxic viral injection, discussed in detail in Chapter 2. In the first set of experiments, we injected AAV5-Cre viral particles into NR1^{2Lox} (n = 7) and GluA2^{2Lox} (n = 8) animals. As a control, we used PBS-injected NR1^{2Lox}/GluA2^{2Lox} animals (n = 9). Three weeks after the injections, we conducted the pheromone detection test to observe any impairment in pheromone detection abilities. Subsequently, pheromone place preference training was performed, and memory was assessed on post-training day 7 and day 15. After memory day 15, three animals from each group were used to quantify Arc expression in the OB, SSC and DG regions. To study the role of AOB microcircuitry in the memory toward pheromonal locations, a new set of adult females underwent stereotaxic surgeries (NR1^{2Lox}: n

= 8, GluA2^{2Lox}; n = 7). The data from the control group of the previous experiment was used for AOB-injected animals (n = 9). These animals also underwent the same sequence of experiments: Pheromone detection test, TrP and MP on day 7 and day 15.

3.3 Results

3.3.1 Generation of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} heterozygous knockouts

Information received from ORNs is heavily modulated by excitatory and inhibitory interneurons in the OB. Furthermore, M/TCs carry odour information to the olfactory cortex. One of the major inhibitory neurotransmitters present in the OB is gamma-aminobutyric acid (GABA). GABA is derived from L-glutamic acid by the enzymatic action of GAD. The adult rodent brain consists of two isoforms of GAD (GAD 67/1 or GAD 65/2). To investigate the role of GAD65-expressing neurons in mediating inhibition in multimodal learning of pheromone locations, we modulated ionotropic glutamate receptors' function in GAD65-expressing neurons. We deleted the NR1 subunit of NMDAR and the GluA2 subunit of AMPAR from GAD65-expressing neurons using the Cre-Lox system (Figure 3.1). As NR1 is an essential subunit of NMDAR, this perturbation results in the reduction of functional NMDARs (Monyer et al., 1992) and the removal of GluA2 results in calcium-permeable AMPARs (CP-AMPA) (Tóth & McBain, 1998). Mice from GAD65-Cre lines were crossed with NR1^{2Lox} or GluA2^{2Lox} animals to generate heterozygous knockouts (KOs) in the F1 generation. DNA was extracted from the tail samples of both male and female animals. The DNA was amplified by using a specific set of primers for NR1 and GluA2 (as discussed in Chapter 2). The PCR products of NR1 were 254 bp and 321 bp in size for F1 generation animals (Figure 3.1). The amplified DNA for GluA2 showed bands at 315 bp and 369 bp for the F1 generation (Figure 3.1.5). To assess the reduction in NR1 and GluA2 protein levels in the OB, immunoblotting of total protein from the entire OB dissected from animals aged 6 to 10 weeks was performed. We observed a ~ 20 % decrease in NR1 levels in the OB of NR1^{GAD65(+/-)} animals compared to controls (Figure 3.1A: N = 6 each, One-tailed unpaired t-test, p = 0.0313), while we observed an almost 30% reduction in GluA2 levels in the OB of GluA2^{GAD65(+/-)} animals compared to controls (Figure 3.1 B: N = 6 each, One-tailed unpaired t-test, p = 0.1034).

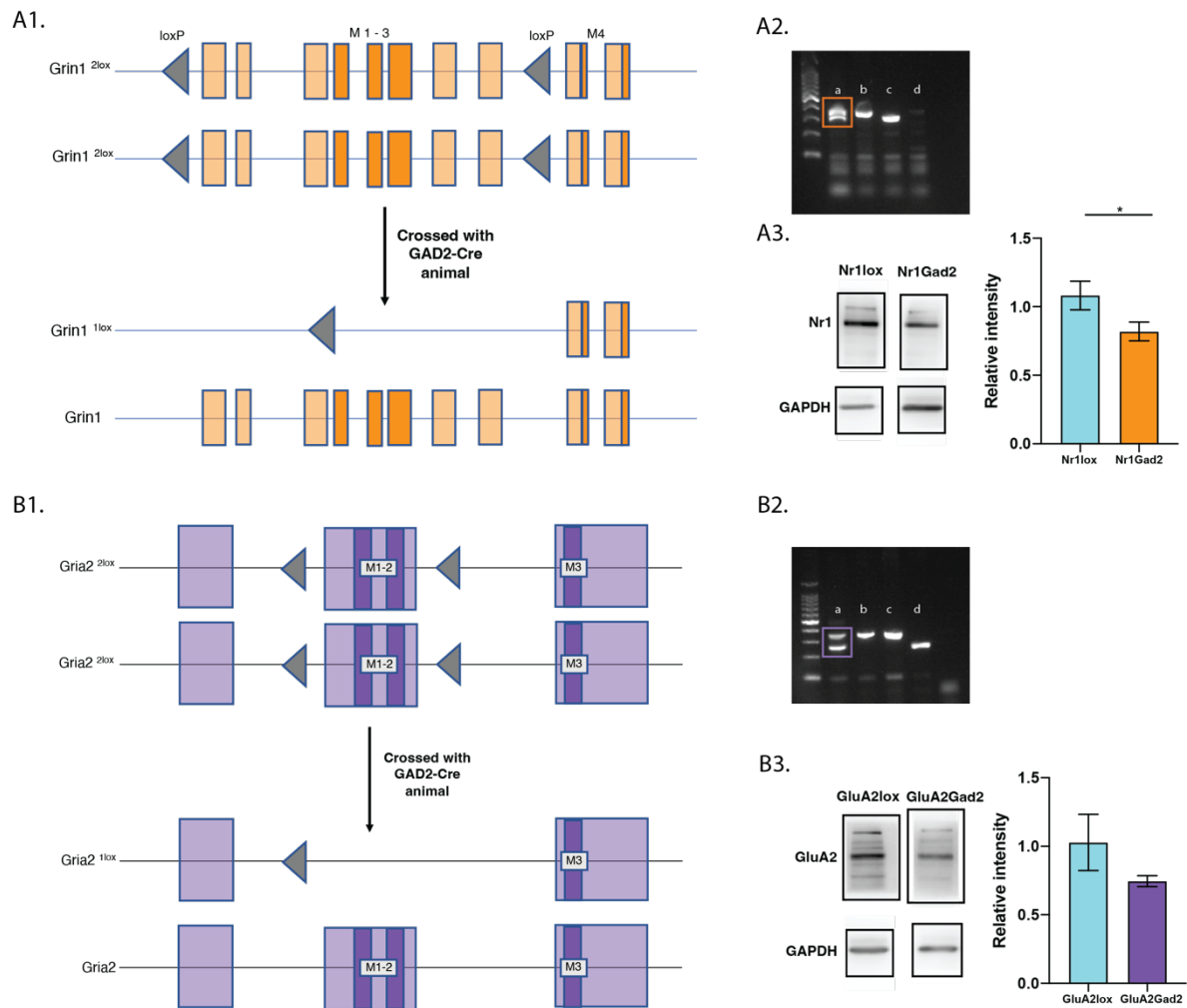


Figure 3.1: Generation of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} heterozygous knockouts

A1. Scheme of genetic crosses used to generate NR1^{GAD65(+/-)} animals.

A2. Agarose gel electrophoresis image of DNA samples taken from a: Heterozygous NR1^{GAD65(+/-)} animal (369 bp and 315 bp), b: NR1-2Lox (369 bp), c: wild-type (315 bp) and d: Blank.

A3. Immunoblots showing expression of NR1 in the olfactory bulb of control and NR1^{GAD65(+/-)} animals. GAPDH was used as a loading control. NR1 expression was reduced in NR1^{GAD65(+/-)} animals. (Control: 1.083 ± 0.1049, NR1^{GAD65(+/-)}: 0.8197 ± 0.0688 mean ± SEM, Unpaired one-tailed t-test, p = 0.0313, N = 6).

B1. Scheme of genetic crosses used to generate GluA2^{GAD65(+/-)} animals.

B2. Agarose gel electrophoresis image of DNA samples taken from a: Heterozygous GluA2^{GAD65(+/-)} KO animal (348 bp and 254 bp), b: GluA2-2Lox (348 bp), c: wild-type (254 bp) and d: Blank.

B3. Immunoblots showing expression of NR1 in the olfactory bulb of control and GluA2^{GAD65(+/-)} animals. GAPDH was used as a loading control. GluA2 expression was reduced in NR1^{GAD65(+/-)} animals (control: 1.027 ± 0.2051, GluA2: 0.745 ± 0.0400, mean ± SEM Unpaired one-tailed t-test, p = 0.1034, N = 6).

3.3.2 Long-term memory of pheromone locations is achieved by the association between olfactory and whisker systems

To investigate the role of the whisker system, along with the olfactory system in forming pheromonal location memory, we conducted whisker deprivation in C57BL6/J females. During this experiment, the whiskers were trimmed and the local anaesthetic gel was applied on the snout of the animal during TP, to ensure the prevention of association between the odour stimulus and the plates with particular diameters. The memory on post-training days 7 and 15 was quantified based on the time spent near the chamber and active attempts made by the animals (Pardasani et al., 2021).

The post-training day 7 memory in whisker-deprived females was significantly reduced compared to whisker-intact females, as shown by the time spent near zone 1 versus zone 2 (Figure 3.2: Paired Wilcoxon t-test, whisker-intact $p = 0.0312$, whisker-deprived females $p = 0.625$), and the number of active attempts made by the females towards respective chambers (Figure 3.2: paired Wilcoxon t-test whisker-intact $p = 0.0312$, whisker-deprived $p = 0.6250$). Similarly, on post-training day 15, memory in whisker-deprived females was significantly reduced compared to whisker-intact females, as indicated by time spent near zone 1 versus zone 2 (Figure 3.5: Paired Wilcoxon t-test whisker-intact $p = 0.0312$, whisker-deprived females $p = 0.3750$), and the number of active attempts made by the females towards respective chambers (Figure 3.2: Paired Wilcoxon t-test whisker-intact, $p = 0.0312$, whisker-deprived, $p = 0.5$). The similar amount of time spent and similar active attempts done by the whisker-deprived females towards both zones indicate an impairment in learning the pheromone locations. These results suggest that the pheromonal location memory is multimodal, involving both the olfactory as well as whisker systems, as previously shown (Pardasani et al., 2021).

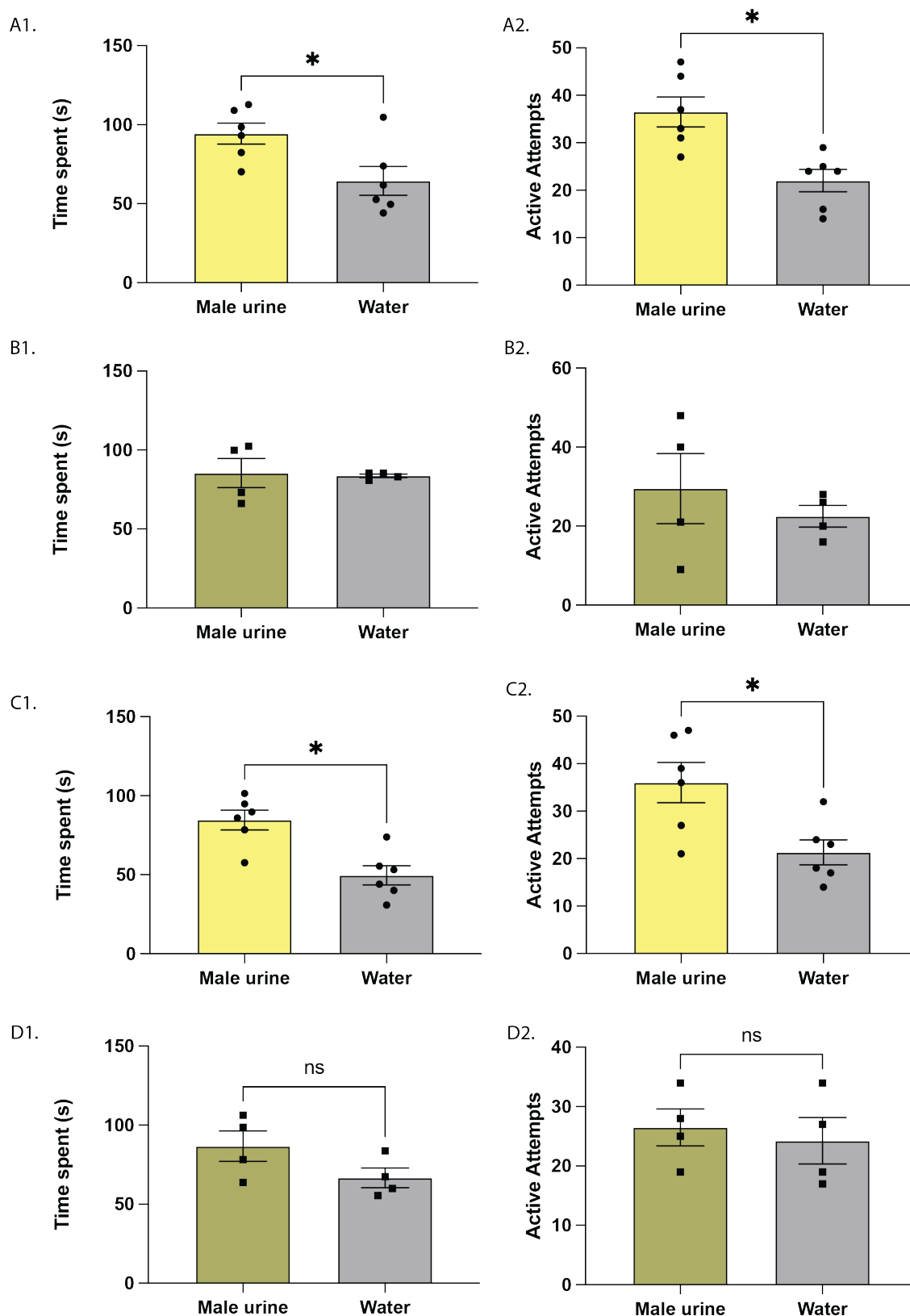


Figure 3.2: Facilitation of long-term memory of pheromone locations by association between olfactory and whisker systems

A. A1. Significantly longer time was spent near to male urine chamber by whisker-intact females on MD 7 ($p = 0.0312$, paired two-tailed student's t-test, $N = 6$ females). A2. Whisker-deprived females

showed similar time spent for both zones on MD 15 ($p = 0.3750$, paired two-tailed student's t-test, $N = 4$ females).

- B. B1. Whisker-deprived females showed similar time spent for both zones on MD 7 ($p = 0.6250$, paired two-tailed student's t-test, $N = 4$ females). B2. Whisker-deprived females displayed a similar number of active attempts for both zones on MD 7 ($p = 0.6250$, paired two-tailed student's t-test, $N = 4$ females).
- C. C1. Significantly longer time was spent near to male urine chamber by whisker-intact females on MD 15 ($p = 0.0312$, paired two-tailed student's t-test, $N = 6$ females). C2. Significantly higher number of active attempts was performed by the whisker-intact females on MD 15 ($p = 0.0312$, paired two-tailed student's t-test, $N = 6$ females).
- D. D1. Whisker-deprived females showed similar time spent for both zones on MD 15 ($p = 0.3750$, paired two-tailed student's t-test, $N = 4$ females). D2. Whisker-deprived females displayed a similar number of active attempts for both zones on MD 15 ($p = 0.5$, paired two-tailed student's t-test, $N = 4$ females).

3.3.3 Ionotropic receptors of GAD65-expressing neurons are required for multimodal learning of pheromone locations

To investigate the role of synaptic inhibition by GAD65-expressing neurons in multimodal learning of pheromone locations, we utilized heterozygous knockouts for NR1 and GluA2. Female mice were trained on a setup designed for multimodal learning of pheromone locations, as detailed in (Pardasani et al., 2021, also explained in Chapter 2). Briefly, during the initial four days, animals explored the setup for 10 minutes each day in the presence of odour stimuli and plates with varying sizes at opposite ends. Subsequently, females underwent training for an additional 15 days with pheromonal cues and water stimulus associated with plates with specific hole sizes. During this training phase, animals were confined in both zones for 15 minutes each, alternating between zone 1 and zone 2 every 5 minutes. Memory was assessed on post-training day 15. The memory in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females were significantly reduced compared to controls, as shown by the time spent near zone 1 vs. zone 2 (Figure 3.3: Control, $p = 0.0001$, NR1^{GAD65(+/-)}, $p = 0.9492$, GluA2^{GAD65(+/-)}, $p = 0.5516$, paired two-tailed student's t-test) and the number of active attempts done by the females towards respective chambers (Figure 3.3: control, $p = 0.0003$, NR1^{GAD65(+/-)}, $p = 0.6574$, GluA2^{GAD65(+/-)}, $p = 0.0819$, paired two-tailed student's t-test). A similar amount of time spent and similar active attempts made by the animals towards both zones prove the impairment in preference towards opposite-sex pheromones for these knockout females.

To address the question of retention of memory towards pheromone locations, we examined the memory on post-training day 7. We trained a new set of females for 15 days and assessed their memory towards pheromone location on post-training 7. We observed that $NR1^{GAD65(+/-)}$ and $GluA2^{GAD65(+/-)}$ females exhibited similar time spent and active attempts towards opposite-sex pheromones, while control females showed a preference towards the male urine chamber, as indicated by increased time and active attempts. This suggests that perturbations in synaptic inhibition through GAD65-expressing neurons lead to impairment in pheromone location learning (Figure 3.4).

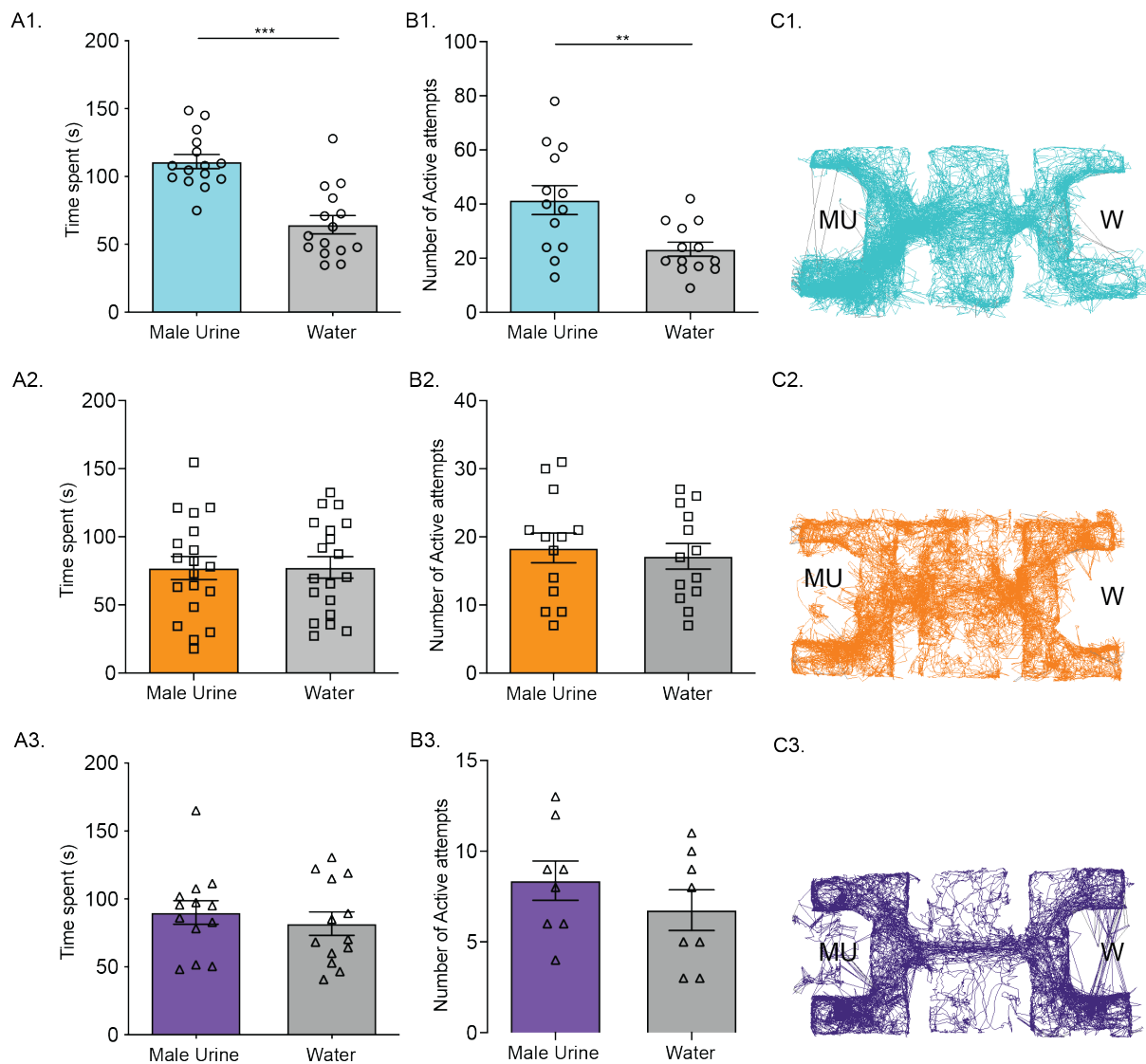


Figure 3.3: Impaired memory of pheromone locations on memory day 15

A1. Significantly longer time was spent near the male urine chamber by control females on MD 15 ($p = 0.0001$, paired two-tailed student's t-test, $N = 15$).

A2. NR1^{GAD65(+/-)} females showed similar time spent near both the chambers on MD 15 ($p = 0.9492$, paired two-tailed student's t-test, $N = 19$).

A3. GluA2^{GAD65(+/-)} females displayed similar time spent near both the chambers on MD 15 ($p = 0.5516$, paired two-tailed student's t-test, $N = 13$).

B1. Control females exhibited a greater number of active attempts to the male urine chamber on MD 15 ($p = 0.0003$, paired two-tailed student's t-test, $N = 13$).

B2. NR1^{GAD65(+/-)} females displayed similar number of active attempts to both chambers on MD 15 ($p = 0.6574$, paired two-tailed student's t-test, $N = 13$).

B3. GluA2^{GAD65(+/-)} females showed a similar number of active attempts to both chambers on MD 15 ($p = 0.0819$, paired two-tailed student's t-test, $N = 8$).

C1,C2,C3. Tracks obtained by the nose-point feature of EthoVision software (8.5 XT). MU represents the male urine chamber and W represents the water chamber.

