NMDAR Plasticity and Metaplasticity in Early Odor Preference Learning in Rats

By

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Abstract

Early odor preference learning is a classical conditioning behavioral model which can be used to understand the molecular mechanism of learning and memory, and synaptic plasticity. In this thesis the research is focused on the role of N-methyl D-aspartate receptor (NMADR) plasticity and metaplasticity in early odor preference learning.

In Chapter 2, we investigated the differential roles of L-type calcium channel (LTCC) and NMDAR in early odor preference learning. The results suggest that the NMDAR is crucial for creating stimulus-specific memory and LTCC is required for maintenance of the memory. Activation of LTCC without NMDAR can cause loss of input specificity and as a result, generalization of the memory.

Chapter 3 depicts that, the effect of altered number of NMDARs in the anterior piriform cortex (aPC) can significantly modifies future learning and synaptic plasticity. Here we show that NMDARs are down regulated at 3hr following the early odor preference learning. Repeated training at 3hr leads to unlearning and this unlearning is mediated by NMDAR itself. Inhibition of the NMDAR prior to retraining at 3hr, blocks unlearning. In continuation of Chapter 3, we have characterized the molecular mechanism underlying the NMDAR mediated unlearning in Chapter 4. Calcineurine and metabotropic glutamate receptor (mGluR) plays a vital role in NMDAR downregulation at 3hr following early odor preference training.

In Chapter 5, we have investigated whether NMDAR plasticity and its mediated metaplasiticity observed in the early odor preference learning can be induced by stronger

trainings that produce prolonged memories. Previous research has shown that infusing trichostatin A (TSA), a histone deacetylation inhibitor, in the olfactory bulb (OB) extends odor preference memory up to 5 days. Our data suggests that OB TSA infusion preventsNMDAR down-regulation and unlearning. These outcomes argue that it is critical to understand the metaplastic effects of training which have implications for learning optimization.

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Since my childhood, I was not a good student at all. It would be better to say that I was below average student but somehow I always managed to pass from one class to another. I continued to a new class and also the problem remained the same as usual. I was unable to understand why I was reading physics, chemistry, and mathematics? Why it is necessary to learn those subjects and what are the implications? Unfortunately, no one was able to tell me answers to those questions. As a result, I scored extremely badly in the higher secondary examination and with that score, it was almost impossible to get into college but some internal connection worked out and I got into Panskura Banamali College in the Microbiology Department. Before, I start "the thanks" list from my college I would like to mention some names during school time. I am really thankful to them for increasing my confidence. Sayantan Ghosh, an excellent student who never disrespected any poor student ever. I would like to thank him for his support for four to five years. Same as Sayantan, Arijit Sett (A grade student) your support worked really well and still it's working. But, especially I would like to give a huge thanks to our biology teacher Ashish Bhowmick. Your enthusiasm and teaching style enhanced my interests in the biological world.

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List of Abbreviations

2-DG	2-Deoxy-D-glucose
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AMPAR	AMPA receptor
AON	Anterior olfactory neuron
aPC	Anterior piriform cortex
ATD	Extracellular amino-terminal domain
cAMP	Cyclic adenosine monophosphate
Ca^{2+}	Calcium ion
CaM	Calmodulin
CaMKII	Calmodulin kinase II
CaMKIV	Calmodulin kinase IV
CaN	Calcineurin
CREB	Cyclic adenosine monophosphate response element-binding protein
CS	Conditioned stimulus
CR	Conditioned response

CTD	Intracellular carboxyl-terminal domain
D-APV	D-amino-5-phosphonopentanoate
DNQX	6,7-Dinitroquinoxaline-2,3-dione
EPSP	Excitatory post-synaptic potential
EPL	External plexiform layer
fEPSP	Field excitatory post-synaptic potential
GABA	Gamma-aminobutyric acid
GC	Granule cell
GCL	Granule cell layer
GL	Glomerular layer
GPCR	G protein coupled receptor
GR	Glutamate Receptor
IHC	Immunohistochemistry
ISO	Isoproterenol
LBD	Ligand binding domain
LC	Locus coeruleus
LOT	Lateral olfactory tract
LTCC	Voltage gated L-type calcium channel
LTD	Long term depression
LTP	Long term potentiation
MC	Mitral cell
MCL	Mitral cell layer
MG^{2+}	Magnesium ion

mGluR	Metabotropic glutamate receptor
NE	Norepinephrine
NMDA	N-methyl-d-aspartate
NMDAR	NMDA receptor
NTX	Naltrexone
OB	Olfactory bulb
OD	Optic density
ORN	Olfactory receptor neuron
ON	Olfactory nerve
ONL	Olfactory nerve layer
OP	Olfactory peduncle
OR	Odor receptor
ORN	Olfactory receptor neuron
OS^+	Odor + stroke training
OS^-	Odor only training
OSN	Olfactory sensory neuron
OT	Olfactory tubercle
PBS	Phosphate buffer solution
pCREB	Phosphorylated CREB
PCX	Piriform cortex
PDZ	First three protein identified with this domain (PSD95, Dlz1 and Zo-1)
PKA	Protein kinase A
pPC	Posterior piriform cortex

PD	Postnatal day
PG	Periglomerular
PSD-95	Postsynaptic density protein 95
ROD	Relative optic density
ROI	Region of interest
SEM	Standard error of measurement
SOM	Somatostatin
TBS	Theta burst stimulation
TC	Tufted cell
TMD	Transmembrane domain
TSA	Trichostatin A
UCS	Unconditioned stimulus

Co-authorship Statement

I, Bandhan Mukherjee, hold a first author status for all the publications used in this thesis as Chapters (Chapter 2-5). However, each publication is co-authored by my supervisors and colleagues, whose contributions have greatly influenced the generation of my hypotheses, experimentation and the writing of the manuscripts.

Contribution from peers in each manuscript is elaborated below.

Manuscript titled “NMDA receptors in mouse anterior piriform cortex initialize early odor preference learning and L-type calcium channels engage for long-term memory" (Chapter 2) in my thesis is co-authored by Qi Yuan. As the first author, I was partly involved in experimental design, conducted all behavioral, immunohistochemistry and Western blot experiments, and took part in writing the first draft of the paper. Qi Yuan conducted the calcium imaging experiments with a minor contribution from me. Research questions and experimental design were originally developed by Qi Yuan. The subsequent adjustment was done by me with proper guidance from Qi Yuan. The whole manuscript was edited and polished by Qi Yuan.

My second paper titled "Unlearning: NMDA receptor-mediated metaplasticity in the anterior piriform cortex following early odor preference training in rats" (Chapter 3) in this dissertation is co-authored by Gillian Morrison, Christine Fontaine, Qinlong Hou, Carolyn W. Harley, and Qi Yuan. Hypothesis and research involved in this manuscript were developed by Qi Yuan. As the

first author of this manuscript, I conducted the majority of the electrophysiological and behavioral experiments in the final figures, data analysis, and first draft writing. Qinlong Hou, Gillian Morrison, and Christine Fontaine contributed to the Western blot and SiRNA experiments. The manuscript was finalzied by Qi Yuan and Carolyn Harley.

My 3rd paper titled “Learning-Induced Metaplasticity? Associative Training for Early Odor Preference Learning Down-Regulates Synapse-Specific NMDA Receptors via mGluR and Calcineurin Activation" (Chapter 4) is co-authored by Carolyn Harley and Qi Yuan. Hypothesis and research design were developed by me and Qi Yuan. As the first author of this manuscript, I performed all experiments and wrote part of the first draft. Final manuscript was written by Qi Yuan and Carolyn Harley.

My 4th paper titled "Revisiting metaplasticity: The roles of calcineurin and histone deacetylation in unlearning odor preference memory in rat pups" (Chapter 5) is co-authored by Sriya Bhattacharya, Jacqueline Blundell, Carolyn Harley, and Qi Yuan. As the co-first author of this article, I and Sriya Bhattacharya equally conducted all the experiments, analyzed data and wrote the method section. Qi Yuan and I designed the research project. Qi Yuan, wrote the manuscript. Carolyn Harley and Jacqueline Blundell edited the manscript. I helped in the manuscript writing and addressing reviewers’ comments.