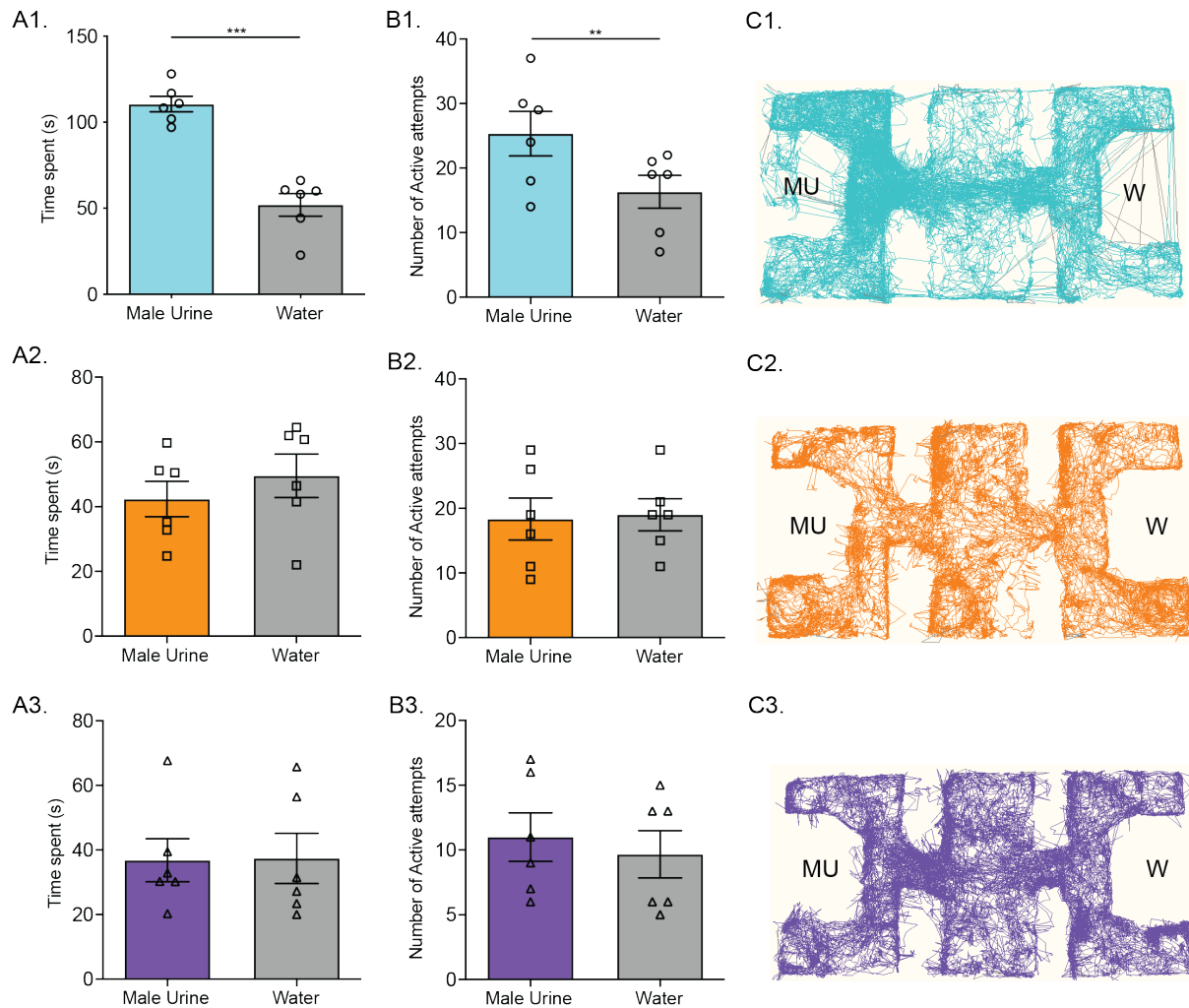


Figure 3.4: NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females showed poor multimodal pheromone memory on memory day 7

- A1. Significantly longer time was spent near male urine chamber by control females on MD 7 ($p = 0.0003$, paired two-tailed student's t-test, $N = 6$ females).
- A2. NR1^{GAD65(+/-)} females showed similar time spent near both the chambers on MD 7 ($p = 0.3655$, paired two-tailed student's t-test, $N = 6$ females).
- A3. GluA2^{GAD65(+/-)} females showed similar time spent near both the chambers on MD 15 ($p = 0.9310$, paired two-tailed student's t-test, $N = 6$ females).
- B1. Control females exhibited a greater number of active attempts to the male urine chamber on MD 7 ($p = 0.0028$, paired two-tailed student's t-test, $N = 6$ females).
- B2. NR1^{GAD65(+/-)} females displayed similar number of active attempts to both chambers on MD 7 ($p = 0.7866$, paired two-tailed student's t-test, $N = 6$ females).
- B3. GluA2^{GAD65(+/-)} females displayed a similar number of active attempts to both chambers on MD 7 ($p = 0.2213$, paired two-tailed student's t-test, $N = 6$ females).
- C1,C2,C3. Tracks obtained by the nose-point feature of EthoVision software (8.5 XT). MU represents the male urine chamber and W represents the water chamber.

3.3.4 NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals did not show any anxiety or despair-like behaviours

To assess the effect of brain-wide modification of GAD65-expressing neurons on anxiety and depression-like behaviours, a battery of tests such as EPMT, OFT, FST, and TST were performed, as detailed in Chapter 2.

Anxiety-like behaviour was further investigated by performing the Elevated-plus maze test (EPMT). The plus-shaped maze consists of a total of four arms, two open arms and two closed arms, elevated from the ground. Two parameters, time spent and number of entries in closed versus open arms, were quantified. We observed similar time spent (Figure 3.5: Ordinary one-way ANOVA, $F = 0.4558$, $p = 0.6437$, control vs NR1^{GAD65(+/-)}, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)}, and control vs GluA2^{GAD65(+/-)} $p > 0.9999$) and the number of entries in open arms (Figure 3.5: Ordinary one-way ANOVA, $F = 0.1422$, $p = 0.8688$, control vs NR1^{GAD65(+/-)}, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)}, and control vs GluA2^{GAD65(+/-)} $p > 0.9999$) vs closed arms (Figure 3.5: Ordinary one-way ANOVA, $F = 3.113$, $p = 0.0786$, control vs NR1^{GAD65(+/-)} $p = 0.3324$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} $p > 0.9999$, and control vs GluA2^{GAD65(+/-)} $p = 0.0889$) by the control group and knockout animals. This indicates that the brain-wide modification of GAD65-expressing neurons did not result in any anxiety-like behaviour in the animals.

To investigate the behavioural phenotype of the C57BL6/J mice upon heterozygously knocking down NR1 or GluA2 from GAD65-expressing neurons across the brain, we conducted a battery of tests, commonly used in the field to observe anxiety or despair-like

behaviour. We performed an open field test (OFT) to assess animal's behaviour in an open, novel arena. Animals spending more time in corners of the arena are considered anxious. Normal wild-type animals spent time exploring the arena, and frequently entered the centre zone. We observed that controls, NR1^{GAD65(+/-)}, and GluA2^{GAD65(+/-)} animals spent an equal amount of time in the centre (Figure 3.5: control vs. NR1^{GAD65(+/-)}: $p = 0.7031$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$, control vs. GluA2^{GAD65(+/-)}: $p = 0.2569$, Ordinary one-way ANOVA, Bonferroni's multiple comparison test) and corner zones (Figure 3.5: control vs. NR1^{GAD65(+/-)}: $p > 0.9999$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p = 0.5046$, control vs. GluA2^{GAD65(+/-)}: $p = 0.5745$, Ordinary one-way ANOVA, Bonferroni's multiple comparison test). Quantification was performed using EthoVision Tracking software (8.5 XT version). The actual tracks taken by the animals were plotted using the "Nose-point" feature of the Ethovision software.

The animals underwent a Forced swim test (FST, Figure 3.5) to observe any despair-like behaviour due to genetic modifications. The time spent by the animals in stationary (immobile) and non-stationary (mobile) states in water was quantified over a total duration of 10 minutes. We observed similar responses across groups, as animals in all groups showed similar immobile time (Figure 3.5: Ordinary one-way ANOVA, Bonferroni's multiple comparison test control vs. NR1^{GAD65(+/-)}: $p = 0.1219$, control vs. GluA2^{GAD65(+/-)}: $p = 0.3684$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$). Additionally, the duration for which animals showed mobility was also similar across groups (Figure: 3.2, control vs. NR1^{GAD65(+/-)}: $p = 0.1219$, control vs. GluA2^{GAD65(+/-)}: $p = 0.3684$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$).

To further confirm the absence of despair-like behaviour, a tail suspension test (TST, Figure 3.4) was conducted. During the TST, the animal was suspended upside-down, and its movements were recorded for 5 mins. The time spent by the animals in stationary versus non-stationary states was quantified. We observed similar behaviour across all groups throughout the experiment. Both the stationary (Figure 3.5: ordinary one-way ANOVA, Bonferroni's multiple comparison test, control vs. NR1^{GAD65(+/-)}, control vs. GluA2^{GAD65(+/-)} and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$) and non-stationary time spent by the animals was similar across groups (Figure 3.5: Ordinary one-way ANOVA, Bonferroni's multiple comparison test, control vs. NR1^{GAD65(+/-)}, control vs. GluA2^{GAD65(+/-)} and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$). These results indicate that the brain-wide modification of GAD65-expressing neurons did not induce anxiety or despair-like behaviour in the animals.

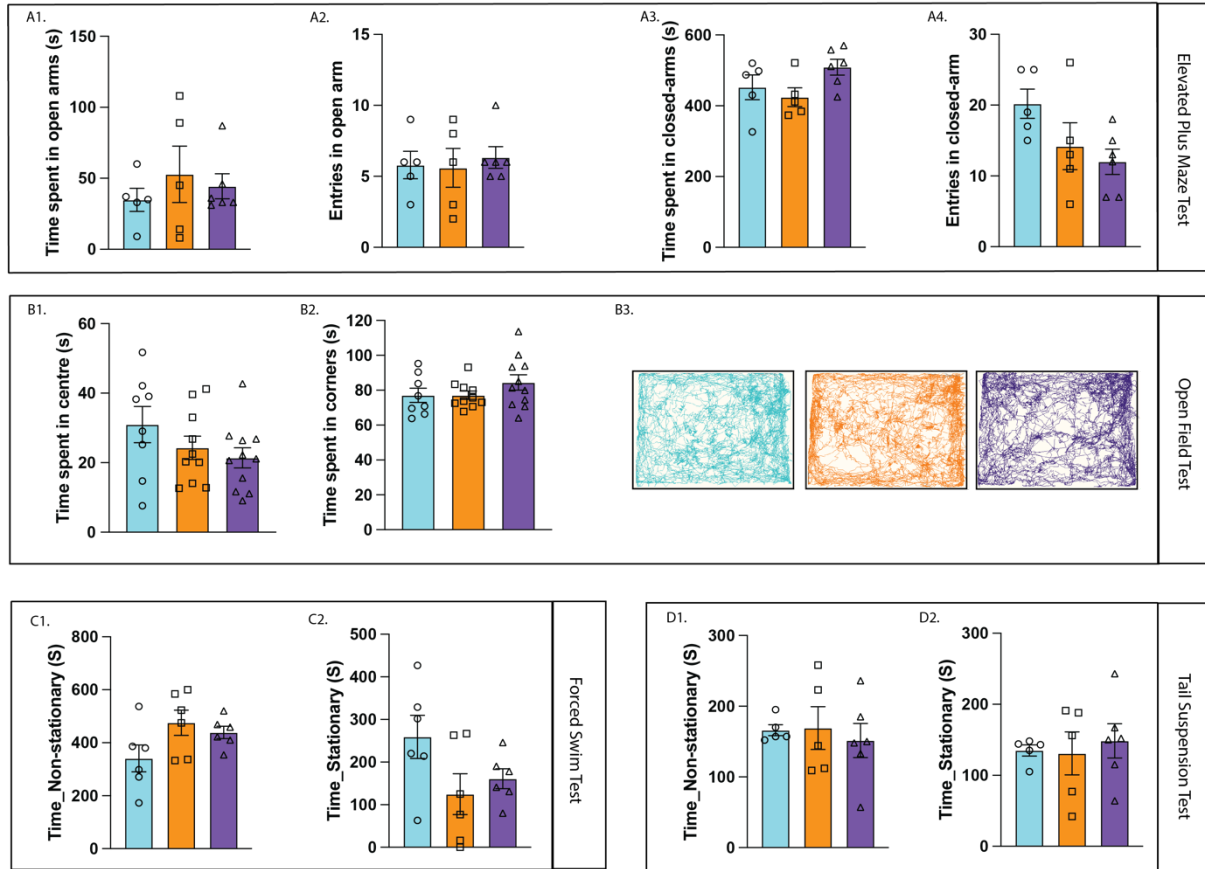


Figure 3.5: The knockouts and control animals did not display any differences in mood disorder-related behaviours

Elevated Plus Maze Test (EPMT):

A1. Time spent in the open arms among groups was similar ($p = 0.6437$, $F = 0.4558$, ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}, control vs. GluA2^{GAD65(+/-)}, and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$, control and NR1^{GAD65(+/-)} $N = 5$, GluA2^{GAD65(+/-)} $N = 6$).

A2. Time spent in the closed arms among groups was similar ($p = 0.1158$, $F = 2.556$, ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}: $p > 0.9999$, control vs. GluA2^{GAD65(+/-)} $p = 0.4935$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p = 0.1402$).

A3. Number of entries in open arms across groups was similar ($p = 0.8688$, $F = 0.1422$, ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}, control vs. GluA2^{GAD65(+/-)} and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$).

A4. Number of entries in close arms across groups was similar ($p = 0.0786$, $F = 3.113$, ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}: $p = 0.3324$, control vs. GluA2^{GAD65(+/-)} $p = 0.0889$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$).

Open Field Test (OFT):

B1. Time spent at the centre was similar across groups ($p = 0.2159$, $F = 1.627$ ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}: $p = 0.7031$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$ control vs. GluA2^{GAD65(+/-)} $p = 0.2569$, control $N = 8$, NR1^{GAD65(+/-)} $N = 10$, GluA2^{GAD65(+/-)} $N = 6$).

B2. Time spent at the corners was similar across groups ($p = 0.2853$, $F = 1.317$ Ordinary one-way ANOVA, Bonferroni's multiple comparison test, control vs. NR1^{GAD65(+/-)}: $p > 0.9999$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p = 0.5046$, control vs. GluA2^{GAD65(+/-)} $p = 0.5745$).

B3. The actual tracks were taken by the animal and analyzed using the "Nose-point" feature of EthoVision software.

Forced Swim Test (FST):

C1. There was no significant difference in the time spent mobile across groups ($p = 0.1006$, $F = 2.687$, ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}: $p = 0.1219$, control vs. GluA2^{GAD65(+/-)} $p = 0.3684$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$, control and NR1^{GAD65(+/-)} $N = 5$, GluA2^{GAD65(+/-)} $N = 6$).

C2. There was no significant difference in the time spent immobile across groups ($p = 0.1006$, $F = 2.687$, ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}: $p = 0.1219$, control vs. GluA2^{GAD65(+/-)} $p = 0.3684$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$).

Tail Suspension Test (TST):

D1. There was no significant difference in the time spent mobile across groups ($p = 0.8402$, $F = 0.1765$, ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}, control vs. GluA2^{GAD65(+/-)}, and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$, control and NR1^{GAD65(+/-)} $N = 5$, GluA2^{GAD65(+/-)} $N = 6$).

D2. There was no significant difference in the time spent immobile across groups ($p = 0.8474$, $F = 0.1677$, ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}, control vs. GluA2^{GAD65(+/-)}, and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$).

Controls are shown in cyan, NR1^{GAD65(+/-)} animals are shown in orange, and GluA2^{GAD65(+/-)} animals are shown in purple. Data are shown in mean $\pm$ SEM.

3.3.5 NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals display similar pheromone detection abilities and sniffing behaviour compared to control animals

To assess any perturbation in the pheromone detection abilities of knockout animals, a pheromone detection test (PDT) was conducted. During the PDT, the animal was allowed to explore an open arena containing male urine and soiled bedding in the centre. The time spent by the animal near the male urine was quantified using EthoVision tracking software (8.5 XT version). We observed similar time spent near the male urine across all animals tested. The actual tracks taken by the animals also followed similar trajectories (Figure 3.6: ordinary one-

way ANOVA, Bonferroni's multiple comparison test, control vs. NR1^{GAD65(+/-)}, control vs. GluA2^{GAD65(+/-)}, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$).

Animals modulate their sniffing during the presentation of odour stimuli, to gather more information (Jordan et al., 2018; P. Liu et al., 2020). To quantify the sniffing modes of both control and knockout animals, we employed a head-restrained setup and recorded sniffing frequencies towards male urine and water over four consecutive days. The sniffing frequencies during the presentation of olfactory stimuli remained similar for all groups across four days. This observation indicates that the sampling strategies employed by the KO animals were not different from those of the control group. Therefore, the lack of memory observed was not due to compromised sampling strategies (Figure 3.7: Ordinary one-way ANOVA, Bonferroni's multiple comparison test, control vs. NR1^{GAD65(+/-)}: $p = 0.7955$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p = 0.5654$, control vs. GluA2^{GAD65(+/-)}: $p > 0.9999$).

These findings demonstrate that the modification of GAD65-expressing interneurons does not lead to alterations in pheromone detection abilities and sniffing strategies of the animals.

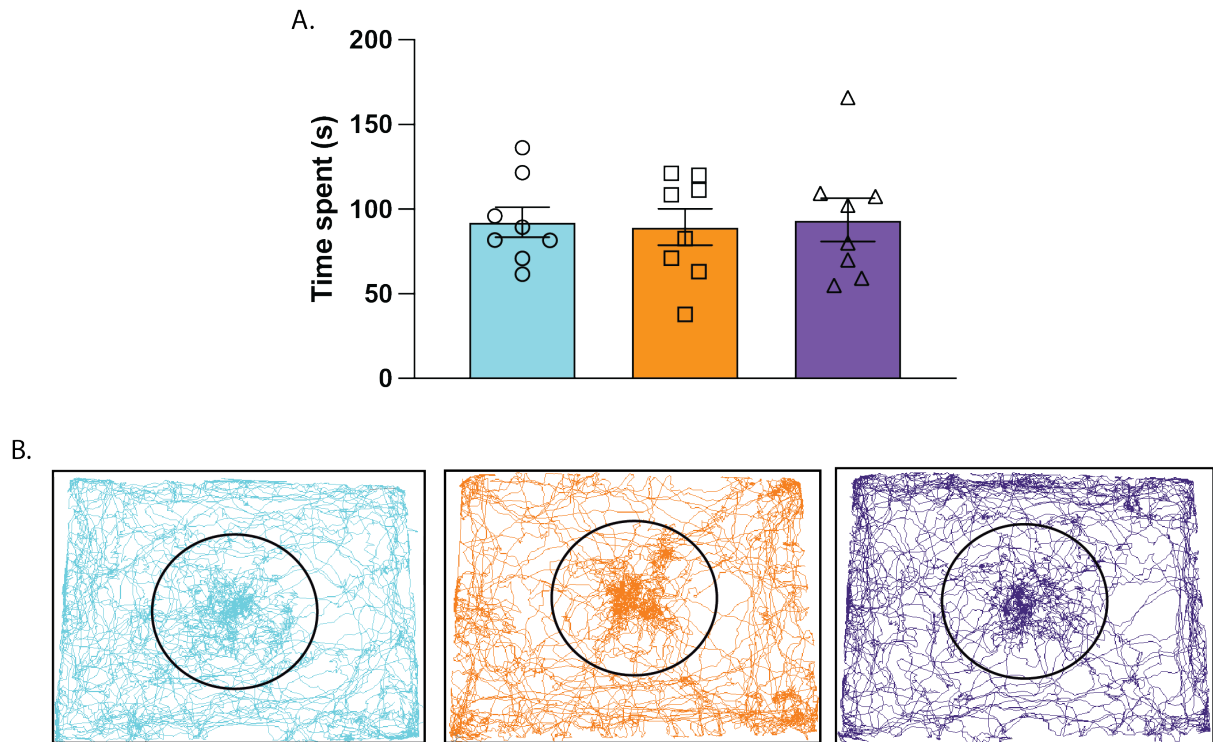


Figure 3.6: Intact pheromone detection abilities of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females

A. Time spent near the male urine was similar across groups ($p = 0.9626$, $F = 0.03817$ Ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}, control vs. GluA2^{GAD65(+/-)}, and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$, $N = 8$).

B. The actual tracks were taken by the animal and analyzed using the “Nose-point” feature of EthoVision software. Tracks are measured for the entire 10-minute duration. As seen by the tracks, females spent similar time near petri-dish containing male urine and soiled bedding shown in black circles.

Controls are shown in cyan, NR1^{GAD65(+/-)} animals are shown in orange, and GluA2^{GAD65(+/-)} animals are shown in purple. Data are shown in mean $\pm$ SEM.

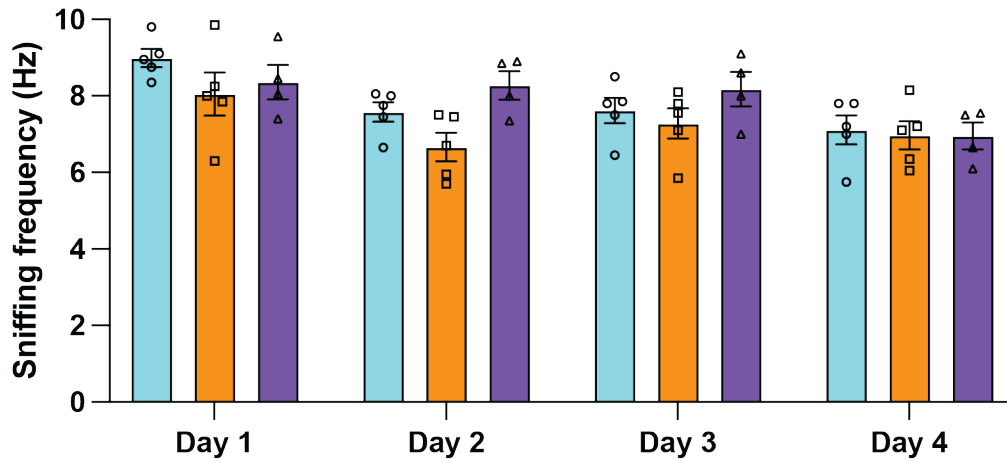


Figure 3.7: Similar sniffing frequency towards male urine displayed by NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females

Sniffing frequency was similar across four days in all the groups. ($p = 0.3552$, $F = 1.164$, Ordinary one-way ANOVA, Bonferroni's multiple comparison tests, control vs. NR1^{GAD65(+/-)}: $p = 0.7955$, NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p = 0.5654$, control vs. GluA2^{GAD65(+/-)} $p > 0.9999$, control, NR1^{GAD65(+/-)} $N = 5$, GluA2^{GAD65(+/-)} $N = 4$). Controls are shown in cyan, NR1^{GAD65(+/-)} animals are shown in orange, and GluA2^{GAD65(+/-)} animals are shown in purple. Data are shown in mean $\pm$ SEM.

3.3.6 Reduced Arc expression in Olfactory Bulb, Dentate Gyrus and Somatosensory cortex on Memory Day 15

As established in the previous section, the formation of pheromonal location memory in our paradigm required both whiskers and nose to associate hole size with the pheromonal cues. To investigate the brain regions involved during memory recalls, we quantified the expression of the Immediate Early Gene (IEG) protein marker, Activity-regulated cytoskeleton (Arc), on memory days. Previously, we demonstrated differential expression of Arc in brain regions such as the OB, DG, and SSC on MD 15 between the whisker-intact and whisker-deprived groups (Pardasani et al., 2021).