Overview

Memory is a very important part of our life. Without memory, our existence would be meaningless and we wouldn't be able to become today's human being. But, understanding the mechanisms involved in forming memory is complicated. Different scientists use different memory-related behavioral models to investigate the process of memory. In my research, I have chosen the early odor preference learning paradigm to investigate the molecular mechanisms involved in learning. These experiments are intended to identify the malleable states of synapses, which can be modified, and the modification of which could affect behavior. These studies add to current learning and memory research by providing a stepwise examination of the pathways involved in both synaptic plasticity (Section 1.3) and metaplasticity (Section 1.3.4). Along with identifying plasticity mechanisms and pathways, these experiments also examine the effect of changes in synaptic plasticity and metaplasticity on behavior.

Calcium plays a crucial role in learning and memory, synaptic plasticity and many different signaling pathways. There are various types of calcium channels present in different cells. Among them, L-type calcium channels (LTCCs) and NMDA receptors (NMDAR) are two calcium-permeable channels that plays a crucial role in learning and memory, and synaptic plasticity. The interactions of LTCCs and NMDARs in memories

are poorly understood. In Chapter 2, I investigated the specific roles of anterior piriform cortex (aPC) LTCCs and NMDARs in early odor preference memory in mice.

Using calcium imaging in aPC slices, LTCC activation is shown to be dependent on NMDAR activation. Behaviorally, in mice that underwent early odor preference training, blocking NMDARs in the aPC prevented short-term (3 hr) and long-term (24 hr) odor preference memory and both memories were rescued when BayK-8644 (a LTCC agonist) was co-infused. However, activating LTCCs in the absence of NMDARs (Pharmacological blockade) resulted in the loss of discrimination between the conditioned odor and a similar odor mixture at 3 hr. Elevated synaptic AMPA receptor expression at 3 hr was prevented by D-APV (NMDAR inhibitor) infusion prior to training, but was restored when LTCCs were directly activated, mirroring the behavioral outcomes. Blocking LTCCs prevented 24 hr memory, but spared 3 hr memory. These results suggest that NMDARs mediate stimulus-specific encoding of odor memory, while LTCCs mediate intracellular signaling leading to long-term memory.

In Chapter 3, we demonstrate a metaplastic change in NMDAR numbers in the aPC in rat pup induced by a 10 min pairing of peppermint odor+stroking, which significantly modifies later learning and memory. Using isolated synaptoneurosomes, we found GLUN1 subunit of NMDAR (GluN1) receptor down-regulation 3 hr after training and up-regulation at 24 hr. Consistent with the GluN1 pattern, the NMDAR-mediated excitatory post synaptic potentiation (EPSP) was smaller at 3 hr and larger at 24 hr.

Subunit composition was unchanged. While long-term potentiation (LTP) was reduced at both times by training, long-term depression (LTD) was facilitated only at 3 hr.

Behaviorally, pups, given a pairing of peppermint+stroking 3 hr after initial peppermint+stroking training, lose the normally acquired peppermint preference 24 hr later. To probe the pathway specificity of this *unlearning* effect, pups were trained first with peppermint and then, at 3 hr, given a second training with peppermint or vanillin. Pups given peppermint training at both times lost the learned peppermint preference. Pups given vanillin re-training had normal peppermint preference when trained with vanillin 3 hr later. Down-regulating GluN1 with siRNA prevented odor preference learning. Finally, the NMDAR antagonist, MK-801 (NMDAR pore blocker), blocked the LTD facilitation seen 3 hr post-training and giving MK-801 prior to the second peppermint training trial eliminated the loss of peppermint odor preference. A training-associated reduction in NMDARs facilitates LTD 3 hr later; while training at the time of LTD facilitation reverses an LTP-dependent odor preference. Experience-dependent, pathway-specific metaplastic effects in a cortical structure have broad implications for the optimal spacing of learning experiences**.**

In a continuation of the previous work (Chapter 3), Chapter 4 showed the underlying molecular pathways involved in the unlearning process. Rat pups readily form a 24 h associative odor preference after a single trial of odor paired with intermittent

stroking. Our previous work (Chapter 3) shows that this training trial, which normally increases AMPA receptor responses in the anterior piriform cortex both 3 h and 24 h following training, induces a down-regulation of NMDA receptors 3 h later followed by NMDA receptor up-regulation at 24 h.