To support our behavioural findings at the molecular level, we examined the expression of Arc, a marker of long-term plasticity, in the MOB, AOB, DG and SSC regions. We found that the reduced memory for opposite-sex pheromones in the multimodal pheromonal place preference assay is associated with decreased Arc expression in the MOB, AOB, DG and SSC regions of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females compared to controls (Figure 3.8: Ordinary one-way ANOVA, $p < 0.05$ for all brain regions). This notable difference in Arc expression among control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females indicates impaired acquisition, retrieval or retention of multimodal pheromonal memory in the NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females.

We examined Arc expression on memory day 7 in a new set of animals and found reduced levels in the MOB, AOB, DG and SSC regions of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females compared to control females (Figure 3.9: Ordinary one-way ANOVA, $p < 0.05$ for all brain regions). The decreased expression of Arc in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} female brains ($N = 3$ each group) strongly correlates with the loss of memory towards the male urine chamber on MD 7 and MD 15.

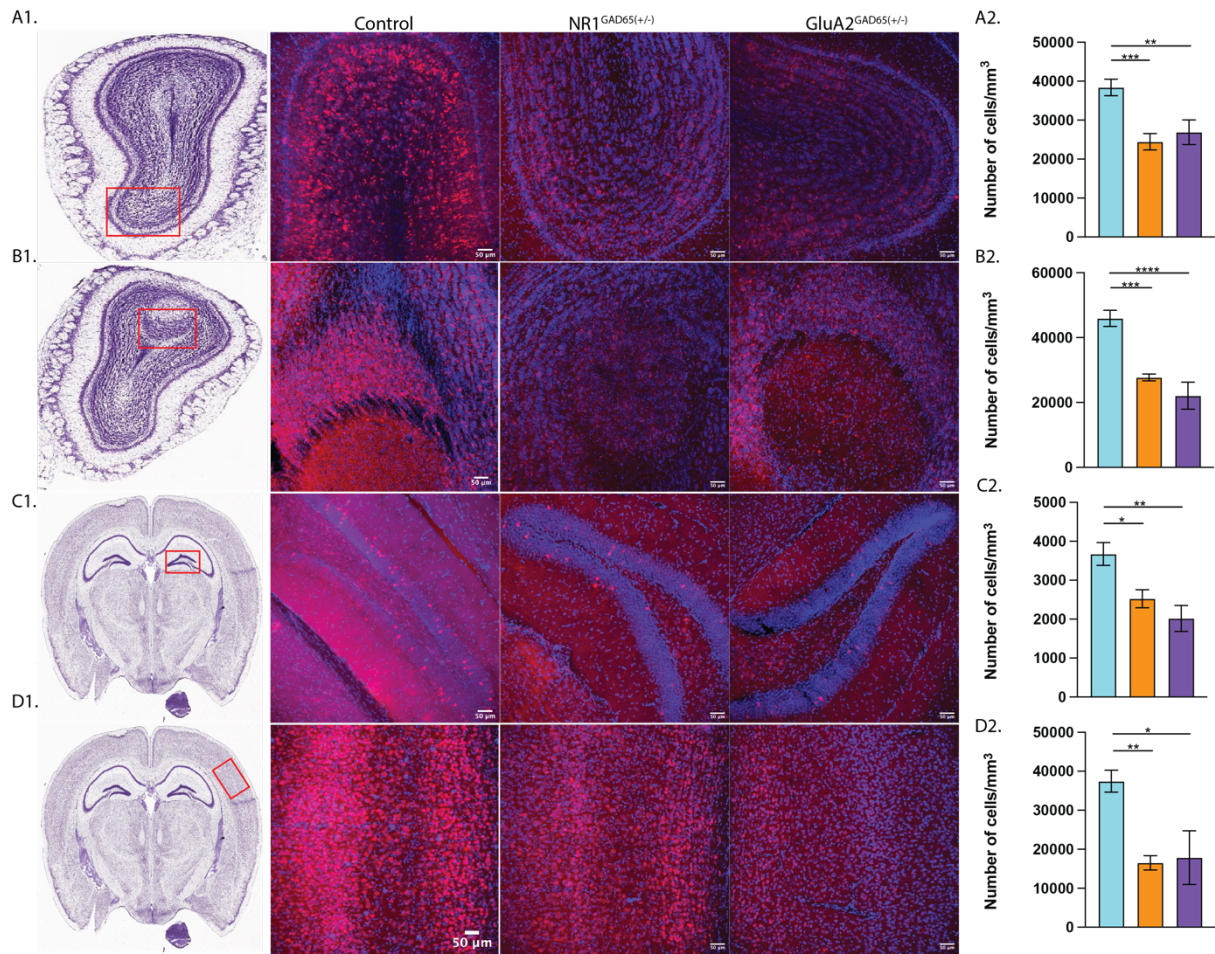


Figure 3.8: Lower Arc expression in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females on memory day 15

A1. Immunofluorescence images of MOB on MD15 of control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)}. (Blue: DAPI, Red: Arc, Scale bar: 50 μ m).

A2. Significantly lower number of Arc positive cells in MOB of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals ($p = 0.0002$, $F = 10.64$, Ordinary one-way ANOVA, Bonferroni's multiple comparisons test, Control vs NR1^{GAD65(+/-)}: $p = 0.0004$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)}: $p > 0.9999$, Control vs GluA2^{GAD65(+/-)}: $p = 0.0062$, $N = 3$).

B1. Immunofluorescence images of AOB on MD15 of control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)}. Significantly lower number of Arc positive cells in AOB of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} heterozygous knockout animals ($p < 0.0001$, $F = 16.52$, Ordinary one-way ANOVA, Bonferroni's multiple comparisons test, Control vs NR1^{GAD65(+/-)}: $p = 0.0007$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)}: $p = 0.5658$, Control vs GluA2^{GAD65(+/-)}: $p < 0.0001$, $N = 3$).

C1. Immunofluorescence images of hippocampus on MD15 of control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)}.

C2. Significantly lower number of Arc positive cells in Dentate Gyrus of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} heterozygous knockout animals ($p = 0.0016$, $F = 8.376$, Ordinary one-way ANOVA,

Bonferroni's multiple comparisons test, Control vs NR1^{GAD65(+/-)} : $p = 0.0352$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} : $p = 0.6882$, Control vs GluA2^{GAD65(+/-)} : $p = 0.0014$, $N = 3$).

D1. Immunofluorescence images of SSC on MD15 of control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)}.

D2. Significantly lower number of Arc positive cells in SSC of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} heterozygous knockout animals ($p = 0.0027$, $F = 7.562$, Ordinary one-way ANOVA, Bonferroni's multiple comparisons test, Control vs NR1^{GAD65(+/-)} : $p = 0.0061$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} : $p > 0.9999$, Control vs GluA2^{GAD65(+/-)} : $p = 0.0105$, $N = 3$).

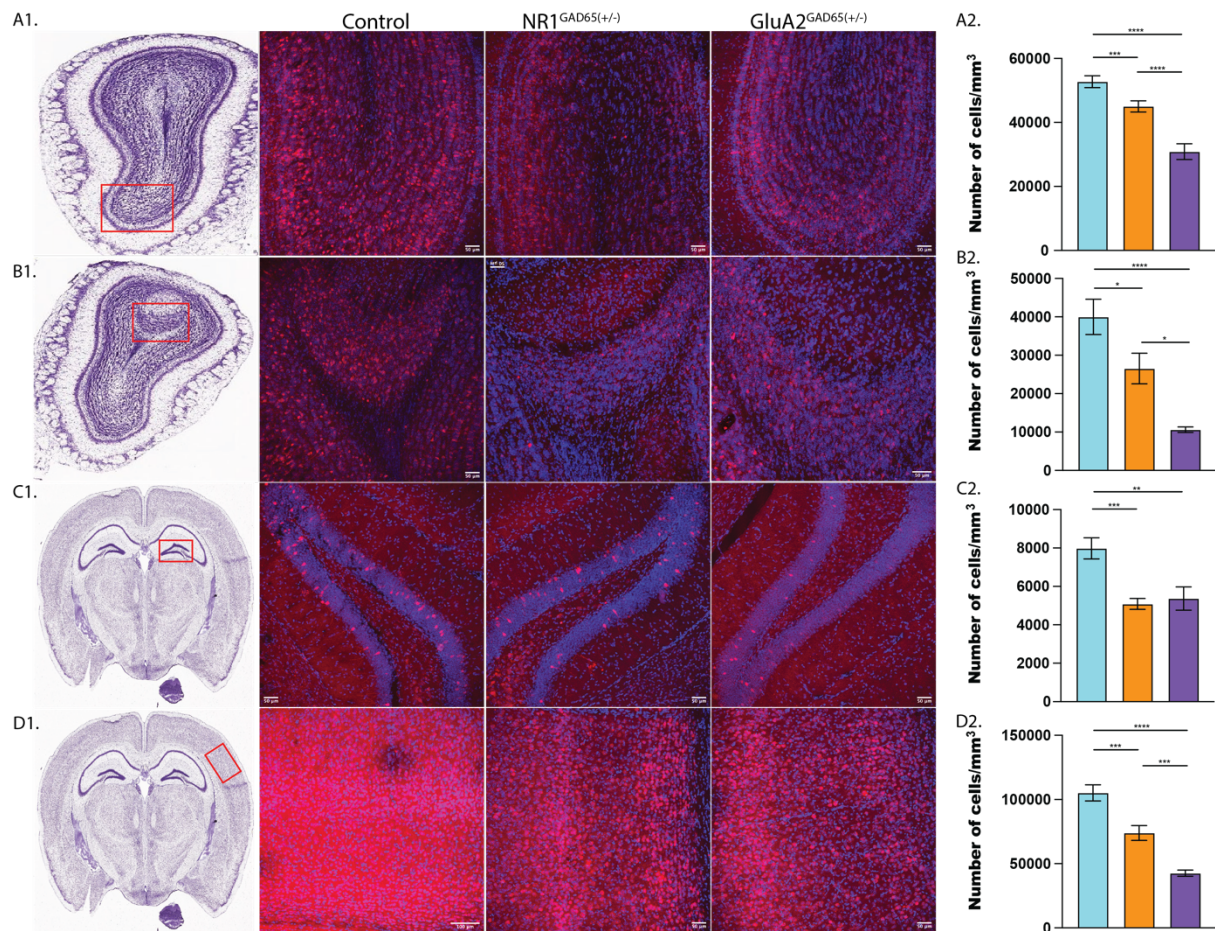


Figure 3.9: Lower Arc expression in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females on memory day 7

A1. Immunofluorescence images of MOB on MD07 of control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)}. (Blue: DAPI, Red: Arc, Scale bar: 50 μ m).

A2. Significantly lower number of Arc positive cells in MOB of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} heterozygous knockout animals ($p < 0.0001$, $F = 29.77$, Ordinary one-way ANOVA, Bonferroni's multiple comparisons test, Control vs NR1^{GAD65(+/-)} : $p = 0.0283$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} : $p < 0.0001$, Control vs GluA2^{GAD65(+/-)} : $p < 0.0001$, $N = 3$).

- B1. Immunofluorescence images of AOB on MD07 of control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females.
- B2. Significantly lower number of Arc positive cells in AOB of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} heterozygous knockout animals ($p < 0.0001$, $F = 14.21$, Ordinary one-way ANOVA, Bonferroni's multiple comparisons test, Control vs NR1^{GAD65(+/-)}: $p = 0.0476$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)}: $p = 0.0235$, Control vs GluA2^{GAD65(+/-)}: $p < 0.0001$, $N = 3$).
- C1. Immunofluorescence images of hippocampus on MD07 of control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)}).
- C2. Significantly lower number of Arc positive cells in the hippocampus of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} heterozygous knockout animals ($p = 0.0002$, $F = 11.55$, Ordinary one-way ANOVA, Bonferroni's multiple comparisons test, Control vs NR1^{GAD65(+/-)}: $p = 0.0004$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)}: $p > 0.9999$, Control vs GluA2^{GAD65(+/-)}: $p = 0.0032$, $N = 3$).
- D1. Immunofluorescence images of SSC on MD07 of control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)}.
- D2. Significantly lower number of Arc positive cells in SSC of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} heterozygous knockout animals ($p < 0.0001$, $F = 35.39$, Ordinary one-way ANOVA, Bonferroni's multiple comparisons test, Control vs NR1^{GAD65(+/-)}: $p = 0.0006$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)}: $p = 0.0002$, Control vs GluA2^{GAD65(+/-)}: $p < 0.0001$, $N = 3$).

3.3.7 Lack of memory for pheromone locations during early recall periods

To examine the pheromonal memory at very early time points, we trained a separate batch of animals and tested their memory on post-training day 1 and day 5. We observed that the NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females spent significantly less time near the male urine chamber compared to control females (Figure 3.10: MD 1: Ordinary one-way ANOVA, $p = 0.0079$, $F = 7.183$, control vs. NR1^{GAD65(+/-)}: $p = 0.0187$, control vs. GluA2^{GAD65(+/-)}: $p = 0.0155$ and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$, MD 5: Ordinary one-way ANOVA, $p = 0.0146$, $F = 5.807$, control vs. NR1^{GAD65(+/-)}: $p = 0.0285$, control vs. GluA2^{GAD65(+/-)}: $p = 0.0293$ and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$). Even during these earlier memory days, the multimodal pheromonal location memory was lacking in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females, hinting towards the impairment in the multimodal learning of pheromone locations.

We then proceeded to investigate the behavioural and molecular changes occurring during the training phase.

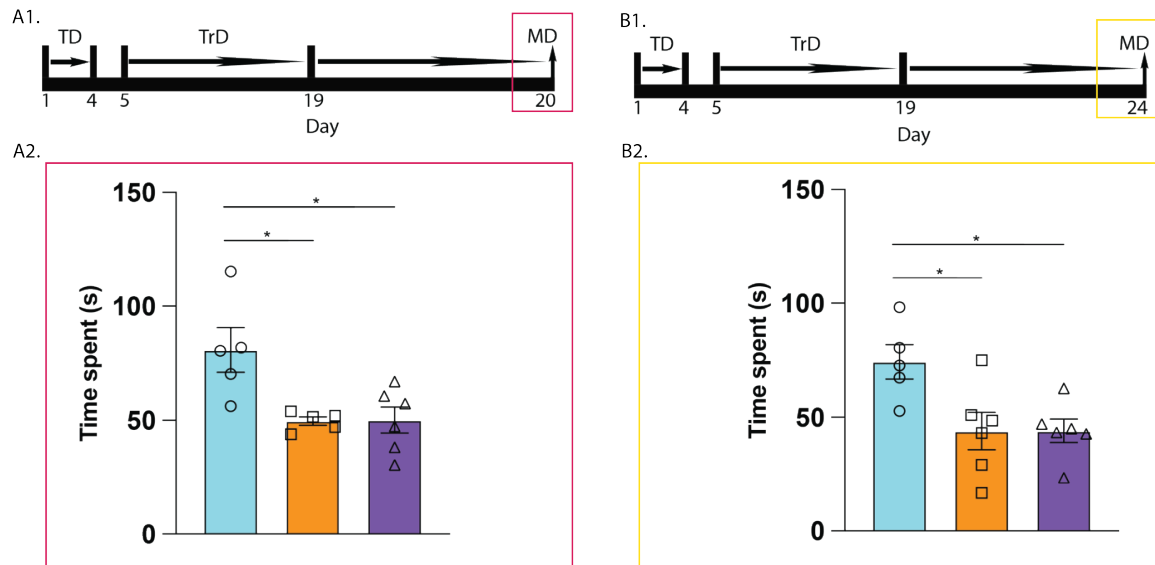


Figure 3.10: Lack of memory for pheromone locations on memory day 1 and day 5

- A. Lower time spent towards the male urine chamber was seen on memory day 1 (Ordinary one-way ANOVA, $p = 0.0079$, $F = 7.183$, control vs. NR1^{GAD65(+/-)}: $p = 0.0187$, control vs. GluA2^{GAD65(+/-)}: $p = 0.0155$ and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$).
- B. On memory day 5, the KOs spent similar time towards both the chambers (Ordinary one-way ANOVA, $p = 0.0146$, $F = 5.807$, control vs. NR1^{GAD65(+/-)}: $p = 0.0285$, control vs. GluA2^{GAD65(+/-)}: $p = 0.0293$ and NR1^{GAD65(+/-)} vs. GluA2^{GAD65(+/-)}: $p > 0.9999$).

3.3.8 Higher c-fos expression in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females on training day 15

As NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females exhibited a lack of memory towards pheromone locations during both late and early recall periods, our subsequent focus was on observing their behaviour during the training phase (TrP). We measured the time spent and the active attempts made by the animals towards the male urine chamber on TrD1, 7, and 15. Since, c-fos, an IEG, serves as a proxy for neuronal activity during learning, we also assessed the expression of c-fos on TrD15 across the groups in different brain regions.

We quantified the behaviour of animals during training days by measuring time spent and active attempts made on training days 1, 7 and 15. Surprisingly, we observed similar sampling behaviour exhibited by the animals across all three groups (Figure 3.11: Two-way ANOVA, $F(2,30) = 0.1240$, $p = 0.8838$, Bonferroni's multiple comparisons, $p > 0.05$ for all days). However, when assessed c-Fos expression on the final training, TrD 15, we found

significantly higher levels of c-Fos in the DG of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} mice compared to controls (Figure 3.11: Ordinary one-way ANOVA, Bonferroni's multiple comparisons, control vs NR1^{GAD65(+/-)} $p = 0.0383$, control vs GluA2^{GAD65(+/-)} $p = 0.0231$). The levels of c-fos in the OB and SSC were similar across all groups ($p > 0.05$). It is well established that c-fos expression tends to be higher during exposure to novel odours compared to familiar odours (Fredericksen et al., 2019). The increased c-fos expression in the DG of TrD15 suggests a lack of odour familiarity in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} female mice compared to controls, indicating difficulty in associating the odour with the hole size of the plates.

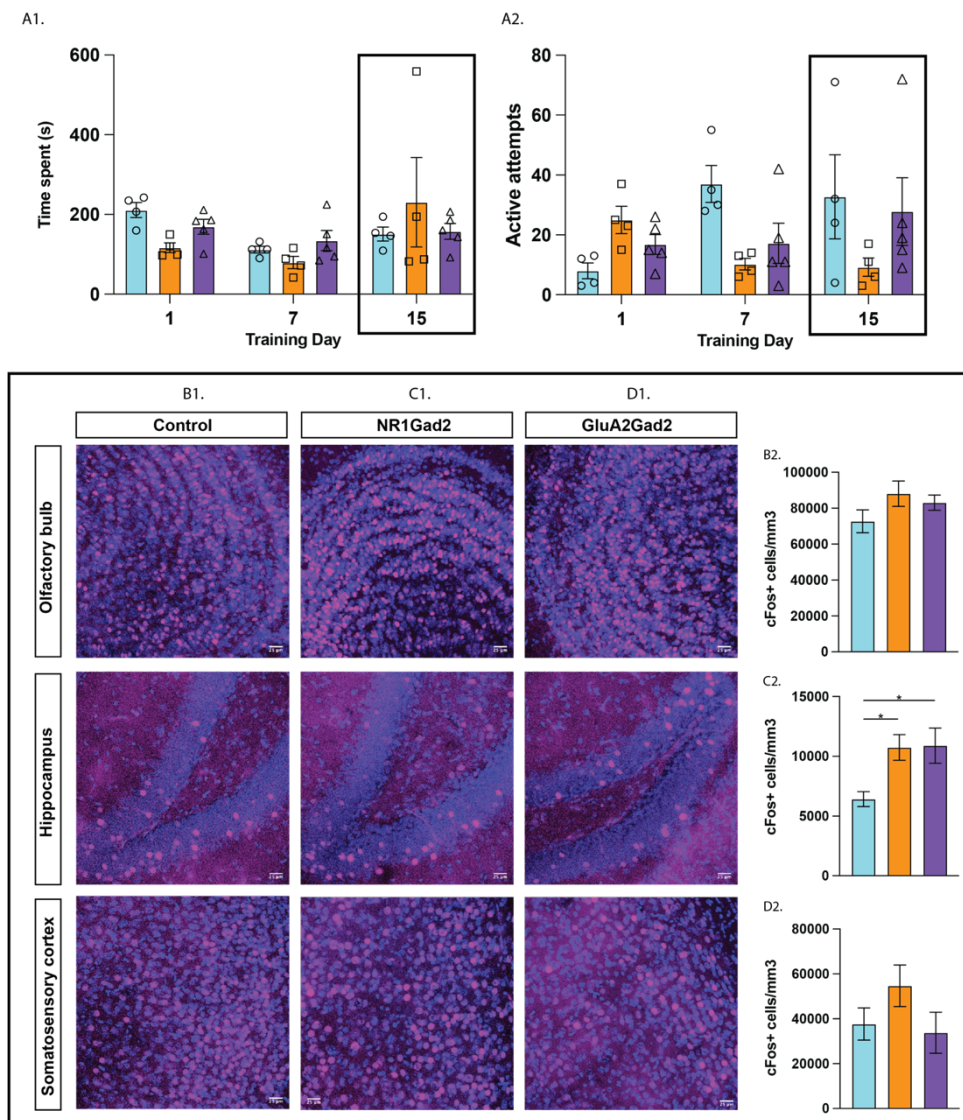


Figure 3.11: Higher c-fos expression in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females on training day 15

A1,A2. Similar sampling pattern shown by animals across groups on TrD1, 7 and 15 (Two-way ANOVA, Bonferroni's multiple comparison test, row factor = 13.71, column factor = 0.5963 for time spent and row factor = 2.862, column factor = 6.892).

B1,C1,D1. Immunofluorescence images of OB, DG and barrel cortical layers (SSC) on TrD15 in control females, NR1^{GAD65(+/-)} females, and GluA2^{GAD65(+/-)} females (Blue: DAPI, Magenta: c-Fos, scale bar = 25 μ m).

B2,C2,D2. Similar expression of c-Fos in OB, DG and SSC (One-way ANOVA, $p > 0.05$ for all brain regions).

3.3.9 Main and Accessory Olfactory Bulb show neural activity upon male urine exposure

Male urine contains a vast array of molecules that play a crucial role in intraspecific chemical communications among rodents. The Major Urinary Protein (MUP) present in male urine has been shown to influence various aspects of female sexual behaviour. Studies have indicated that MUP can impact puberty onset (Mucignat-Caretta et al., 1995), ovulation (Morè, 2006), and the estrus cycle (Marchlewska-Koj et al., 2000) in female rodents.

To investigate the regions within OB that exhibit neural activity upon exposure to male urine, we conducted an experiment where female mice were exposed to male urine and soiled bedding for 10 minutes. Subsequently, we performed staining for c-fos expression in the OB. Our findings revealed c-fos expression not only in the AOB but also in various layers of the MOB, including GL and GCL, as depicted in Figure 3.12.

In our next phase of experiments, we modulated the microcircuitry within OB and AOB using a stereotaxic viral injection approach, as outlined in Chapter 2. This approach allowed us to gain further insights into how specific neural circuits within the OB contribute to multimodal learning of pheromone locations.

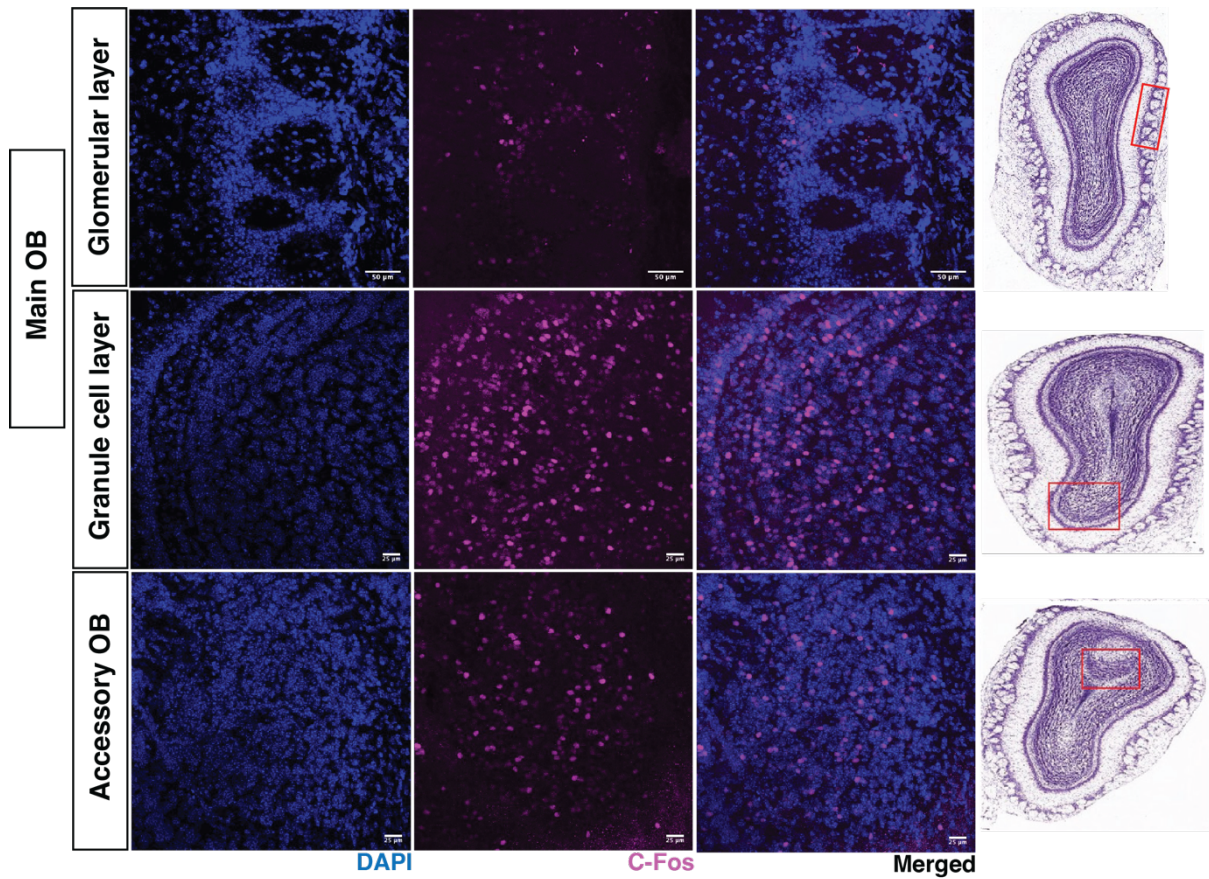


Figure 3.12: c.Fos expression in MOB and AOB of a female brain on male urine exposure

Immunofluorescence images showing c-fos expression in different regions of the main OB (glomerular layer and granule cell layer) and accessory OB. (Blue: DAPI, Magenta: c-Fos, scale bar = 25 μ m).

3.3.10 Ionotropic glutamate receptors in the Olfactory Bulb are crucial for pheromonal location memory

Stereotaxic injections of AAV 5-Cre viral particles were performed in the GCL of the MOB of NR1^{2Lox} and GluA2^{2Lox} animals. The AAV-Cre gene was fused with a reporter gene, EGFP, to track the expression of EGFP and confirm the location of the viral injection site. Following the surgery, animals were allowed a recovery period of three weeks with ad libitum access to food and water. The detailed experimental procedure is outlined in Chapter 2. Before conducting the multimodal pheromone place preference assay, the mice were subjected to a pheromone detection assay. We evaluated their pheromone detection abilities and compared them to the control group. The results showed similar pheromone detection abilities in the females that had undergone surgery compared to the control, shown by time spent near the petri plate containing male urine and soiled bedding (Figure 3.13: Ordinary one-way ANOVA, $p =$

0.6247, $F = 0.4811$, Bonferroni's multiple comparison tests, control vs. NR1^{ΔOB}, control vs. GluA2^{ΔOB}, and NR1^{ΔOB} vs. GluA2^{ΔOB}: $p > 0.9999$).

After confirming intact pheromone detection abilities, we proceeded with the multimodal pheromone place preference assay and assessed the memory on post-training days 7 and 15. The memory performance of NR1^{ΔOB} and GluA2^{ΔOB} females was significantly reduced compared to controls, as evidenced by time spent near zone 1 versus zone 2 (Figure 3.14: Paired two-tailed student's t-test control, $p = 0.0073$, NR1^{ΔOB}, $p = 0.1167$, GluA2^{ΔOB}, $p = 0.9689$) and the number of active attempts done by the females towards respective chambers (Figure 3.14: Control, $p = 0.0039$, NR1^{ΔOB}, $p > 0.9999$, GluA2^{ΔOB}, $p = 0.4531$). The similarity in time spent and similar active attempts towards both zones indicates an impairment in preference towards opposite-sex pheromones for these female mice. These results underscore the active role of the OB circuitry in the multimodal learning of pheromone locations.

The memory was also evaluated on post-training day 15, yielding similar results. The memory performance of NR1^{ΔOB} and GluA2^{ΔOB} females remained significantly reduced compared to controls, as indicated by the time spent near zone 1 versus zone 2 (Figure 3.15: Paired two-tailed student's t-test, control, $p = 0.0001$, NR1^{ΔOB}, $p = 0.4677$, GluA2^{ΔOB}, $p = 0.9689$.) and the number of active attempts made by the females towards respective chambers (Figure 3.15: Paired two-tailed student's t-test control, $p = 0.0039$, NR1^{ΔOB}, $p = 0.5625$, GluA2^{ΔOB}, $p > 0.9999$).

The neural plasticity was assessed on post-training day 15 by quantifying the expression of Arc in sham, NR1^{ΔOB}, and GluA2^{ΔOB} females using immunohistochemistry techniques outlined in Chapter 2. Briefly, animals were sacrificed within 30 minutes after completing the task on memory day 15. thin sections of 50 μm were obtained using the cryostat and stained with an Arc antibody. Imaging was conducted using Leica SP8 confocal imaging, focusing on the OB, DG and SSC brain regions. Our findings revealed a significant decrease in Arc expression in the OB of NR1^{ΔOB} females compared to control and GluA2^{ΔOB} females (Figure 3.16: Ordinary one-way ANOVA, Bonferroni's multiple comparisons test, control vs. NR1^{ΔOB} $p < 0.0001$, NR1^{ΔOB} vs. GluA2^{ΔOB} $p = 0.0002$). Arc expression in control and GluA2^{ΔOB} females was similar (Control vs. GluA2^{ΔOB} $p = 0.4217$). In SSC, NR1^{ΔOB} females exhibited increased Arc expression compared to control and GluA2^{ΔOB} (Control vs. NR1^{ΔOB} $p = 0.2142$, NR1^{ΔOB} vs. GluA2^{ΔOB} $p < 0.0001$). Conversely, GluA2^{ΔOB} animals displayed significantly lower arc expression in SSC compared to controls (Control vs. GluA2^{ΔOB} $p = 0.0025$). Interestingly, DG showed reduced Arc expression in NR1^{ΔOB} and GluA2^{ΔOB} females compared

to controls (Control vs. NR1^{ΔOB} $p = 0.0137$, control vs. GluA2^{ΔOB} $p = 0.0025$, NR1^{ΔOB} vs. GluA2^{ΔOB} $p = 0.3236$).

Given that the hippocampus is a site for memory and Arc serves as a proxy for neural plasticity, the decreased Arc expression in DG of NR1^{ΔOB} and GluA2^{ΔOB} females aligns with the observed lack of memory towards pheromonal locations. These results suggest the involvement of ionotropic glutamate receptors of the mouse OB in facilitating multimodal learning of pheromone locations.

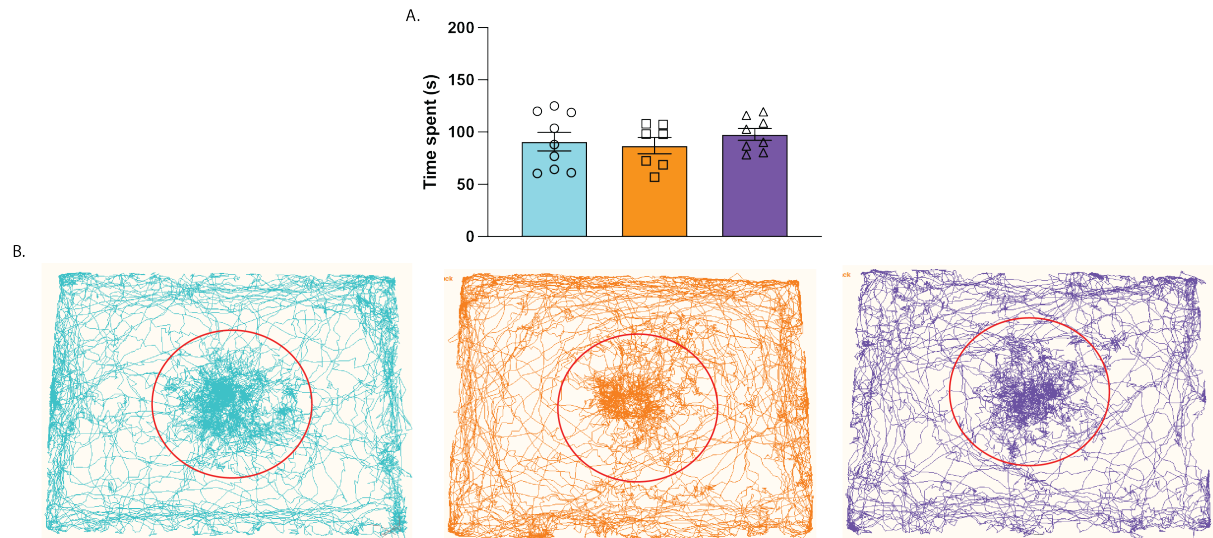


Figure 3.13: No impairment in pheromone detection abilities of NR1^{ΔOB} and GluA2^{ΔOB} females

- A. Similar time was spent by the females across groups (Ordinary one-way ANOVA, $p = 0.6247$, $F = 0.4811$, Bonferroni's multiple comparison tests, control vs. NR1^{ΔOB}, control vs. GluA2^{ΔOB}, and NR1^{ΔOB} vs. GluA2^{ΔOB}: $p > 0.9999$). Data are shown in mean $\pm$ SEM. (Control $N = 9$, NR1^{ΔOB} $N = 7$, GluA2^{ΔOB} $N = 8$).
- B. The actual tracks were taken by the animal and analyzed using the "Nose-point" feature of EthoVision software. As seen by the tracks, females spent similar time near petri-dish containing male urine and soiled bedding shown in a red circle.

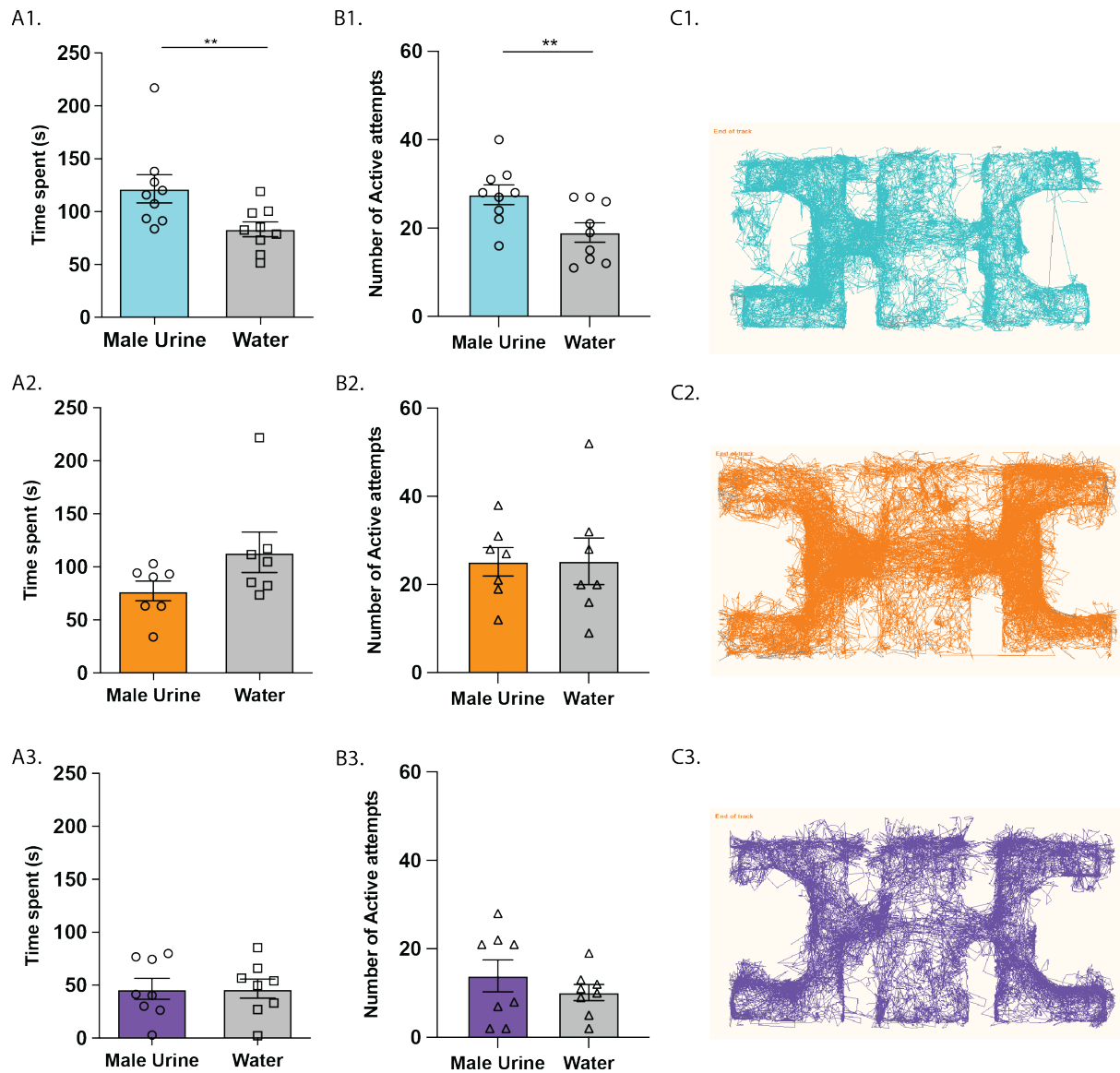


Figure 3.14: Lack of multimodal learning revealed in a pheromone preference assay demonstrated by NR1A^{OB} and GluA2A^{OB} females on memory day 7

- A1. Control females spent significantly more time near the male urine chamber on Memory Day 7 ($p = 0.0073$, paired two-tailed student's t-test, $N = 9$).
- A2. A similar amount of time was spent near the male urine chamber and water chamber on MD 7 by NR1A^{OB} females ($p = 0.1167$, paired two-tailed student's t-test, $N = 7$).
- A3. A similar amount of time was spent near the male urine chamber and water chamber on MD 7 by GluA2A^{OB} females ($p = 0.9689$, paired two-tailed student's t-test, $N = 8$).
- B1. Higher number of active attempts on MD 7 by control females (paired two-tailed student's t-test, $p = 0.0039$, $N = 9$).
- B2. Similar number of active attempts by NR1A^{OB} females on MD 7 (paired two-tailed student's t-test, $p > 0.9999$, $N = 7$).

B3. Similar number of active attempts by GluA2^{ΔOB} females on MD 7 (paired two-tailed student's t-test, $p = 0.4531$, $N = 08$ females).

C1,C2,C3. The actual tracks were taken by the animal and analyzed using the “Nose-point” feature of EthoVision software.

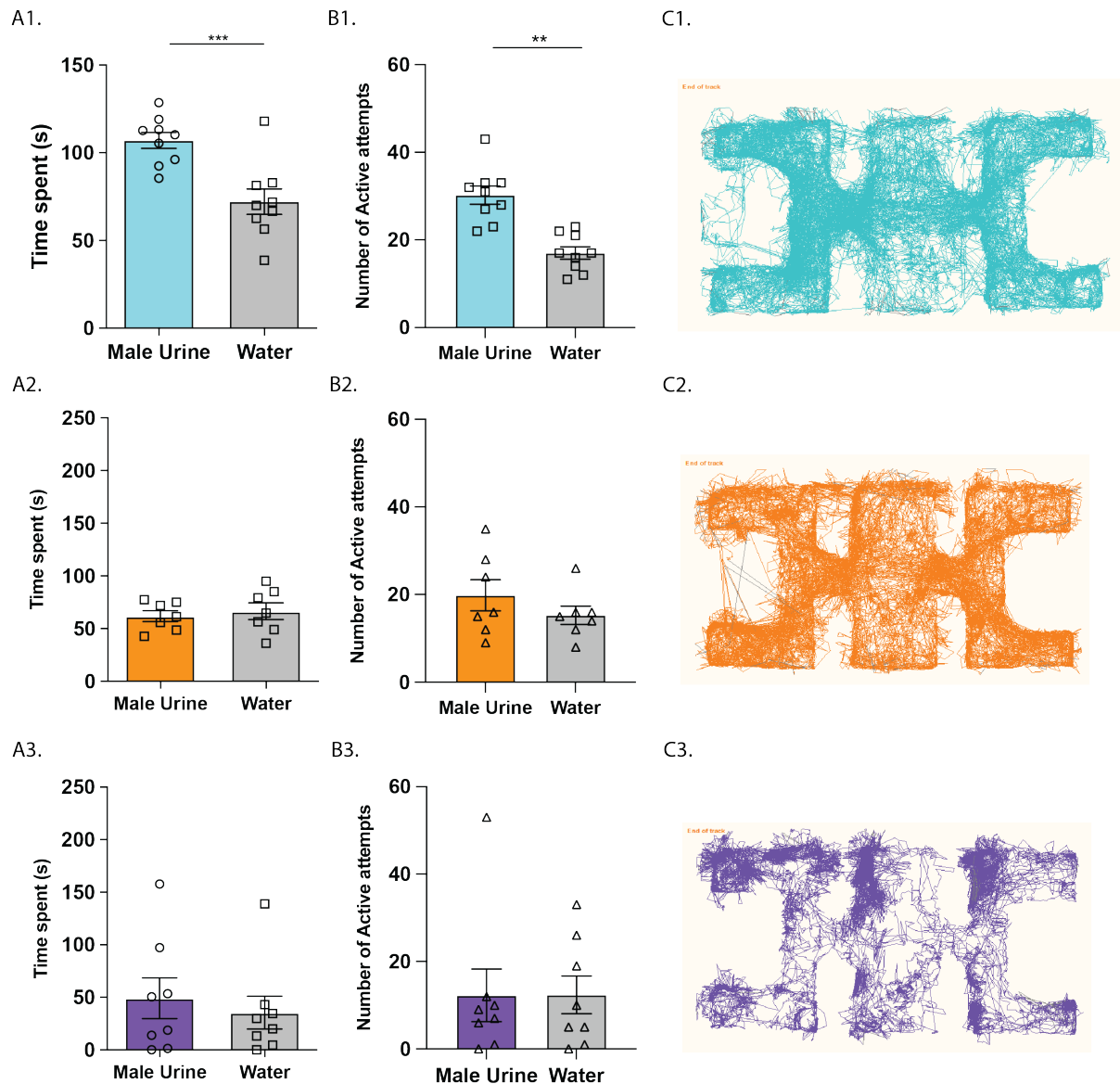


Figure 3.15: Similar sampling pattern displayed by NR1^{ΔOB} and GluA2^{ΔOB} females towards male urine and water chamber on memory day 15

A1. Significantly more time was spent near the male urine chamber by control females in MD 15 (paired two-tailed student's t-test, $p = 0.0001$, $N = 9$).

A2. NR1^{ΔOB} females exhibited memory impairment, as they spent a similar amount of time near both chambers on MD 15 (paired two-tailed student's t-test, $p = 0.4677$, $N = 7$).

- A3. GluA2^{ΔOB} females displayed a lack of memory by spending similar time near both chambers on MD 15 (paired two-tailed student's t-test, $p = 0.9689$, $N = 8$).
- B1. Higher number of active attempts was made by control females on MD 15 ($p = 0.0039$, paired two-tailed student's t-test, $N = 9$).
- B2. NR1^{ΔOB} females performed a similar number of active attempts towards both chambers on MD 15 (paired two-tailed student's t-test, $p = 0.5625$, $N = 7$).
- B3. GluA2^{ΔOB} females performed a comparable number of active attempts towards both chambers on MD 15 (paired two-tailed student's t-test, $p > 0.9999$, $N = 8$).
- C1,C2,C3. The actual tracks were taken by the animal and analyzed using the “Nose-point” feature of EthoVision software.