When retrained with the same odor at 3 h, rat pups unlearn the original odor preference. Unlearning can be prevented by blocking NMDA receptors during retraining. In the fourth Chapter the mechanisms that initiate NMDA receptor down-regulation are assessed. Blocking mGluR receptors or calcineurin during training prevents down-regulation of NMDA receptors 3 h following training. Blocking NMDA receptors during training does not affect NMDA receptor down-regulation. Thus down-regulation can be engaged separately from associative learning. When unlearning occurs, AMPA and NMDA receptor levels at 24 h are reset to control levels. Calcineurin blockade during retraining prevents unlearning consistent with the role of NMDA receptor down-regulation. The relationship of these events to the metaplasticity and plasticity mechanisms of long-term depression and depotentiation is discussed. We suggest a possible functional role of NMDA receptor down-regulation in the offline stabilization of learned odor representations.

Previous work had shown that 24 h duration odor preference learning, induced by one-trial training, generates a down-regulation of the GluN1 receptor in aPC at 3 h, and results in metaplastic unlearning if a second training trial is given at 3 h. The GluN1 receptor upregulates at 24 h so 24 h spaced training is highly effective in extending

memory duration. Chapter 5 replicates the piriform cortex unlearning result in the olfactory bulb circuit and further studies the relationship between the initial training strength and its associated metaplastic effect. Intrabulbar infusions that block calcineurin or inhibit histone deacetylation normally produce extended days-long memory. If given during training, they are not associated with GluN1 downregulation at 3 h and do not recruit an unlearning process at that time. The two memory strengthening protocols do not appear to interact, but are also not synergistic. These outcomes argue that it is critical to understand the metaplastic effects of training in order to optimize training protocols in the service of either memory strengthening or of memory weakening.

Chapter 1: Introduction

1.2 Learning and Memory

Learning and memory are two distinct but interconnected concepts. It is impossible to learn or develop language, relationships, and personal identity without previous memories [1]. Learning and memory are one of the most intensively studied subjects in the field of neuroscience.

Memory is a process by which we gather information from past experiences and use that information in the present [2]. On the other hand, learning is described as a process of acquiring new, or modifying, existing memories [3]. A memory can be achieved by some

series of procedures. The initiation process is called acquisition of memory or learning. The following step is called consolidation of memory. The consolidation process is dependent on the initial learning phase. Depending on the stimulus, memory can be consolidated into short-term memory (STM) or long-term memory (LTM) (LTM will be explained more thoroughly in section 1.2.1). The last and crucial step is called memory retrieval. A memory is meaningless if we cannot retrieve the information for later use. There are two other procedures also involved in learning and memory, which are memory extinction and forgetting. In both operant conditioning and classical conditioning behavior, memory extinction is possible. In the case of operant conditioning, the behaviour gradually stops occurring when the operant behaviour that has been previously reinforced is now unable to produce reinforcing consequences.[4]. In case of classical conditioning, when a conditioned stimulus (CS) is presented alone so that it no longer predicts the coming of the unconditioned stimulus (UCS), the conditioned response gradually diminishes. Memory extinction is a process when an animal learns to uncouple a response from a stimulus. For example in contextual fear memory, a rodent learns to show no freezing when placed repeatedly in the same context without shock, and after multiple trials, the rodent learns that the same context is not associated with shock anymore. Similarly, forgetting is a spontaneous or gradual process in which an individual is unable to recall information already encoded and stored in an individual's LTM [5].