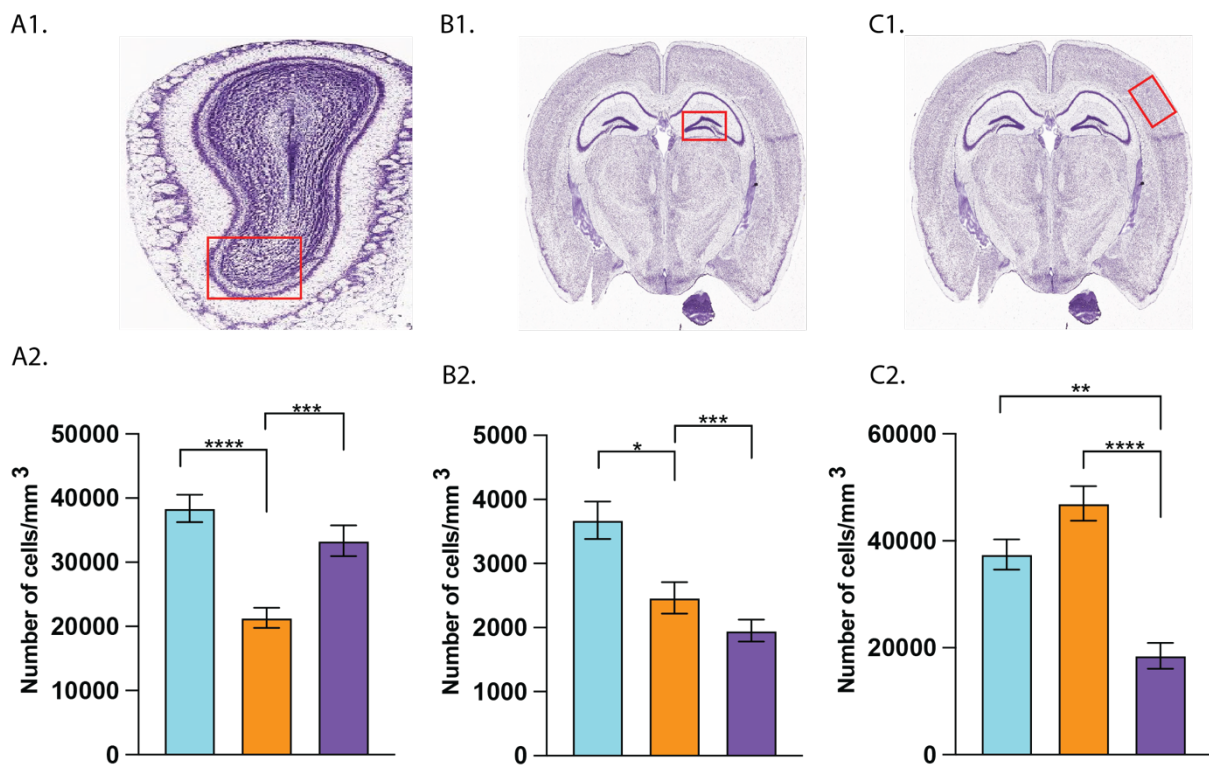


Figure 3.16: Arc expression in OB, DG and SSC on memory day 15

- A. A1. Atlas map images of Nissl staining taken from the Allen mouse brain atlas depicting MOB. The red box demonstrates the regions used for quantification of Arc expression. A2. Lower expression of Arc was seen in NR1^{ΔOB} females compared to sham and GluA2^{ΔOB} females (Ordinary one-way ANOVA, $p < 0.0001$, $F = 21.53$, Bonferroni's multiple comparisons test, Sham vs NR1^{ΔOB}: $p < 0.0001$, NR1^{ΔOB} vs GluA2^{ΔOB}: $p = 0.0002$, Sham vs GluA2^{ΔOB}: $p = 0.4217$, $N = 3$).
- B. B1. Atlas map images of Nissl staining taken from the Allen mouse brain atlas depicting DG. B2. Lower expression of Arc was seen in NR1^{ΔOB} and GluA2^{ΔOB} females compared to sham animals

(Ordinary one-way ANOVA, $p = 0.0008$, $F = 8.170$, Bonferroni's multiple comparisons test, Sham vs NR1 Δ^{AOB} : $p = 0.0137$, NR1 Δ^{AOB} vs GluA2 Δ^{AOB} : $p = 0.3236$, Sham vs GluA2 Δ^{AOB} : $p = 0.0005$, $N = 3$).

C. C1. Atlas map images of Nissl staining taken from the Allen mouse brain atlas depicting SSC. C2. Lower expression of Arc was seen in GluA2 Δ^{AOB} females compared to sham and NR1 Δ^{AOB} females (Ordinary one-way ANOVA, Bonferroni's multiple comparisons test, $p < 0.0001$, $F = 25.42$, Sham vs NR1 Δ^{AOB} : $p = 0.2142$, NR1 Δ^{AOB} vs GluA2 Δ^{AOB} : $p < 0.0001$, Sham vs GluA2 Δ^{AOB} : $p = 0.0025$, $N = 3$).

3.3.11 Ionotropic glutamate receptors of accessory olfactory bulb are crucial for pheromonal location memory

Stereotaxic injections of AAV5-Cre viral particles were conducted in the internal cell layer (ICL) of the AOB in NR1-Lox and GluA2-Lox animals. The ICL comprises granule cells known as internal granule cells (IGCs), which abundantly express GAD65-isoform (Maksimova et al., 2019). These viral particles were fused with EGFP as a reporter fluorophore to confirm the location and extent of the infection. Following the surgical procedure, the animals were allowed a recovery period of 3 weeks with ad libitum access to food and water.

Before proceeding with the multimodal pheromone place preference assay, the mice underwent a pheromone detection assay. We found similar pheromone detection abilities in the females undergone surgery compared to the control, as evidenced by the time spent near the petri plate containing male urine and soiled bedding (Figure 3.17: Ordinary one-way ANOVA $F = 0.6139$, $p = 0.5507$, Bonferroni's multiple comparisons test, control vs NR1 Δ^{AOB} $p > 0.9999$, control vs GluA2 Δ^{AOB} $p = 0.8574$, NR1 Δ^{AOB} vs GluA2 Δ^{AOB} $p > 0.9999$).

After confirming the integrity of pheromone detection capabilities, we proceeded with the multimodal pheromone place preference assay and assessed memory on post-training days 7 and 15. The memory performance of NR1 Δ^{AOB} and GluA2 Δ^{AOB} females was notably diminished compared to controls, as evidenced by the time spent near zone 1 versus zone 2 (Figure 3.18: Control, $p = 0.0073$, NR1 Δ^{AOB} , $p = 0.1185$, GluA2 Δ^{AOB} , $p = 0.9760$, paired two-tailed student's t-test) and the number of active attempts towards respective chambers (Figure 3.18: Control, $p = 0.0039$, NR1 Δ^{AOB} , $p = 0.0781$, GluA2 Δ^{AOB} , $p = 0.5$, paired two-tailed student's t-test). The similar time spent and active attempts toward both zones by these female mice indicate an impairment in their preference towards opposite-sex pheromones. This underscores the active involvement of AOB circuitry in the multimodal learning of pheromone locations.

The memory of the same female mice was also assessed on post-training day 15, yielding similar results. The memory performance of NR1 Δ^{AOB} and GluA2 Δ^{AOB} females remained significantly reduced compared to controls, as indicated by the time spent near zone 1 versus zone 2 (Figure 3.19: Paired two-tailed student's t-test, control $p = 0.0001$, A2: NR1 Δ^{AOB} $p = 0.5734$, A3: GluA2 Δ^{AOB} $p = 0.2159$) and the number of active attempts towards respective chambers (Figure 3.19: Paired two-tailed student's t-test, control, $p = 0.0039$, B2: NR1 Δ^{AOB} , $p = 0.3906$, B3: GluA2 Δ^{AOB} , $p = 0.4062$). This consistent observation further solidifies the pivotal role of ionotropic glutamate receptors within the microcircuitry of the mouse accessory olfactory bulb in facilitating the memory of complex, multimodal information regarding pheromone locations.

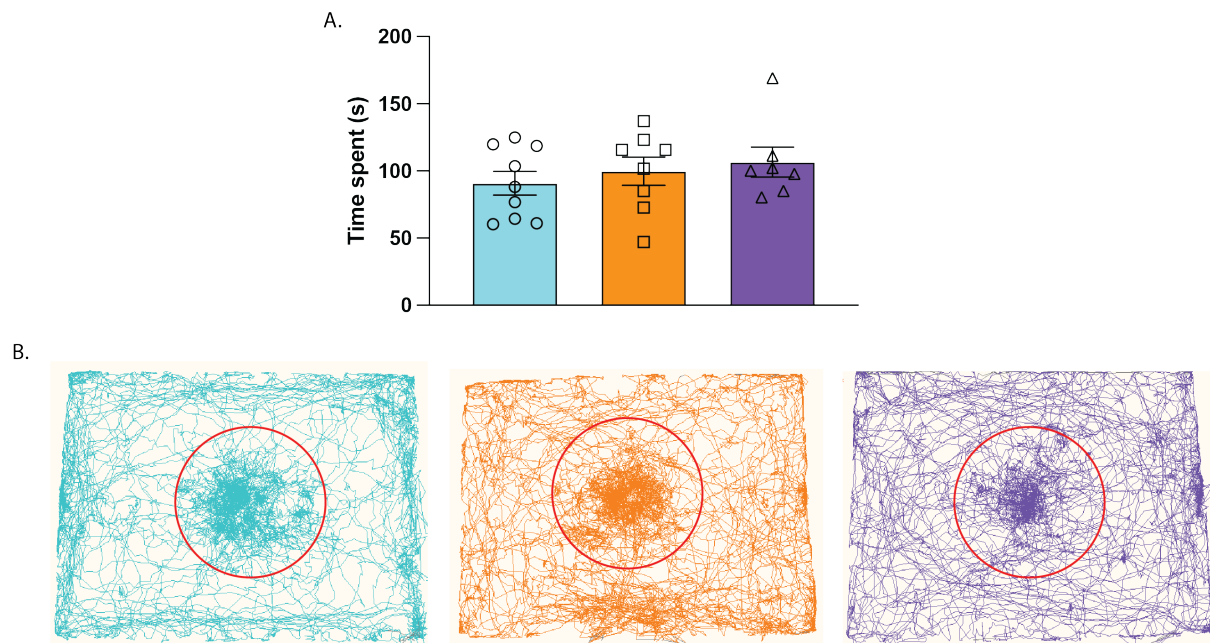


Figure 3.17: No impairment in pheromone detection abilities of NR1 Δ^{AOB} and GluA2 Δ^{AOB} females

- A. A similar amount of time was spent near male urine across groups (Ordinary one-way ANOVA, $p = 0.6139$, $F = 0.5507$ Bonferroni's multiple comparison tests, control vs. NR1 Δ^{AOB} : $p > 0.9999$, control vs. GluA2 Δ^{AOB} $p = 0.8574$, NR1 Δ^{AOB} vs. GluA2 Δ^{AOB} : $p > 0.9999$). Data are mean $\pm$ SEM. (Control $N = 9$, NR1 Δ^{AOB} $N = 8$, GluA2 Δ^{AOB} $N = 7$).
- B. The actual tracks were taken by the animal and analyzed using the "Nose-point" feature of EthoVision software. As the tracks show, females spent similar time near petri-dish containing male urine and soiled bedding shown in a red circle.

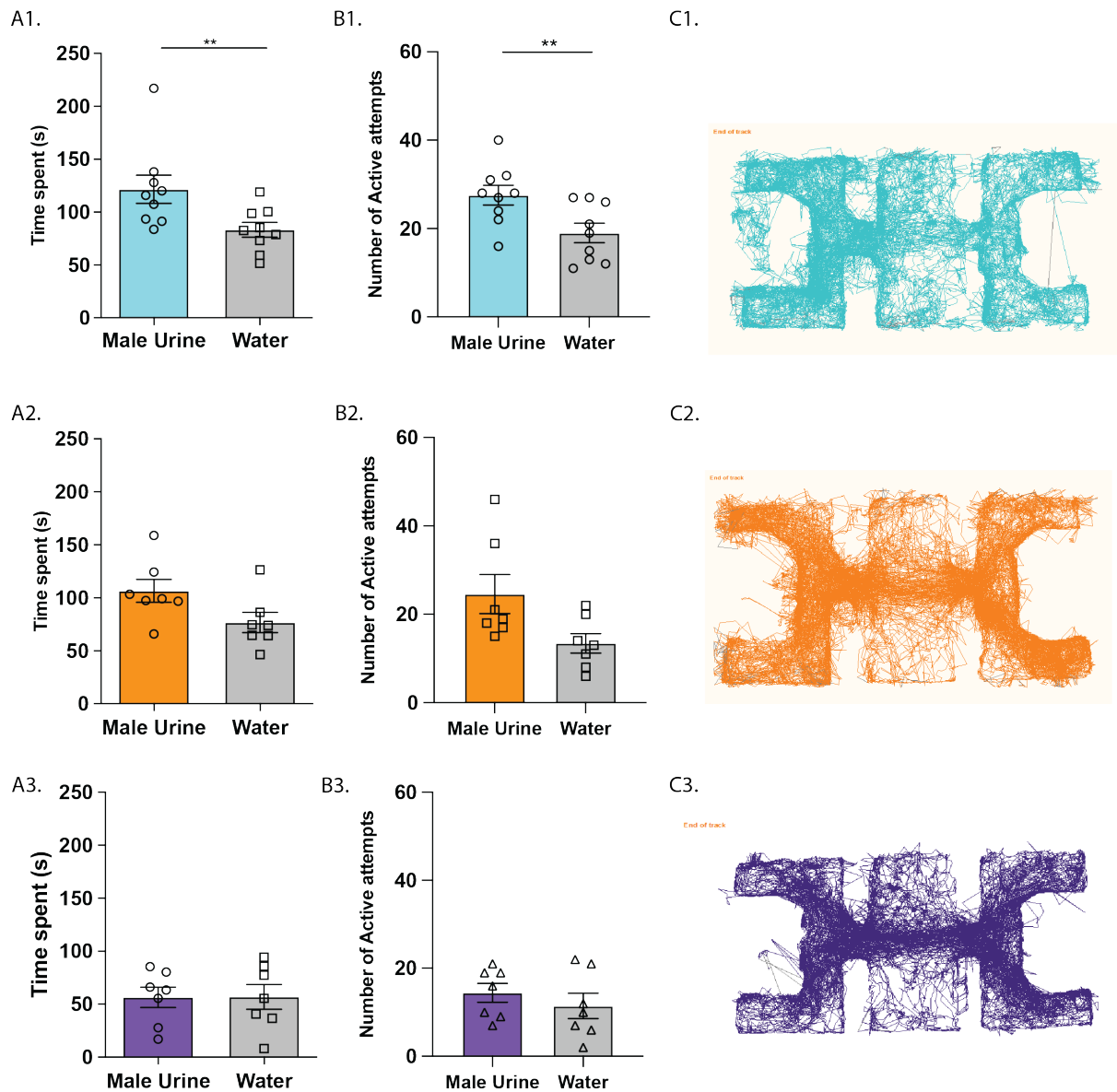


Figure 3.17: Loss of memory in NR1 Δ AOB and GluA2 Δ AOB on memory day 7 of pheromone place preference assay

A1. Control females spent a significantly greater amount of time near the male urine chamber on MD 7 (Paired two-tailed student's t-test, $p = 0.0073$, $N = 9$).

A2. NR1 Δ AOB females did not show preference towards male urine chamber on MD 7 (Paired two-tailed student's t-test, $p = 0.1185$, $N = 7$).

A3. GluA2 Δ AOB females spent an equal amount of time near both chambers on MD 7 (Paired two-tailed student's t-test, $p = 0.9760$, $N = 7$).

A4. On MD 7, control females demonstrated a preference for the male urine chamber through a greater number of active attempts (paired two-tailed student's t-test, $p = 0.0039$, $N = 9$).

A5. NR1 Δ AOB females made a similar number of active attempts for both chambers on MD 7 (Paired two-tailed student's t-test, $p = 0.0781$, $N = 7$).

B3. Similar number of active attempts by GluA2^{ΔAOB} females for both the chambers on MD 7 (Paired two-tailed student's t-test, $p = 0.5$, $N = 7$).

C1,C2,C3. The actual tracks were taken by the animal and analyzed using the “Nose-point” feature of EthoVision software.

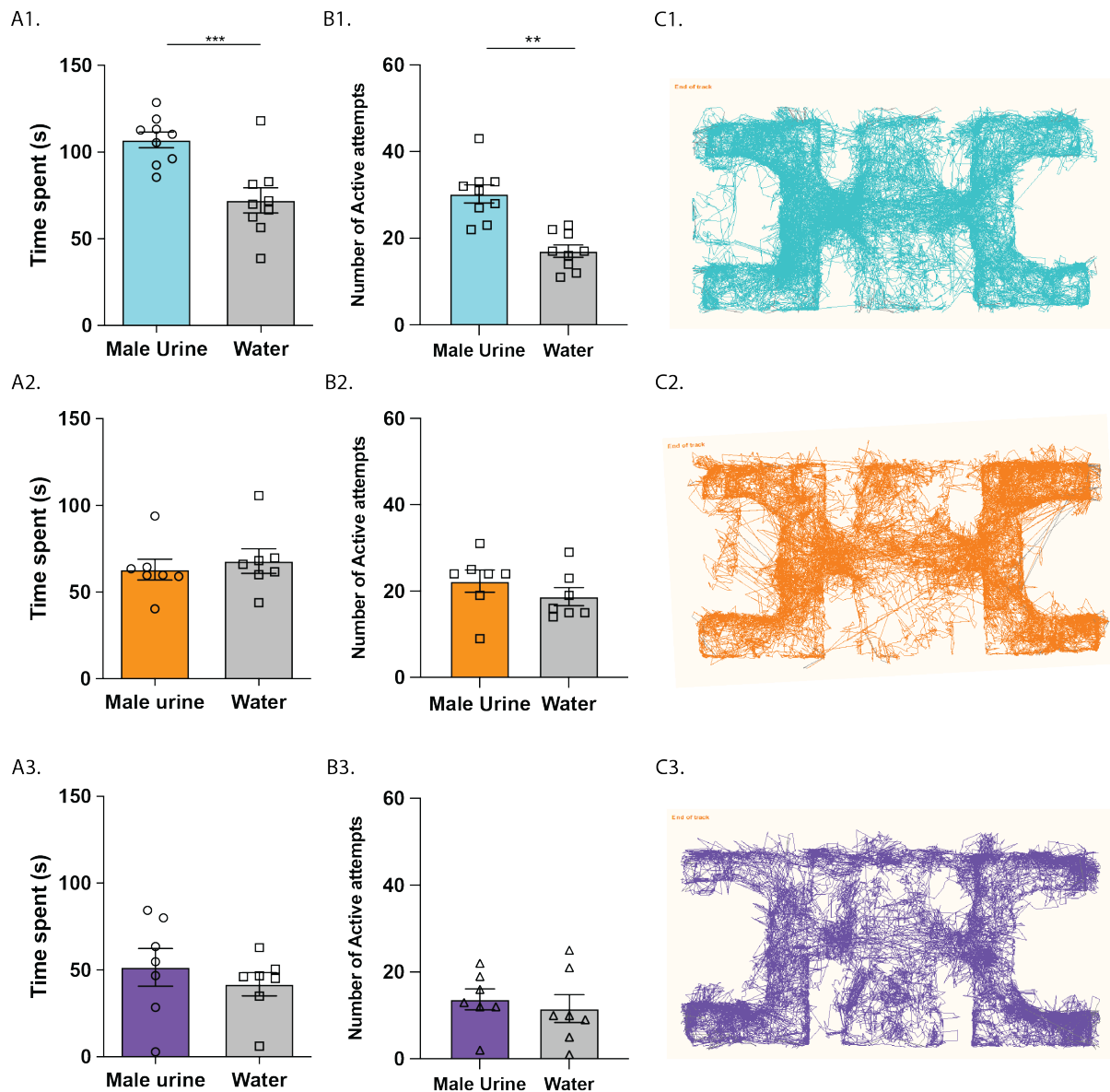


Figure 3.18: Loss of memory in NR1^{ΔAOB} and GluA2^{ΔAOB} on memory day 15 of pheromone place preference assay

A1. On MD 15, control females spent significantly more time near the male urine chamber compared to the time spent near the water chamber (paired two-tailed student's t-test, $p = 0.0001$, $N = 9$).

A2. Similar amount of time was spent near both chambers on MD 15 by NR1^{ΔAOB} females (Paired two-tailed student's t-test, $p = 0.5734$, $N = 7$).

- A3. Similar amount of time was spent near both chambers on MD 15 by GluA2^{ΔAOB} females (Paired two-tailed student's t-test, $p = 0.2159$, $N = 7$).
- B1. Greater number of active attempts were achieved by control females on MD 15 (Paired two-tailed student's t-test, $p = 0.0039$, $N = 9$).
- B2. NR1^{ΔAOB} females showed a similar number of active attempts for both chambers on MD 15 (Paired two-tailed student's t-test $p = 0.3906$, $N = 7$).
- B2. On MD 15, GluA2^{ΔAOB} females exhibited an equal number of active attempts for both chambers (Paired two-tailed student's t-test, $p = 0.4062$, $N = 8$).
- C1,C2,C3. The actual tracks were taken by the animal and analyzed using the “Nose-point” feature of EthoVision software.

3.4 Discussion

3.4.1 Involvement of ionotropic glutamate in multimodal learning of pheromone locations

Male mice display urinary scent-marking behaviour, which is beneficial for social communication (Hurst, 1987b, 1989), the demonstration of social hierarchy (Hurst, 1993; R. B. Jones & Nowell, 1974), and the determination of the reproductive health of potential mates (Barnard & Fitzsimons, 1988; Mossman & Drickamer, 1996). Female mice tend to remember such locations displaying pheromonal cues and frequently revisit these places where they have previously encountered urine scent marks to increase the probability of successful mating in future (Hurst, 1986, 1987a; Potts et al., 1991; Roberts et al., 2010). In a natural habitat, such scent marking is found streaked on stones or foliage of varying sizes and shapes. We hypothesized that females may use both their olfactory and whisker systems to learn the locations of pheromonal cues deposited by males. To investigate this behaviour under laboratory conditions, we employed a novel assay termed the ‘Pheromone place preference assay’ (Pardasani et al., 2021). Briefly, we subjected the animals to a 4-day testing phase (TP), and a 15-day training phase (TrP) and investigated the memory after 15 days (MD 15) in the absence of any stimuli. During the TrP, female mice were trained to associate odour stimuli with a particular dimension of the orifice. On MD 15, the preference towards respective chambers (male urine chamber and water chamber) was accessed. During MD 15, the duration and number of active behaviours directed towards the male urine chamber and water chamber by the female mice were quantified. Our observations, consistent with a previous study, also confirmed the involvement of the whisker system in addition to the olfactory system, in

learning the pheromonal location (Pardasani et al., 2021). The correlation between pheromones and the size of entities could facilitate the formation of memories associated with pheromone locations within a complex natural environment. Consequently, our objective was to explore the neural mechanisms underlying the multimodal learning of pheromone locations.

To investigate the neural mechanisms involved in the multimodal learning of pheromone locations and memory formation in female mice, we targeted one of the most abundant interneuron populations in the mouse brain, specifically the GAD65-expressing neuronal population (Maksimova et al., 2019; Parrish-Aungst et al., 2007). We generated heterozygous knockouts (KOs) of the NR1 subunit of NMDA receptors and the GluA2 subunit of AMPA receptors in GAD65-expressing interneurons, resulting in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals respectively. The NR1 subunit is an essential subunit of NMDAR. Deletion of the NR1 subunit causes a reduction in functional NMDARs. On the other hand, knocking out of the GluA2 subunit from AMPAR leads to an increase in calcium-permeable AMPARs (CP-AMPARs). Before conducting the pheromonal place preference assay, we subjected the animals to a variety of mood disorder-related tests to assess for anxiety or despair-like behaviours. We observed no anxiety or despair-like behavioural readouts in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals. Furthermore, the KOs displayed similar pheromone detection abilities compared to control females.

In performing a pheromone place preference assay with these heterozygous KO animals, we observed a loss of preference towards the urine chamber on MD 15. In contrast, the control group spent more time and made more active attempts towards the urine chamber. This indicates the vital role of NR1 and GluA2 subunits in GAD65-expressing neurons towards memory for social cues.