Broadly, memory can be classified as "declarative memory" which requires the conscious effort of our mind [6] and "non-declarative memory" which is not available to our consciousness [7]. Declarative memory help us to remember about facts and events. In

other words, we can verbally "declare" these type of memories. On the other hand non-declarative memory is a type of memory that we are unable to "declare" verbally [6]. This type of memory dosen't need conscious effort to recall the memory for example motor skills, habituation in particular environment, associative (classical and operant conditioning) and non-associative learning (reflex). Clinical observations showed that patients with an impaired declarative memory could have their procedural memory completely spared, which suggests that these two types of memory are independent of each other [8]. Thus, neuroscientists concluded that there must be separate mechanisms involved for each type of memory [8]. These different memories appear to be encoded by distinct brain areas and circuitries [9]. For example, basal ganglia are involved in procedural learning and memory, such as habit learning [10]. On the other hand, the medial temporal lobes are more involved in declarative memory [10]. Scientists have shown that the hippocampus is implicated in both spatial and contextual memory [11] which would fit into the explicit type memory category. The olfactory bulb (OB) and olfactory cortex (OC) are two areas involved in olfactory learning and memory [12] [13] [14] and will be further explored in this thesis.

1.2.1 STM and LTM

Temporally, and mechanistically, memories have been characterized as having multiple phases, typically, STM, intermediate-term memory (ITM) and LTM[15–17]. By definition STM refers to the capacity for holding a small amount of information in mind in an active, readily available state for a short time period (minutes to hrs depending on

the behavioral models studied). On the contrary, LTM holds the information for the longer time period for later manipulation. ITM is a distinct stage of memory separated from sensory memory, working memory, STM and LTM [18,19]. ITM is a simultaneous process, rather than sequential, and requires mRNA translation, but not transcription [19].

Memories can be divided into STM and LTM based on their duration [20]. Studies on patient H.M. suggest that the recall of these two types of memory may be mediated by different areas of the brain [21]. Henry Molaison (H.M), the patient, was unable to form any new declarative memories whereas he was able to recall LTM that formed well before his surgery. One of the major questions in the field of memory research is whether STM is qualitatively distinct from LTM or whether they can be represented as a single quantitative continuum. Various neuropsychological case studies and their data influenced this debate. In one study, researchers showed that patients with damage to the medial temporal lobe demonstrated deficits in LTM, although their ability to form STM was intact [22]. On the other hand, patients with perisylvian cortex damage demonstrated a deficit in maintaining short-term phonological information, even though their LTM remained intact [23].

The memory retrieval process generates information on the basis of a retrieval cue, remembered details of a past event, and the strength of the memory [24]. The decision process determines how we act on this information [24]. The broadly classified memory categories are not only temporally different; there are differences in their dependence on protein synthesis as well. For example, STM does not require protein synthesis, whereas

ITM depends on translation, but does not require mRNA transcription. LTM requires both translation and transcription [25–31].

For both STM and LTM, neuroscientists believe memory is supported by synaptic alterations in the brain. Synapses are where neurons communicate. A change in the transmission efficacy at the synapse (synaptic plasticity) has been considered to be the cellular mechanism of memory. A particular pattern of synaptic usage or stimulation, called the conditioning stimulation, is believed to induce synaptic plasticity (discussed in section 1.3).

1.2.2 Stimulus Specificity of Memory

Another unique property of memory is stimulus-specificity. Generally, memory is specific and retrieved in the presence of specific stimuli. This stimulus-specific nature of memory has been investigated for decades in synaptic plasticity-related studies in which the focus is the strengthening of memory-related connections. Researchers have also hypothesized that weakening specific synapses may prevent recall. Different properties of long-term potentiation (LTP) will be discussed in a later section (Synaptic plasticity section 1.3). One of LTP's properties is input specificity which makes our memory stimulus specific. The input specificity of synaptic plasticity is currently explained by the synaptic tagging hypothesis.

An individual neuron has thousands of synapses, which may process minimally 100s of inputs. Evidence suggests single synapses can be modified in a highly selective and independent manner [32]. The highly selective modification of a synapse for LTM requires both transcription and translation [33]. Synaptic modification is also required for STM but it is independent of transcription and translation [34] consistent with its transitory nature. Translation occurs both in local synaptic areas as well as the cell body, while transcription is confined to the nuclear part of a neuron [33]. With respect to synapse specificity, the question arises of how gene products from the nucleus are transported and targeted to only the relatively few activated synapses [35]? To address this question, the synaptic tagging hypothesis has been put forward. According to the hypothesis, the products of gene expression are shipped throughout the cell, but they are delivered to specific synapses which have been "tagged" by previous activity [36]. In other words, activated synapses create a protein complex (tag setting), which serves as a synaptic tag for delivery of the gene products supporting synaptic strengthening.

Several molecules have been identified which may serve as synaptic tags and, thus, could be responsible for the input specificity of memory, these include: CaMKII, phosphorylation of AMPARs and different protein kinases [37] [38]. It has been shown that late-phase LTP (L-LTP) in the hippocampus requires both gene expression and protein synthesis [39–42]. According to the synaptic tag hypothesis, as noted, gene products can only be captured by a synapse which was previously activated and where a tag setting in the synapse has been created [43–45]. CaMKII has been considered as an integral candidate in mediating LTP and memory. Inhibition of CaMKII results in disruption of the LTP

generating ability of CaMKII [46,47] and impairs hippocampus-dependent spatial learning [48,49]. CaMKII remains autophosphorylated for longer than other molecules and thus it could serve as a synaptic tag. Although there is a debate about the time window for the CaMKII autophosphorylation state, some report that it remains autophosphorylated for an hour and while others suggest that it remains phosphorylated for 10-20 min. For example, Modarresi et al showed that CaMKII is rapidly phosphorylated in the OB and remains phosphorylated for 10 min [50]. In this study, it was shown that blocking CaMKII in presence of PKA activation can produce a long-term memory, but the memory is not specific to the trained odor. This result suggests that CaMKII activation provides the tag that confers stimulus specificity for memory. In my thesis, I introduce the roles of NMDAR and LTCCs in the input specificity of memory (Chapter 2).

Actin is another molecule which might serve as a synaptic tag because it helps in stabilization of cytoskeletal structure for recruiting postsynaptic proteins to the spine [51]. There is evidence that activation of CaMKII controls the status, activation, and content of spine actin filaments, which ultimately controls the structural plasticity of the spine and supports synaptic strengthening [51].

Other than production of individual protein molecules, degradation of protein molecules is also important for synaptic plasticity. It has been shown that inhibition of the proteasome impairs synaptic plasticity, the formation of LTMs [52], and the maintenance of LTP [53]. Cai et al. (2010) showed that proteasome activity is required for synaptic tagging

and the capture of plasticity-related proteins at the activated synapse [54]. However, the specific proteins that are degraded by proteasome tagging were not clear.

1.2.3 Spaced vs Massed Learning and Its Effect on Memory

Different learning paradigms have been used by researchers to investigate the physiological and molecular basis of learning and memory. Scientists are trying to understand learning and memory in order to improve educational training and teaching.

There are multiple reports which suggest that items studied at spaced time intervals are recalled better in the long term than those studied repeatedly with no intervening delay [55–59]. Morris and colleagues in studies of late LTP and LTM in various contexts and species [60] demonstrate that repeated stimuli given at spaced intervals can create intracellular settings which influence the plasticity and learning. Their work demonstrated that protein synthesis was important for the enhancement of both LTP and LTM in the spaced training paradigm [61,62]. Again, the synthesized proteins strengthen activated synapses, triggering LTP [36,63,64]. In a behavioral study, Menzel et al., showed that the space between repetitions was critical for LTM encoding [65]. In their study, they used three different intertrial interval times: 30 sec, 3 min, and 10 min. Their results showed that with 3 min and 10 min intertrial intervals memory lasts for 3 days whereas at 30 sec intervals it lasts only for minutes up to one day [65]. Spaced training has large and robust positive effects on LTM as has been demonstrated across domains, such as verbal learning [66,67], conditioning (even in animals as simple as Aplysia; see [68,69]), the learning

of educational materials [69,70] and motor learning [71]. Although, E.Z. Rothkopf cautions that "spacing is the friend of recall, but the enemy of induction" [72]. According to Rothkopf, massing allows one to notice the similarities between successive events or examples, whereas spacing reduces the ability to detect such similarities.