GAD is predominantly expressed in GCs and PGCs of the MOB and AOB (Maksimova et al., 2019; Parrish-Aungst et al., 2007), which are involved in the circuits mediating recurrent and lateral inhibition (Quaglino et al., 1999). GABAergic interneurons of the MOB respond to social stimuli (Wacker et al., 2010). GABAergic neuronal cell population has been directly implicated in the processing of pheromone selectivity in the AOB (Hendrickson et al., 2008). Previous studies have shown that GAD67-expressing (an isoform of GAD) interneurons play an important role in rodent social behaviours. Haplo-deficiency of GAD67-expressing neurons led to disturbance in the processing of socially relevant odour stimuli such as pheromones. In these animals, reduced levels of c-fos expression were observed in the medial and cortical amygdala, bed nucleus of the stria terminalis, medial preoptic area and lateral septum. Interestingly, c-fos expression remained unchanged in the main and accessory OB (Sandhu et

al., 2014). These observations highlight the significance of GABAergic neurons in processing socially relevant odorant cues. In our study, modifications of iGluRs in GAD65-expressing interneurons reveal a plausible mechanism by which mice memorize the location of pheromonal cues.

Reduction in the NR1 subunit from GABAergic interneurons in the hippocampus and cortical regions has been shown to cause social memory deficit (Lukas et al., 2011). Jacobs and Tsien have demonstrated that forebrain-specific NR1-containing NMDA receptors modulate social motivation and recognition (Tsien et al., 1996). Chiang et al. showed that deletion of the NR1 subunit gene in the ventral, but not in the dorsal, CA3 pyramidal cells results in deficits in social memory, while mice lacking the same gene in the DG granule cells showed intact social memory (Chiang et al., 2018). In rodents, olfaction plays an important role in forming a social memory towards conspecifics. Several studies have demonstrated the significance of NMDA-receptor-mediated neurotransmission in the regulation of social interaction in rodents (reviewed in Zoicas & Kornhuber, 2019). AMPA receptors in the AOB of female mice have also been demonstrated to play a role in forming memories of mating males (P. A. Brennan, 1994). These studies emphasize the importance of iGluRs in forming memories related to conspecifics in mice. These memories heavily rely on the olfactory abilities of these animals. Furthermore, memorizing the locations of pheromones is essential for female mice to enhance the probability of mating, and this process involves both the olfactory and whisker systems (Pardasani et al., 2021). Our study focused on the OB circuitry to investigate the role of iGluRs in multimodal learning of pheromone locations. Despite unimpaired pheromonal detection, we observed a loss of memory for pheromone locations in females with an altered composition of NMDAR and AMPAR in the OB. The loss of the ability to memorize the location of social odour cues provides a plausible explanation for deficits in social behaviour observed in animals with modified NMDA and AMPA receptor functions. In 2021, Pardasani et al, developed an assay termed “Pheromone place preference assay” to study a behaviour displayed by female mice to frequent the male pheromone locations (Pardasani et al., 2021; Roberts et al., 2012). We showed that this memory towards the pheromone locations is multimodal in nature involving olfactory as well as whisker systems. Previously researchers have focused on the female mice memory towards pheromones of stud male. They confirmed that the site of such memory is in the AOB (Brennan et al., 1990). Our aim through this study was to deduce the involvement of ionotropic glutamate receptors in pheromone location learning and memory through olfactory subsystems. As OB is the first relay station where chemical cues such as

pheromones are processed, we targeted our study to iGluRs in the olfactory bulb. Such modulations lead to changes in synaptic inhibition at the level of OB (Abraham et al., 2010).

The GluA2 subunit of AMPAR plays a crucial role in various stages of memory, including memory consolidation, retrieval, and updating (Bast et al., 2005; Torquatto et al., 2019). A previous study demonstrated that the forgetting of consolidated memory ensues as dorsal hippocampal GluA2-containing AMPAR receptors are lost at synapses (Migues et al., 2016). A recent study in *C. elegans* showed that the removal of GluA2-containing AMPA receptors could lead to the dissolution of synaptic infrastructure crucial for memory persistence due to their interaction with cell adhesion and cytoskeletal proteins (Hadziselimovic et al., 2014). Shimshek et al., in 2005 concluded that olfactory discrimination is enhanced by an increase in AMPAR-mediated Ca^{2+} permeability due to the deletion of the GluA2 subunit in the forebrain region. Conversely, olfactory memory becomes impaired upon genetically induced GluA2 ablation in higher brain centres, specifically in the piriform cortex (Shimshek et al., 2005).

Females with modified NMDA and AMPA receptors in the OB exhibited a lack of preference towards the male urine chamber on MDs 7 and 15 during the pheromonal place preference assay, suggesting a plausible role of iGluRs in the multimodal learning of pheromone locations. Previous studies have demonstrated changes in Ca^{2+} permeability upon removal of the GluA2 subunit from AMPA receptors of GCL of the OB. This alteration led to a faster odour discrimination ability while keeping odour memory intact (Abraham et al., 2010). However, deleting the GluA2 subunit from the forebrain has been shown to impair the olfactory memory (Shimshek et al., 2005), suggesting a role of GCL in early events of odour processing while involving higher forebrain regions in odour memory.

In our study, we modulated iGluRs in the OB, including the AOB which is considered a site of memory for the pheromones of the mating male (Nakazawa et al., 1995). Ca^{2+} signalling plays a vital role in the process of learning and memory, and perturbing the Ca^{2+} dynamics can lead to impairments in learning and memory, as evidenced by various age-related neurological disorders such as Alzheimer's disease (Khachaturian, 1989; Thibault et al., 2007), which also display deficits in social interactions (Hsiao et al., 2018). Previous studies have demonstrated that changes in other forms of plasticity such as theta-burst pairing potentiation, which are heavily dependent on postsynaptic intracellular Ca^{2+} (Hoffman et al., 2002), may play a crucial role in pheromonal memory. Phosphorylation of GluA2 is required for long-term stabilization of memory (Chung et al., 2003), which might have been disturbed in these KOs due to the deletion of the GluA2 subunit from AMPA receptors, potentially causing perturbed

CaMKII activity (W. Lu et al., 2014) and leading to impairment in multimodal learning of pheromone locations. Additionally, it has been observed that in CP-AMPA, an AMPAR-dependent, NMDAR-independent form of LTP is induced (Hoffman et al., 2002; Jensen et al., 2003). This is likely due to calcium influx by CP-AMPA leading to non-Hebbian forms of plasticity, which are solely induced by post-synaptic action potentials. However, for memory storage, the Hebbian form of plasticity i.e. simultaneous pre- and post-synaptic activity is necessary (Chater & Goda, 2014; Chung et al., 2003). Altered iGluR subunit composition may thus result in deficits in memory formation rather than memory storage, as it has been previously shown that the non-Hebbian plasticity is mainly affected by the Ca^{2+} influx through NMDA and AMPA receptors. In summary, our findings suggest the putative role of ionotropic glutamate receptors of mouse OB in multimodal learning of pheromone locations.

Our paradigm is testing the long-term memory of pheromone locations. Two iGluRs namely NMDAR and AMPAR might play distinct roles at different stages of memory. For example, it has been shown that NMDARs are important for the memory retrieval (Alaghband et al., 2014) while AMPARs majorly play a role during memory maintenance (Migues et al., 2014). Hence, the phenotype which we are seeing during memory days of pheromonal place preference assay, is similar in both the cases. To delineate precisely which memory stage is getting modified, we need to employ the methods with more spatio-temporal control.

The c-fos expression in DG of NR1-KO animals was non-significantly higher compared to controls and GluA2-KO animals. This slight increase in the c-fos activity in the DG of NR1GAD65 KO animals might be due to calcium influx through non-NMDA receptors (Lerea et al., 1992). Another plausible explanation to the slight increase in the c-fos expression in SSC could be less inhibition onto excitatory neurons. It has been demonstrated that the less inhibition from interneurons lead to more activation of MTCs which corresponds to more c-fos expression in the OB (Wilson et al., 1996). Similar phenomenon could occur in the SSC leading to non-significant increase in the c-fos expression as seen in figure 3.11 D2. NR1-KO in the SSC have been seen to disrupt the localized c-fos expression leading to widespread expression of c-fos across barrel cortex (Lo et al., 2013).

Rodents highly depend on chemical cues to navigate, forage food and find potential mates, mainly because vision and audition are less commonly used sensory modalities (Aboitiz & Montiel, 2015; Reep et al., 2007). Hence, an hypothesis termed olfactory spatial hypothesis was introduced which suggests that the primary function of olfaction is navigation (Jacobs, 2012). Animals in their vicinity learn the relationship between various cues such as olfactory or somatosensory and landmarks which allow them to perform behaviours more flexibly and

adaptively (O'Keefe & Nadel, 1978). Integrating chemosensory information into cognitive map is crucial for animals. Animals use odor memory to guide spatial choices (Gire et al., 2016). Brain regions for odour perception and spatial memory are developmentally linked, which form the allocortex (consisting of olfactory, hippocampal and entorhinal cortices) (Poo et al., 2022). Thus, olfaction and spatial memory systems are intimately related.

3.4.1 Immediate early gene Arc expression correlates with impairment in memory toward pheromone location

Strong synaptic activation of neurons leads to the expression of immediate early genes (IEGs). One such IEG is the Activity-regulated cytoskeleton-associated (Arc) protein, also known as Arg 3.1. It has been observed that newly formed Arc mRNA accumulates at the synapse experiencing a strong activity. Therefore, we probed the expression of Arc during MDs of the pheromonal place preference assay. Lower Arc expression was observed in the main olfactory bulb (MOB), accessory olfactory bulb (AOB), the somatosensory cortex (SSC), and dentate gyrus (DG) of female mice with modified iGluRs in the OB when compared to control females. This finding aligned with the deficits noted during the memory phase of the pheromonal place preference assay.

The signal transduction leading to the localization of Arc mRNA to active synapse involves NMDARs and AMPARs (Steward & Worley, 2001). Some previous studies have also discussed the potential role of NMDAR activation in Arc induction (Lyford et al., 1995). Steward et al., in 2001 also showed that massive depolarization caused by AMPARs is not sufficient to mediate induction and localization of Arc (Steward & Worley, 2001). Arc mRNA is induced by Ca^{2+} influx through voltage-gated calcium channels and NMDARs and is trafficked to dendrites and synthesized at synaptic sites (Korb & Finkbeiner, 2011). NMDAR hypofunction has also been coupled with LTP deficits in the hippocampus, dendritic spine density, spatial, nonspatial learning and memory (Balu et al., 2012, 2013; DeVito et al., 2011; Shimizu et al., 2000; Tsien et al., 1996). In our study, we observed a similar outcome on deleting an essential subunit of NMDA receptors known as NR1. This deletion resulted in impaired preference display toward pheromone locations during memory days, indicating a deficiency in associative memory related to pheromone locations. Furthermore, we noticed reduced Arc expression in the MOB, AOB, SSC, and DG in animals with modified iGluRs. Previous research has shown that NMDAR activity regulates the expression of Arc protein under basal conditions in the hippocampus (Balu & Coyle, 2014). Arc positively modulates spine density in the hippocampus (Peebles et al., 2010). Arc protein acts as an effector IEG widely implicated

in experience-dependent synaptic plasticity and memory consolidation (Guzowski et al., 2000; Holloway & McIntyre, 2011; Steward et al., 1998). In our study, we observed that NR1^{GAD65(+/-)} heterozygous KO females showed reduced Arc expression in the MOB, AOB, DG, and SSC, indicating an active role of NMDARs in inducing Arc during pheromonal location memory.

The function of Arc at active synapses is still not clear; however, it has been speculated that it regulates synaptic strength by modulating the trafficking of AMPARs (Mokin et al., 2006). Arc is necessary for the maintenance of synaptic potentiation and long-term consolidation of memory (Guzowski et al., 2000; McIntyre et al., 2005). AMPARs regulate Arc activation. Inhibiting AMPARs increases Arc transcription, but translation and Arc stability are unaffected (Rao et al., 2006). Neurons tend to regulate the ratio between NMDA and AMPA at synapses during changes in synaptic strength to ensure fixed currents through these channels (Watt et al., 2000, 2004). This homeostatic regulation helps maintain the information content of synaptic transmission (Watt et al., 2004). In primary cultured neurons, Rao et al. (2006) also found that Arc expression is determined by the relative extent of NMDA and AMPA activation, which indicates a possible integration of pathways from NMDAR and AMPAR to control Arc transcription (Rao et al., 2006). Arc is expressed upon synaptic activity, which leads to the surface expression of AMPARs. This causes a decrease in the NMDA/AMPA ratio, inhibiting Arc transcription. Later, delayed NMDAR potentiation resets the NMDA/AMPA ratio allowing subsequent expression of Arc and synaptic plasticity. This is one of the speculative explanations for the negative feedback control of Arc expression. Arc but not c-Fos is regulated by AMPA receptor inhibition (Rao et al., 2006). These observations display the intricate relation between iGluRs and Arc protein, during long-term neural plasticity.

The behavioural study and the expression of Arc in the OB, SSC and DG display a plausible role of ionotropic glutamate receptors of OB in the multimodal pheromonal location paradigm. Further experiments, such as in-vivo Ca²⁺ imaging during the behavioural assays, will provide more insights into the functioning of the circuit as the learning progresses.

3.5 Future directions

3.5.1 Multimodal learning of pheromone location under nature-like settings

To study the multimodal learning of pheromone locations under nature-like conditions by female mice, we constructed a setup. The outer arena dimensions were 60 cm x 60 cm x 30 cm (L x W x H). The inner arena was movable, measuring 16.5 cm x 12 cm x 20 cm. It included various objects with different shapes, sizes and textures such as wooden sticks, pebbles,

artificial grass, and sandpaper, placed in the inner arena (Figure 3) for tactile enrichment. These objects helped enhance somatosensation through whisker stimulation.

The paradigm involved a training phase of 11 days, during which each female was introduced to the arena. Once inside the inner arena, a male mouse was introduced, and they were allowed to interact for 10 minutes. Throughout the entire training phase, the pair and the soiled bedding remained constant. On post-training day 15, the females were allowed to explore the setup in the absence of the male mouse and soiled bedding. During the memory phase, the time spent by the female inside the inner arena was quantified using a tracking software, Ethovision 8.5 XT (Nodulus).

We trained control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females in this setup and quantified the time spent in the inner arena as an indicator of the memory towards the location where the female had encountered the male, on post-training day 15. We observed similar time spent by the control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} females ($p = 0.4870$, $F(10,13) = 1.004$, two-way ANOVA, control vs NR1^{GAD65(+/-)} $p = 0.2405$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} $p = 0.4918$, and control vs GluA2^{GAD65(+/-)} $p = 0.8276$, control $N = 10$, NR1^{GAD65(+/-)} $N = 11$, GluA2^{GAD65(+/-)} $N = 5$).

We did not observe any impairment in location memory when the females were trained in the presence of a male mouse (Figure 3.19). Although olfaction is a primary sensory modality, mice use various communication modes such as acoustic stimuli to communicate with conspecifics (R. C. Liu et al., 2003; Portfors, 2007). We employed a naturalistic setting in which a female interacts with a male over a period of 15 days, with sessions lasting 10 minutes each day. During these sessions, a female may receive various sensory signals that help form the memories of the location. Similar to male urine, acoustic stimuli also convey information about an animal's fitness (Egnor & Seagraves, 2016; Hoffmann et al., 2012). It has been observed that adult male mice produce rhythmic sequences of discrete, frequency-modulated pure tone elements when they sense the presence of a fertile female (Chabout et al., 2012, 2017; Holy & Guo, 2005). Sexually receptive females get attracted to such courtship songs (Pomerantz et al., 1983). Hence, along with olfaction and somatosensation, audition also may play a major role in forming a memory towards the location where a female encounters a potential mate. Thus, it would be interesting to investigate the contribution of other sensory modalities such as audition in multimodal learning of pheromone locations.

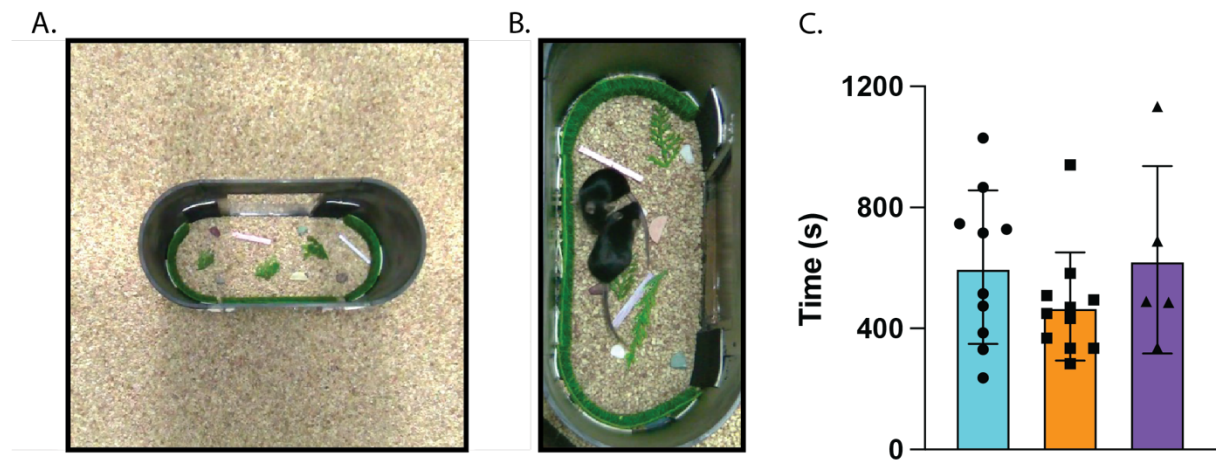


Figure 3.19: Multimodal learning of pheromone location under nature-like settings

- A. The setup with the outer arena, and inner arena containing objects with varying shapes, sizes and textures to stimulate the whisker system.
- B. Interaction of male and female mice during the training phase.
- C. Similar time spent by the animals in the inner arena on memory day 15 ($p = 0.4870$, $F(10,13) = 1.004$, two-way ANOVA, control (cyan) $N = 10$, NR1^{GAD65(+/-)} (orange), and $N = 11$, GluA2^{GAD65(+/-)} (purple) $N = 5$).

CHAPTER 4

Role of ionotropic glutamate receptors in odour and airflow discrimination

4.1. Introduction

The rodent olfactory system is designed to process highly complex odour mixtures in the environment. Rodents utilize this ability to make informed decisions, resulting in learned and innate behaviours. Olfactory information processing starts with the excitation of OSNs by odour stimuli. M/TCs receive odour-specific information from OSNs (Shepherd et al., 2004) and convey it to higher olfactory cortical regions (Igarashi et al., 2012; Nagayama, 2010). It is known that the inhibitory circuit of the OB plays an important role in odour discrimination (Abraham et al., 2010; Cleland & Linster, 2005; Gschwend et al., 2015; Leon & Johnson, 2003). The responses of M/TCs are modulated by inhibition exerted by interneurons present in GL and GCL. This includes circuits between M/TCs and PGCs at GL or M/TCs and GCs at GCL. PGCs establish reciprocal dendrodendritic synapses with primary dendrites of M/TCs, while GCs form reciprocal dendrodendritic synapses with secondary dendrites of M/TCs. This excitation-inhibition interplay helps delineate the overlapping odour responses during odour discrimination (Leon & Johnson, 2006; Mori et al., 1999; N. Urban, 2002; Yokoi et al., 1995). The GAD65-expressing interneuron population constitutes the majority of the GABAergic interneuron population in mouse OB (reviewed in Burton, 2017; Parrish-Aungst et al., 2007). To elucidate the contribution towards odour discrimination specifically from the GAD65-expressing inhibitory interneurons, we modified these interneurons' functions by knocking out the NR1 subunit from NMDAR and the GluA2 subunit from AMPAR as explained in Chapter 2.

Rodents inhabit a multi-sensory environment in nature, requiring their brains to process a variety of sensory stimuli from the external world. They are exposed to numerous odour mixtures in diverse concentrations, carried by odour plumes that guide them to the scent's location (Marin et al., 2021; Vickers, 2000; Young et al., 2020). We aimed to investigate the role of ionotropic glutamate receptors in GAD65-expressing interneurons of the OB in processing odour discriminations under various complexities, concentrations, and airflow discriminations. Odour discrimination has been extensively studied using a Go/No-go operant conditioning paradigm with an automated olfactometer (Abraham et al., 2004, 2010; Bodyak,

1999; Pardasani et al., 2023; Slotnick, 2001; Walker & O'Connell, 1986). Different parameters such as learning accuracy, discrimination time (DT), and inter-trial interval (ITI) have been quantified, reflecting the animals' discrimination ability, the time required to discriminate between stimuli, and their motivation levels during the training, as discussed in Chapter 2.

4.2 Materials and Methods

A total of 121 adult animals were utilized in the experiments discussed in this section. All odour discrimination tasks were performed with adult male mice aged 6 to 12 weeks and the number of animals varied across the tasks.

Simple odour discrimination task: control (n = 8), NR1^{GAD65(+/-)} (n = 7) and GluA2^{GAD65(+/-)} (n = 8)

Complex odour discrimination task: control (n = 6), NR1^{GAD65(+/-)} (n = 5) and GluA2^{GAD65(+/-)} (n = 5)

Multi-component odour discrimination task: control (n = 6), NR1^{GAD65(+/-)} (n = 8) and GluA2^{GAD65(+/-)} (n = 5)

Odour detection threshold task: control (n = 7), NR1^{GAD65(+/-)} (n = 7) and GluA2^{GAD65(+/-)} (n = 8)

Odour discrimination threshold task: control (n = 7), NR1^{GAD65(+/-)} (n = 7) and GluA2^{GAD65(+/-)} (n = 8)

All airflow discrimination tasks were performed with adult male mice aged 6 to 12 weeks (n = 12 for each). The detailed protocol for airflow discrimination has been discussed in Chapter 2. The sequence of airflow discrimination training was as follows: 0.4 vs 0.5 LPM, 0.25 vs 0.3 LPM and 0.1 vs 0.15 LPM.

4.3 Result

4.3.1 Simple and complex odour discriminations remained unaffected by functional variations in GAD65-expressing interneurons

Male mice from NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} groups, as well as control mice, were trained on a GNG operant conditioning task using custom-built olfactometers to investigate the involvement of ionotropic glutamate receptors (iGluRs) of GAD65-expressing interneurons in odour discrimination. The details of the olfactometer and paradigm are described in Chapter 2.

The animals were sequentially trained for simple (monomolecular/simple: Ci vs Eu) and complex (binary/complex: 60:40 vs 40:60 mixture of Ci and Eu) odour mixture tasks. Prior

to the discrimination learning, animals underwent task habituation training. After completing the Ci/Eu simple odour discrimination task, mice underwent training for Ci/Eu complex (60/40) odor mixture discrimination task. For a simple odour discrimination task, the NR1^{GAD65(+/-)}, GluA2^{GAD65(+/-)} animals and controls achieved $\geq 80\%$ accuracy within 600 trials. The final learning accuracies of different groups remained similar (Figure 4.1 A: Two-way ANOVA, $F(2,180) = 0.8763$, $p = 0.4181$, control: 81.10 ± 4.8 , NR1^{GAD65(+/-)}: 80.86 ± 4.7 and GluA2^{GAD65(+/-)}: 79.38 ± 4.5). The time taken by the animals to discriminate between the odours was calculated as discrimination time (DT). Animals across groups displayed similar DTs for the simple odour discrimination task (Figure 4.1 B: Ordinary one-way ANOVA, $F = 2.389$, $p\text{-value} = 0.1173$, control: 14.47 ± 1.1 , NR1^{GAD65(+/-)}: 16.36 ± 2.2 and GluA2^{GAD65(+/-)}: 17.43 ± 2.6). The motivation level of the animals was observed with time during inter-trial interval (ITI). The ITI was similar across groups (Figure 4.1 C: Ordinary one-way ANOVA, $F = 0.5366$, $p = 0.5929$, control: 407.5 ± 19.98 , NR1^{GAD65(+/-)}: 382.2 ± 12.22 and GluA2^{GAD65(+/-)}: 395.6 ± 29.40).

All of the animals reached $\geq 80\%$ accuracy within 700 trials and reached around 90 % by the end of 1200 trials (Figure 4.2 A: Two-way ANOVA, $F(2,156) = 1.102$, $p = 0.3348$, control: 75.99 ± 4.1 , NR1^{GAD65(+/-)}: 76.42 ± 4.4 and GluA2^{GAD65(+/-)}: 78.07 ± 4.2). For complex odour discrimination task, the DTs were found to be similar across different groups (Figure 4.2B: Ordinary one-way ANOVA, $F = 0.7194$, $p = 0.5055$, control: 536 ± 59.51 , NR1^{GAD65(+/-)}: 484 ± 34.29 and GluA2^{GAD65(+/-)}: 456 ± 42.14). We also calculated the ITI which is a proxy for motivation during training sessions. For both the simple and complex odour discrimination tasks, the ITI (Figure 4.2C: Ordinary one-way ANOVA, $F = 0.4900$, $p = 0.6235$, control: 16.10 ± 1.0 , NR1^{GAD65(+/-)}: 14.78 ± 2.0 and GluA2^{GAD65(+/-)}: 18.47 ± 4.1) was similar across the groups. The data are shown in mean $\pm$ SEM.

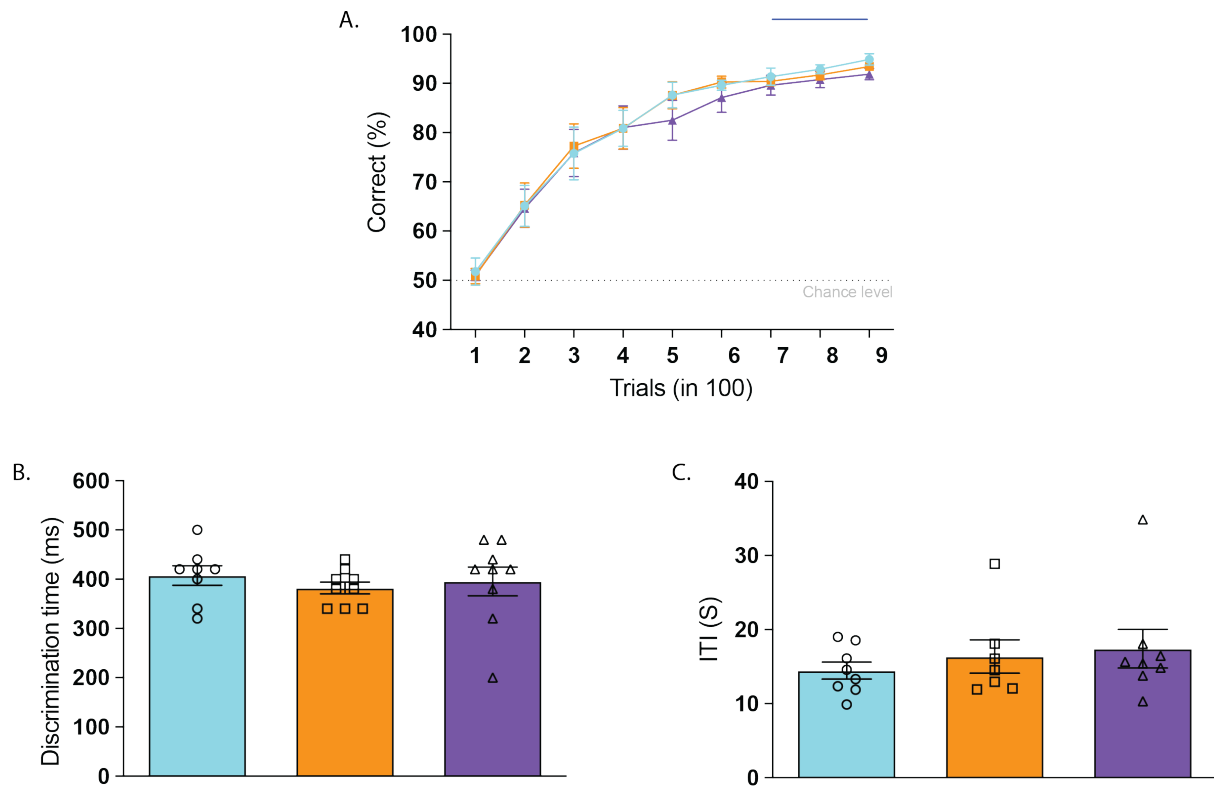


Figure 4.1: NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals showed similar odour discrimination abilities for simple odour pair compared to control animals

- A. Animals across groups showed similar learning accuracy throughout the simple odour discrimination training (Ordinary two-way ANOVA, $F(2,180) = 0.8763$, $p\text{-value} = 0.4181$). Each data point represents correct responses averaged across 100 trials. Each point is represented as mean $\pm$ SEM (Control $N = 8$, NR1^{GAD65(+/-)} $N = 8$, and GluA2^{GAD65(+/-)} $N = 9$).
- B. Similar odour discrimination times for simple odour discrimination across groups. Data is shown as mean $\pm$ SEM (Ordinary one-way ANOVA, $F = 2.389$, $p\text{-value} = 0.1173$).
- C. The inter-trial interval (ITI) for simple odour discrimination across groups was similar. Data is shown as mean $\pm$ SEM (Ordinary one-way ANOVA, $F = 0.5366$, $p\text{-value} = 0.5929$).

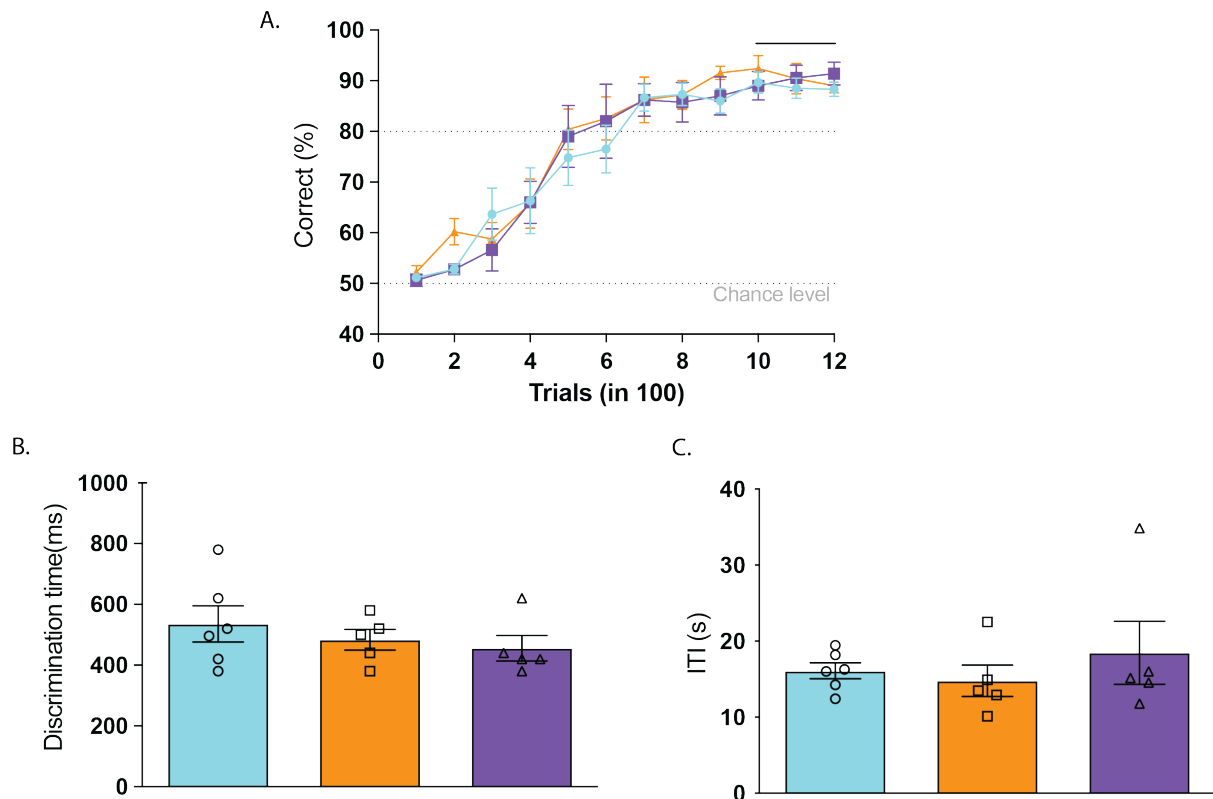


Figure 4.2: NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals showed similar complex odour discrimination compared to control animals

- A. For complex odour pair mice across three groups showed similar learning accuracy throughout the training. Each data point represents correct responses averaged across 100 trials. Each point is represented as mean $\pm$ SEM (Ordinary two-way ANOVA, $F(2,156) = 1.102$, $p\text{-value} = 0.3348$, control $N = 6$, NR1^{GAD65(+/-)} $N = 5$ and GluA2^{GAD65(+/-)} $N = 5$).
- B. Odour discrimination times for complex odour discrimination across groups were similar. (Ordinary one-way ANOVA, $F = 0.7194$, $p\text{-value} = 0.5055$).
- C. Similar motivation levels were displayed by ITIs for animals across groups. (Ordinary one-way ANOVA, $F = 0.49$, $p\text{-value} = 0.6235$).

Rodents live in an olfactory world, made not only of monomolecular or bimolecular stimuli but also of more complex olfactory mixtures such as ‘multi-component’ odour mixtures that span the olfactory stimulus space (Weiss et al., 2012). We observed similar learning across different groups for simple and complex odour discriminations, we further sought to elucidate the role of GAD65-expressing interneurons in even more complex environments. To achieve this, we challenged the animals to discriminate between two stimuli, each made by combining 20 odours mixed in similar concentrations. The odour composition is as follows: 2-Pentanone, 1-Heptanol, Octanol (-), Octanol (+), Heptanal, Valeraldehyde, Geraniol, Isoamyl acetate,

Eugenol, Carvone (-), Carvone (+), Limonene-, Hexanal, 1,4-Cineole, Nonanal, Ethyl butyrate, Amyl acetate, Pentanol, 3-Hexanone, Acetophenone/Nonanol. The odours which differed in both mixtures were Acetophenone and Nonanol. During the multi-component odour discrimination task, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals exhibited similar learning accuracies compared to controls (Figure 4.3A: Two-way ANOVA, $F(2,144) = 6.275$, $p = 0.0024$, control: 72.24 ± 4.2 , NR1^{GAD65(+/-)}: 72.82 ± 4.8 and GluA2^{GAD65(+/-)}: 78.2 ± 4.7). The DTs were calculated based on lick patterns. The DTs were similar for the last task (300 trials) across the groups (Figure 4.3B: Ordinary one-way ANOVA, $F = 0.5351$, $p\text{-value} = 0.5957$, control: 280 ± 18.62 , NR1^{GAD65(+/-)}: 292.5 ± 40.34 and GluA2^{GAD65(+/-)}: 332 ± 29.39).

The motivation levels throughout the training were observed during the time of ITL. ITIs were similar across groups, indicating no difference in the motivation levels. (Figure 4.3C: Ordinary one-way ANOVA, $F = 1.896$, $p\text{-value} = 0.1824$, control: 14.60 ± 1.3 , NR1^{GAD65(+/-)}: 11.43 ± 0.5409 and GluA2^{GAD65(+/-)}: 11.43 ± 0.54). The data are shown in mean $\pm$ SEM.

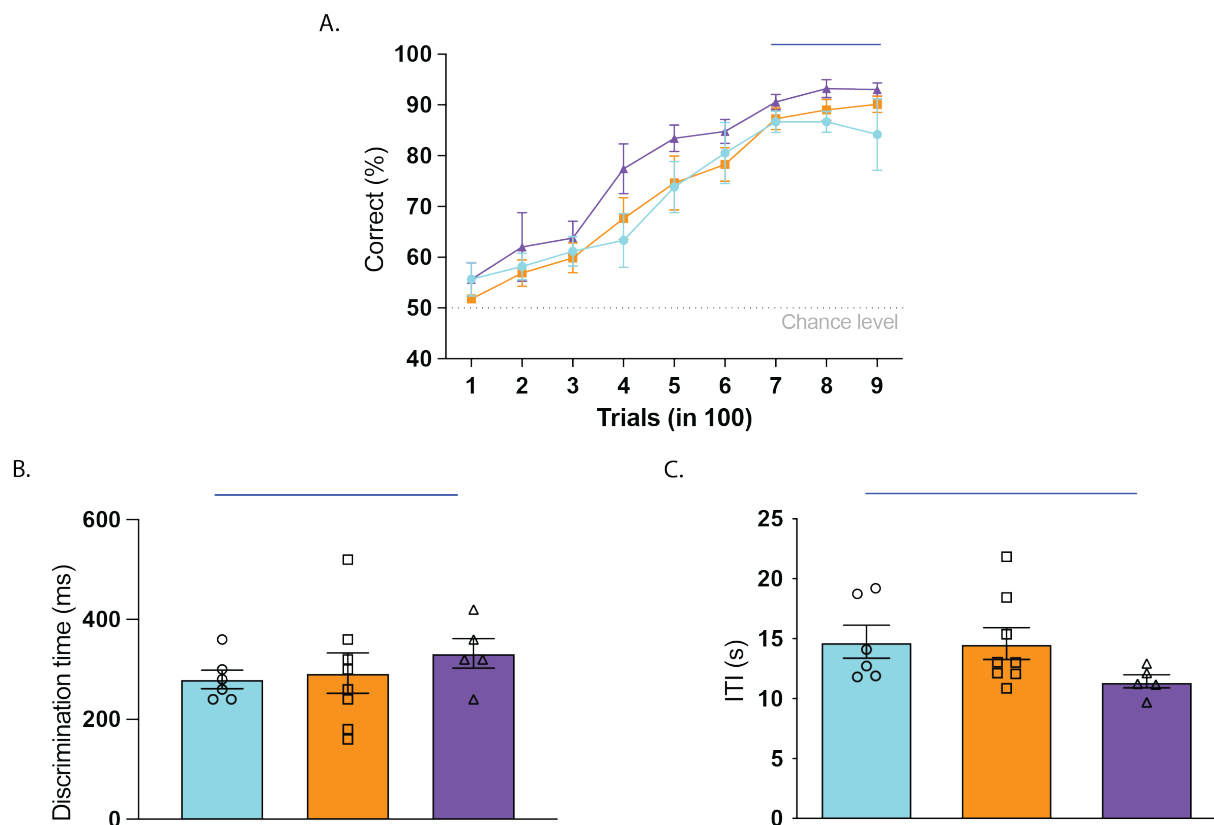


Figure 4.3: NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals showed similar multi-component odour discrimination compared to control animals

- A. For 20-component odour mixture discrimination, mice across three groups showed similar learning accuracy throughout the training. (Ordinary two-way ANOVA, $F(2,144) = 6.275$, $p\text{-value} = 0.0024$, control $N = 6$, $NR1^{GAD65(+/-)} N = 8$ and $GluA2^{GAD65(+/-)} N = 5$).
- B. Odour discrimination times for complex odour discrimination across groups were similar. (Ordinary one-way ANOVA, $F = 0.5351$, $p\text{-value} = 0.5957$).
- C. ITI for complex odour discrimination across groups were similar. (Ordinary one-way ANOVA, $F = 1.896$, $p\text{-value} = 0.1824$).

4.3.2 Modifications in GAD65-expressing interneurons cause changes in the threshold of detecting and discriminating odours

Thus far, the knockout of GluA2 and NR1 from GAD65-expressing interneurons has shown no significant impact on odour learning behaviour at supra-threshold levels. We further investigated the threshold of odour detection and discrimination in $NR1^{GAD65(+/-)}$ and $GluA2^{GAD65(+/-)}$ animals. Since odorant molecules in the environment are always under the influence of turbulent airflows, thereby making the odour concentration profile dynamic, we hypothesized that the above-mentioned subunits may have a more crucial role in preserving odour quality under dynamic scenarios. To test this, we subjected a new set of animals to undergo odour detection and odour discrimination at different concentrations.

First, we trained them to detect the Limonene (+) odour at the following concentrations: 10^{-10} %, 10^{-9} %, and 10^{-8} %, against mineral oil (MO). At 10^{-10} % odour concentration, $NR1^{GAD65(+/-)}$ and $GluA2^{GAD65(+/-)}$ and control animals displayed accuracies closer to chance level, i.e., 50 % accuracy (Figure 4.4A: Two-way ANOVA, $F(2,62) = 0.8468$, $p = 0.4337$, control: 51.96 ± 1.2 , $NR1^{GAD65(+/-)}$: 50.67 ± 1.9 and $GluA2^{GAD65(+/-)}$: 50.92 ± 1.4). However, at 10^{-9} % odour concentration, control animals exhibited discrimination learning and reached ≥ 80 % accuracy by the end of the task, while $NR1^{GAD65(+/-)}$ and $GluA2^{GAD65(+/-)}$ animals showed impairments in odour discrimination learning (Figure 4.4A: Two-way ANOVA, $F(2,102) = 28.56$, $p < 0.0001$, control: 71.6 ± 5.1 , $NR1^{GAD65(+/-)}$: 60 ± 3.3 and $GluA2^{GAD65(+/-)}$: 66.33 ± 3.7).

When we increased the odour concentration to 10^{-8} %, all animals including $NR1^{GAD65(+/-)}$ and $GluA2^{GAD65(+/-)}$ animals learned the task with similar accuracies as controls (Figure 4.4A: Two-way ANOVA, $F(2,42) = 03.850$, $p = 0.0292$, control: 82.17 ± 2.6 , $NR1^{GAD65(+/-)}$: 80.67 ± 3.1 and $GluA2^{GAD65(+/-)}$: 77.2 ± 4.2). The results demonstrate changes in the threshold for odour detection in $NR1^{GAD65(+/-)}$ and $GluA2^{GAD65(+/-)}$ animals.

Further, we continued training the same set of animals on odour discrimination tasks at different concentration levels to determine the threshold for odour concentration during

discrimination. The odours Ci/Eu were used in the following concentrations: 10^{-10} %, 10^{-8} %, and 10^{-6} %.

At a 10^{-10} % odour concentration, the accuracies of controls, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals remained near chance level performance (Two-way ANOVA, $F(2,48) = 0.4546$, $p = 0.6374$, control: 53.9 ± 0.33 , NR1^{GAD65(+/-)}: 55.06 ± 0.58 and GluA2^{GAD65(+/-)}: 53.17 ± 0.5). When the odours concentration was increased to 10^{-8} %, the learning accuracies of the animals across groups stayed near 50%, indicating lack of discrimination learning (Two-way ANOVA, $F(2,57) = 1.941$, $p = 0.1530$, control: 53.38 ± 0.62 , NR1^{GAD65(+/-)}: 55.33 ± 1.33 and GluA2^{GAD65(+/-)}: 54.81 ± 0.53).

At 10^{-6} % odour concentration, the animals in the control group reached an accuracy of ≥ 85 % by the end of the task. However, the NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals displayed impairment in the discrimination accuracy (Two-way ANOVA, $F(2,114) = 21.69$, $p < 0.0001$, control: 81.21 ± 2.76 , NR1^{GAD65(+/-)}: 70.76 ± 3.89 and GluA2^{GAD65(+/-)}: 69.25 ± 3.07). These findings offer valuable insights into the potential mechanisms utilized by the olfactory system when processing stimuli at sub-threshold levels.

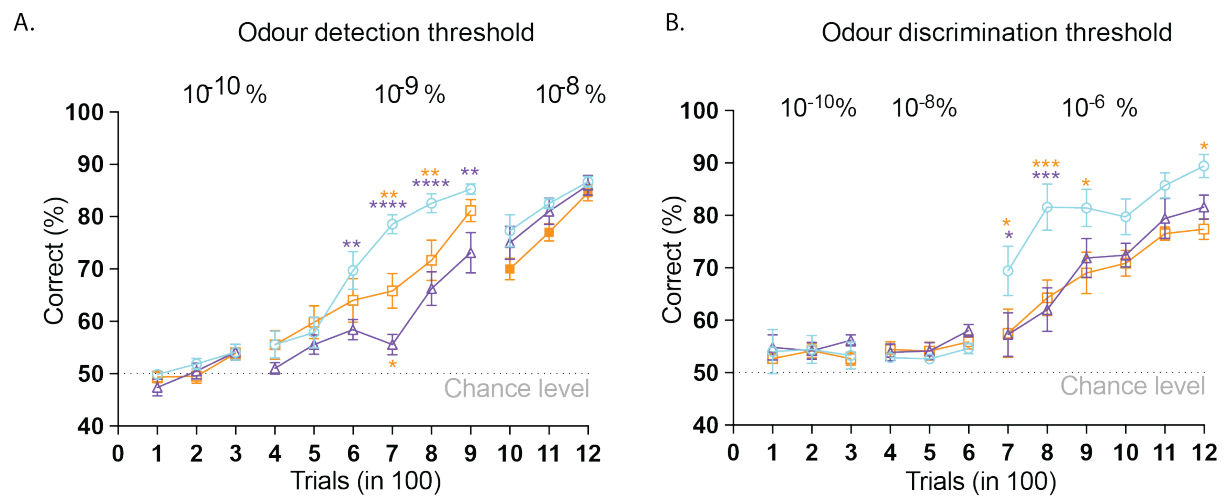


Figure 4.4: Odour detection and discrimination threshold is perturbed in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals

- A. Odour detection learning for simple odour pair Limonene (+) vs MO starting from dilution 10^{-10} % (v/v) to 10^{-8} %. Animals across groups did not learn at the odour dilution of 10^{-10} %. At an odour dilution of 10^{-9} %, control animals learned the task, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals showed impaired detection. Animals learned the detection task at an odour concentration of 10^{-8} %.
- B. Odour discrimination for simple odour pair Ci vs Eu for dilutions 10^{-10} % (v/v), 10^{-8} %, and 10^{-6} %. Control, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals did not learn the discrimination task at 10^{-10} %

and 10^{-8} % odour dilutions. At 10^{-6} % odour concentration, controls reached $\geq 85\%$ accuracy. However, NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals showed impaired discrimination learning.

4.3.3 Inhibition via the GAD65-expressing interneurons in the olfactory bulb modulates the airflow discrimination behaviour

In nature, odour plumes are always accompanied by airflows, and the complexity of airborne odour plumes has been well-documented (Celani et al., 2014). Wind helps to carry the odour molecules from a source to different parts, thereby providing an additional mechanical component to odours in the form of airflows. One pioneering study performed in vitro demonstrated OSN activity upon airflow stimulation, displaying the dual functions of OSNs as odour molecules as well as mechanical detectors (Grosmaître et al., 2007). Some studies indicate that airflow information can be processed through the olfactory system (Iwata et al., 2017; R. Wu et al., 2017). In the lab, it has been shown that bullectomy in mice leads to impairment in airflow discrimination abilities, indicating the importance of the OB in processing mechanical stimuli (Mahajan et al., 2024, submitted). However, the neural mechanisms of airflow information through the olfactory system remain unknown. Here, we sought to deduce the role of ionotropic glutamate receptors of GAD65-interneurons and thereby the role of synaptic inhibition in processing mechanical stimuli such as airflow.

We subjected NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} mice to airflow discrimination tasks (0.4 vs 0.5 LPM, 0.25 vs 0.30 LPM and 0.25 vs 0.30 LPM). Firstly, animals were trained on 0.4 vs 0.5 LPM airflow discrimination task for 1200 trials. Controls and NR1^{GAD65(+/-)} animals learned the discrimination task and reached $\geq 80\%$ accuracy. However, impaired airflow discrimination ability was seen in GluA2^{GAD65(+/-)} animals (Figure 4.5 A1: Two-way ANOVA, $F(2,306) = 71.70$, $p < 0.0001$, control: 79.74 ± 1.3 , NR1^{GAD65(+/-)}: 81.87 ± 2.1 and GluA2^{GAD65(+/-)}: 64.44 ± 1.7). Further, mice were trained on the 0.25 vs 0.30 LPM and 0.1 vs 0.15 LPM airflow discrimination tasks, which yielded similar outcomes. We observed impairment in airflow discrimination learning in GluA2^{GAD65(+/-)} animals compared to controls and NR1^{GAD65(+/-)} animals (Figure: 4.5A2, Two-way ANOVA, $F(2,126) = 17.06$, $p < 0.0001$, 4.5A3, $F(2,90) = 5.625$, $p = 0.0050$).

We calculated the DTs using the lick patterns from the last task. GluA2^{GAD65(+/-)} animals demonstrated significantly higher DTs compared to controls and NR1^{GAD65(+/-)} animals (Figure 4.5 B1,B2,B3: Ordinary one-way ANOVA, control vs GluA2^{GAD65(+/-)} $p < 0.05$, control vs NR1^{GAD65(+/-)} $p > 0.05$). As indicated by the time during ITI, the differences in learning and DTs were not due to variations in the motivation of the animals, as animals across groups showed

similar ITIs (Figure 4.5 C1, C2, C3: Ordinary one-way ANOVA, $p > 0.05$). Hence, taken together, our results provide conclusive evidence for the role of iGluRs in fine odour discriminations as well as airflow discriminations.

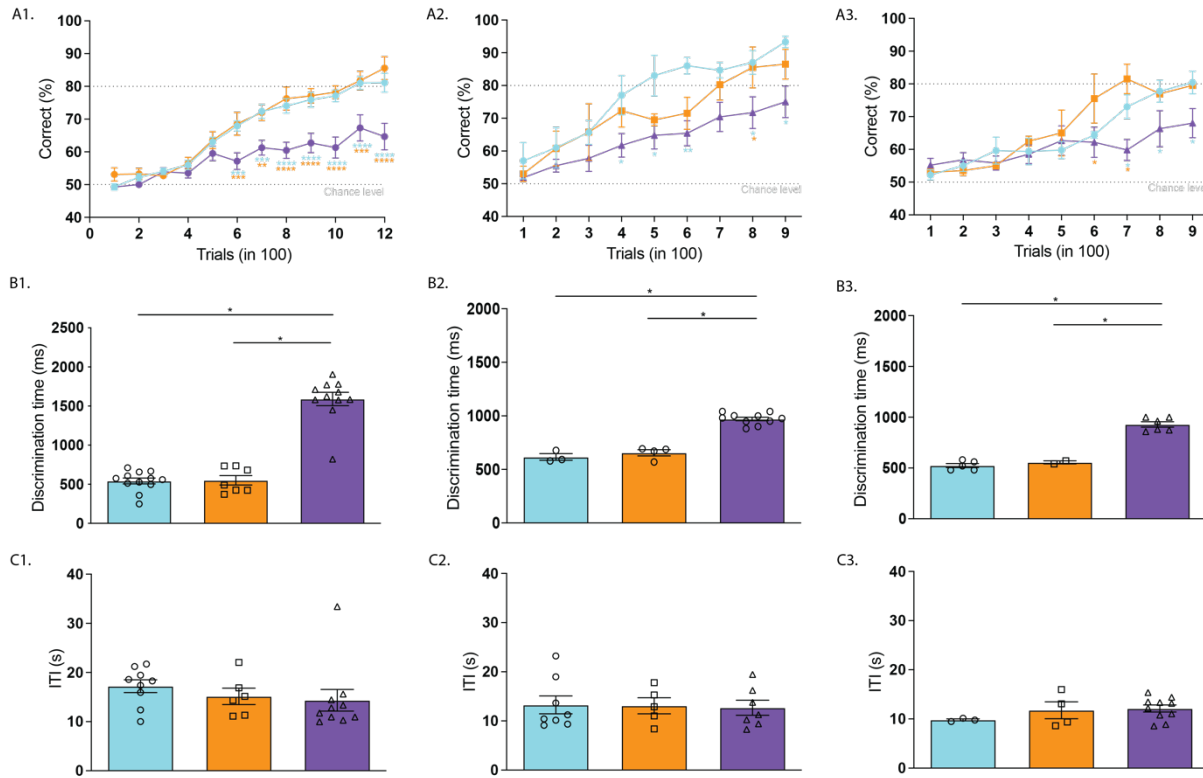


Figure 4.5: Increased inhibition of MTCs results in impairment in airflow discrimination

A1,B1,C1. On comparing the learning accuracies of NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} mice with WT control mice, we observed no change in NR1^{GAD65(+/-)} animals' airflow discrimination abilities. However, GluA2^{GAD65(+/-)} mice showed lower airflow discrimination abilities. (A1. 0.4 vs 0.5 LPM ordinary two-way ANOVA, $F(2,306) = 71.70$, $p \leq 0.0001$, A2. 0.25 vs 0.3 LPM, ordinary two-way ANOVA, $F(2,126) = 17.06$, $p \leq 0.0001$, A3. 0.1 vs 0.15 LPM, ordinary two-way ANOVA, $F(2,90) = 5.625$, $p = 0.005$).

A2,B2,C2. GluA2^{GAD65(+/-)} mice showed higher (DTs) compared to control and NR1^{GAD65(+/-)} animals. Data are presented as mean $\pm$ SEM. (A2.Ordinary one-way ANOVA, $F = 89.97$, Control vs NR1^{GAD65(+/-)} $p = 0.9353$, Control vs GluA2^{GAD65(+/-)} $p \leq 0.0001$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} $p \leq 0.0001$, A2. Ordinary one-way ANOVA, $F = 77.94$, Control vs NR1^{GAD65(+/-)} $p = 0.3638$, control vs GluA2^{GAD65(+/-)} $p \leq 0.0001$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} $p \leq 0.0001$. A3.Ordinary one-way ANOVA, $F = 83.76$, control vs NR1^{GAD65(+/-)} $p = 0.5142$, control vs GluA2^{GAD65(+/-)} $p \leq 0.0001$, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} $p \leq 0.0001$).

A3,B3,C3. The ITIs were similar across groups, implying similar motivation levels. Data are presented as mean $\pm$ SEM. (A3. Ordinary one-way ANOVA, $F = 4.916$, control vs NR1^{GAD65(+/-)} p

= 0.4568, control vs GluA2^{GAD65(+/-)} p = 0.0113, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} p = 0.1635, B3. Ordinary one-way ANOVA, F = 1.071, control vs NR1^{GAD65(+/-)} p = 0.3073, control vs GluA2^{GAD65(+/-)} p = 0.1669, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} p = 0.8028. C3. Ordinary one-way ANOVA, F = 0.6973, control vs NR1^{GAD65(+/-)} p = 0.4728, control vs GluA2^{GAD65(+/-)} p = 0.2586, NR1^{GAD65(+/-)} vs GluA2^{GAD65(+/-)} p = 0.7774).

4.4 Discussion

4.4.1 Role of ionotropic glutamate receptors in odour information processing

In rodents, the olfactory system serves as a fundamental sensory modality. The capacity to detect and differentiate odours is essential for their survival, given that tasks like feeding, foraging, and mating are heavily influenced by the animal's olfactory abilities (Firestein, 2001; Shipley & Ennis, 1996). These behaviours demand that animals extract and perceive different features of the odour stimulus such as odour identity, odour concentrations, and airflow, which help carry the odorant molecules. In this chapter, the role of ionotropic glutamate receptors (iGluRs) in influencing odour detection, discrimination, discrimination threshold concentration, and airflow discrimination abilities of animals has been studied.

We observed that the odour discrimination abilities of the NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals were unhampered when subjected to optimal odour concentrations. However, the odour detection and discrimination threshold concentration was shifted in the KOs. Perceiving small changes in odour concentration is a useful property of the rodent olfactory system as it helps them to locate the source of the odour (P. W. Jones & Urban, 2018; Parabucki et al., 2019). They can locate the odour source either by comparing simultaneous samples from the two nostrils or by evaluating samples taken sequentially from various locations (Catania, 2013; Y. Wu et al., 2020). It has been shown that the information conveyed from the OB to the OC through M/TCs is responsible for the perceptions of “odour objects”, which represent various features of the odour such as identity and concentration (Gottfried, 2010; D. A. Wilson & Sullivan, 2011). The input to the glomeruli at the GL is highly concentration-dependent. In other words, exposure to odours of higher concentrations activates more glomeruli. This effect is partly due to the inhibition of GABA release, resulting in an increase in input strength at the GL (Abraham et al., 2014; Rubin & Katz, 1999; Spors & Grinvald, 2002), while OB output is normalized over a wide range of odour concentrations (Banerjee et al., 2015; Economo et al., 2016; Roland et al., 2016; Sirotin et al., 2015). Interestingly, it has been demonstrated that information about odour concentration reappears in the PCx (Stettler & Axel, 2009; Sugai et al., 2005; Xia et al., 2015). This suggests the

potential for OB circuitry, particularly downstream of the GL, to perform computations ensuring an accurate representation of odour concentration information reaching the PCx. The inhibitory network of the GCL may contribute significantly to this process. Additionally, studies have demonstrated that the odour identity and odour concentration are represented by different and independent features of neural response such as a subset of activated glomeruli, latency of M/TC activation etc (Miura et al., 2012).

Odorants activate different glomeruli and M/TCs during specific phases of the respiratory cycle. Prior studies have demonstrated that increasing the odour concentration does not alter the spiking in the activated M/TCs but rather decreases their onset latencies (Cang & Isaacson, 2003; Chalansonnet & Chaput, 1998; Fukunaga et al., 2012; Margrie et al., 2001; Sirotin et al., 2015). This implies that the same subset of M/TCs may be activated by a specific odour at different concentrations but with shorter latencies at higher concentrations. This suggests that the perception of odour concentration is mainly dependent on the temporal coding of M/TCs activity at the GL (Bolding & Franks, 2017; Haddad et al., 2013). The latency of M/TCs activity at the GL is observed at various odour concentrations. However, the OB circuitry downstream to the GL may compensate for low odour concentration while keeping the odour identity unchanged. Still, at PCx, this odour concentration information is maintained due to the inhibitory network at the PCx (Bolding & Franks, 2017). In the modifications we have done, we may have disrupted the ‘odour identity’ at low concentrations, and hence we observed impairment in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals during odour discrimination at lower concentrations. Nevertheless, to encode a broad range of odour concentrations while maintaining the same odour identity, the OB circuitry downstream of GL likely plays a crucial role. Therefore, disrupting the inhibitory circuit of GAD65-expressing interneurons could potentially result in a loss of fidelity in representing odour concentration as observed at the GL.

Here, we showed that changing the ionotropic glutamate receptor composition of GAD65-expressing interneurons resulted in loss of odour discrimination at sub-threshold concentrations, keeping odour discrimination at supra-threshold concentrations intact. This suggests the possibility of an altered temporal code of odours at lower concentrations in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals, leading to impaired detection and discrimination. Further experiments are needed to investigate the perturbations in the temporal codes of M/TCs in NR1^{GAD65(+/-)} and GluA2^{GAD65(+/-)} animals.

4.4.2 Role of ionotropic glutamate receptors in airflow information processing

Odour information is received by OSNs, processed in the OB, and then conveyed to the olfactory cortex (Lledo et al., 2005; Shepherd et al., 2004). The OB is the first relay station where odour information processing and coding occur. Previous studies have linked the rhythmic activity of the OB with respiration (Fontanini et al., 2003; Macrides & Chorover, 1972; Onoda & Mori, 1980; M. E. Phillips et al., 2012; Ueki & Domino, 1961), indicating that the OB can also sense airflow information. A study conducted in 2007, demonstrated for the first time, that approximately 70% of OSNs in the septal organ (SO) and around 50% of OSNs in the MOE respond to mechanical stimulation (Grosmaître et al., 2007). This suggests that OSNs are capable of sensing both odours as well as airflows. It has been speculated that both odour and airflow detection are mediated by the same cAMP cascade. A study has shown that different OSNs expressing different receptors have varied responses to mechanical stimulation such as air puff. This suggests that GPCRs might function as mechanoreceptors as well (Connelly et al., 2015).

The responses elicited by odour and airflow exposure in the OB have been shown to be different. A BOLD-fMRI study of the OB upon odour exposure revealed odour-specific and sparse activity, while airflow exposure resulted in broadly distributed OB activity. The activity maps corresponding to different airflows are stereotypic (R. Wu et al., 2017). Previous results from the lab have shown that the optogenetic activation of GAD65-expressing interneurons enhances odour discrimination learning, while optogenetic inhibition of these interneurons leads to impairment in the odour discrimination abilities of animals. However, in the case of airflow discrimination, activation of GAD65-expressing interneurons results in the impairment of task learning, and inhibition of GAD65-expressing interneurons enhances airflow discrimination learning (Mahajan et al., 2024, submitted). These outcomes hint at differential mechanisms employed by the OB for the discrimination of odours and airflows.

Here, we genetically modulated GAD65-expressing interneurons and observed the loss of airflow discrimination abilities in GluA2^{GAD65(+/-)} animals. Removal of GluA2 from AMPARs causes glutamate-mediated Ca²⁺ influx (Brusa et al., 1995; Feldmeyer et al., 1999; Z. Jia et al., 1996), leading to increased GABA release and stronger inhibition of the M/TCs (Abraham et al., 2010; Chen et al., 2000; Halabisky et al., 2000b; Isaacson, 2001; Isaacson & Strowbridge, 1998). This modification mimics the optogenetic activation of GAD65-expressing interneurons. Hence, the differential results obtained in odour discriminations and airflow discriminations point towards a possibility of differential optimal inhibition for odour and airflow discriminations.

4.5 Future experiments

The olfactory world is first represented at the OB. The M/TCs of OB are synaptically coupled via inhibitory interneurons arranged in a two-stage network (Aungst et al., 2003). Granule cells (GCs) at the GCL are the most numerous types of inhibitory interneurons in the OB. Researchers have previously demonstrated that optogenetic stimulation of GCs improves odour discrimination learning by enhancing pattern separation (Gschwend et al., 2015). Hence, we aimed to understand the role of GCs in airflow discrimination. To answer the question, we conducted some pilot experiments in which we injected ChR2-viral particles in the GCL of GAD65-Cre animals and optogenetically activated GCL interneurons during airflow discrimination tasks. After the surgical procedure, as explained in Chapter 2, animals were subjected to task habituation. After task habituation, optimization of LED power was performed to decide the light intensity for ChR2 stimulation (Figure 4.6).

Further, we performed an airflow discrimination task (0.4 vs 0.5 LPM) under no-light conditions. We observed that the animals started learning with chance-level accuracy and reached $\geq 80\%$ accuracy by the end of the task. We continued with the training of the animal on the (0.1 vs 0.15 LPM) airflow discrimination task under light-on conditions. We observed a rapid increase in the learning accuracy of the animals (Figure 4.7). This increase might result from the extent of training. Hence, we trained the animals on the next (0.25 vs 0.30 LPM) airflow pair under no-light conditions. The animals followed a particular learning trajectory, starting with chance-level performance and reaching $\geq 80\%$ accuracy by the end of the task. To reproduce the result we obtained in the first light-on condition, we trained the animals for the next airflow discrimination task (0.35 vs 0.45 LPM) under the light-on condition and observed an increase in accuracy, reaching $\geq 90\%$ accuracy by the end of the task. As we had limitations with these experiments, further studies are needed to probe the role of GCs in the airflow discrimination abilities of rodents.

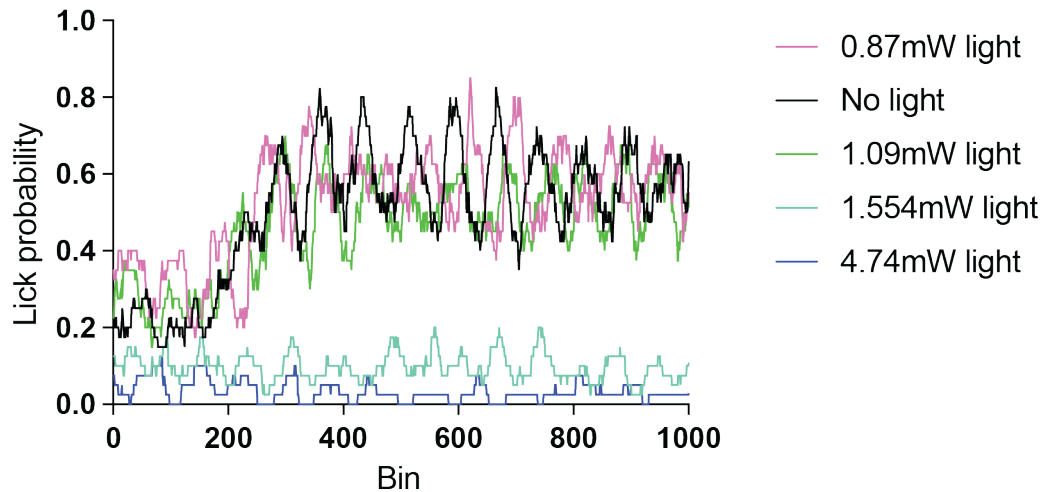


Figure 4.6: Optimization of LED power for optogenetic modulation of GAD65-expressing interneurons in the granule cell layer during airflow discrimination task

Adjustment of LED power for optogenetic stimulation of GAD65 interneuron in the GCL expressing Chr2. This is a representative graph for the licking pattern evoked by odour presentation with different light intensities. We observed that increasing LED power progressively suppressed licking behaviour compared to the licking during no-light trials (Black line). For each mouse, the highest power which preserved licking response during odour presentation was selected for odour discrimination tasks.

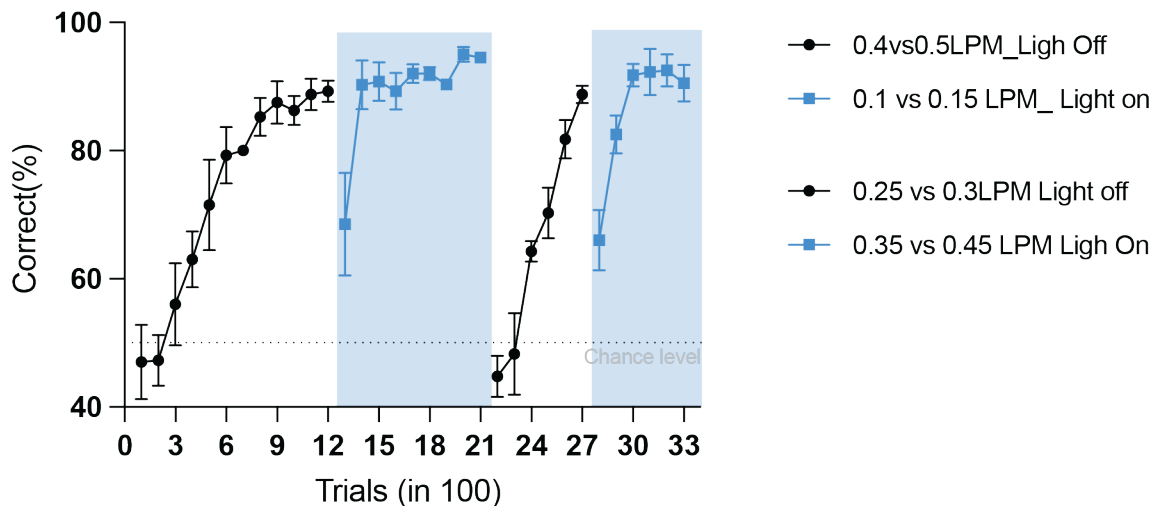


Figure 4.7: Optogenetic activation of GAD65 interneurons in the Granule cell Layer enhances airflow discrimination abilities

Light stimulation on GAD65-expressing interneurons in the GCL expressing Chr2 increased the learning pace as compared to the learning pace under no light conditions.

Publications

1. Pardasani, M., **Marathe, S. D.**, Purnapatre, M. M., Dalvi, U., & Abraham, N. M. (2021). Multimodal learning of pheromone locations. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 35(9), e21836.
2. Mahajan S, Sen D, Sunil A, Srikanth P, **Marathe SD**, Shaw K, Sahare M, Galande S, Abraham NM. Knockout of angiotensin converting enzyme-2 receptor leads to morphological aberrations in rodent olfactory centers and dysfunctions associated with sense of smell. *Front Neurosci*. 2023 Jun 19;17:1180868.

Manuscripts under review:

1. Mahajan S, Tamboli S, Das S, Bhattacharjee AS, Pardasani M, Srikanth P, **Marathe SD**, Adlakha A, Ranjan L, Pandey S, Abraham NM. Mouse olfactory system acts as an anemo-detector and -discriminator (submitted).
2. Paul, R., Sarkar, S., **Marathe, S. D.**, Murali, K., Das, S., Abraham, N. M., & Varma, H. M. (2023, March). Functional imaging of olfactory bulb and somatosensory cortex in mice using small-animal blood flow imaging platform. In *Dynamics and Fluctuations in Biomedical Photonics XX* (Vol. 12378, pp. 45-48). SPIE.
3. Rajdeep Bhowmik, Meenakshi Pardasani, Sarang Mahajan, Anindya S. Bhattacharjee, Sasank Konakamchi, Shambhavi Phadnis, Thasneem Musthafa, Eleanor McGowan, Priyadharshini Srikanth, **Shruti D. Marathe**, Nixon M. Abraham (2022) Uncertainty revealed by delayed responses during olfactory matching. *bioRxiv* 2022.09.11.507462.

Manuscripts under preparation:

1. **Marathe, S. D.**, Meenakshi Pardasani & Abraham, N. M. Role of synaptic inhibition in multimodal learning of pheromone locations.
2. **Marathe, S. D.**, Sanyukta Pandey & Abraham, N. M. Modifying rodent olfactory circuits: a strategy to create animal models of brain dysfunctions.

Appendix

Copyright forms



Multimodal learning of pheromone locations

Author: Meenakshi Pardasani, Shruti D. Marathe, Maitreyee Mandar Purnapatre, et al

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