In my research, I have shown a mechanism that causes memory to be negatively regulated by repeated training with shorter-intervals while being enhanced by repeated training with longer-intervals (Chapters 2 and 3).

1.3 Synaptic Plasticity

Neurons communicate with other neurons, muscle cells and gland cells by synapses. Synapses are the junctions where neurons pass signals to other types of cells. Most of the signaling between neurons rely on chemical synapses which use neurotransmitters for signal transmission. Neurotransmitters which are synthesized in the cytosol or axon terminal, are the key components of chemical synapses. There are several steps involved in neurotransmitter release at the synaptic junction, including the fusion of synaptic vesicles with the membrane and subsequent exocytosis. After the neurotransmitters undergo exocytosis from synaptic vesicles into the synaptic cleft, they bind to specific receptors on the plasma membrane of a postsynaptic cell. The binding of neurotransmitters onto postsynaptic cell membrane-associated receptors typically causes a change in permeability to ions. The change in the membrane ion permeability alters the

potential of the postsynaptic plasma membrane. If the postsynaptic potential is sufficiently depolarized, an action potential is then generated in the postsynaptic neuron.

One of the most important and fascinating properties of the mammalian brain is the synaptic plasticity which underlies learning and the formation of memory. Associative memory is formed when synapses get strengthened due to the pairing of presynaptic and postsynaptic activity [73]. Synaptic plasticity following coincident activation of pre and postsynaptic neurons is the cellular basis for learning such as Pavlovian classical conditioning [74]. Bliss and colleagues [75,76] first reported that repetitive activation of excitatory synapses in the hippocampus that led to strong post-synaptic depolarization caused a potentiation of synaptic strength that could last for hours or even days. This long-lasting strengthening of the synapse was termed LTP. The opposite of LTP, the weakening of synapse, is called long-term-depression (LTD).

1.3.1 Receptors Involved in Synaptic Plasticity

When neurotransmitters bind to the membrane-bound receptors, receptors change their conformations to allow different ions into the postsynaptic cells, which causes postsynaptic depolarization or hyperpolarization. Besides transmitter binding to receptors, different post-translational modifications of receptors can play a critical role in conformational changes, receptor mobility in the membrane and receptor translocation for subcellular localization [77].

Of course, synaptic receptors are necessary for normal neuronal function as well as for synaptic plasticity. There are two broad types of glutamatergic receptors implicated in the process of synaptic plasticity: (a) ionotropic receptors and (b) metabotropic receptors or G-protein-coupled receptors. The ionotropic receptors get activated when glutamate binds to the receptors and open up ionic channels in the membrane. On the other hand, the metabotropic receptors can be activated by glutamate as well as a variety of molecules including odors, hormones, small or large peptides, and neurotransmitters. G-protein coupled receptors alter the production of intracellular second messengers.

1.3.1.1 Glutamatergic Receptors

Glutamate is the most abundant neurotransmitter in the nervous system. It is the main excitatory neurotransmitter in the brain. As highlighted, the receptors which get activated due to the binding of glutamate in the ligand binding domain are called glutamatergic receptors. The alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), N-methyl-D-aspartate receptor (NMDAR) and kainate receptor (KAR) belong to the ionotropic receptor category. The most widely investigated ionotropic receptor proteins in plasticity research are AMPAR and NMDAR. Although, recent evidence is showing that the KAR also has some roles in synaptic plasticity [78]. These receptors form a pore when glutamate binds to the ligand-binding domain and activates the receptors.

Specifically, the iGluRs consist of four large subunits which create a central ion channel pore. These subunits are formed with more than 900 amino acid residues. All the subunits in different types of iGluRs have some sequence similarities, including AMPAR, NMDAR, and KAR. The subunits of GRs have four semi-autonomous domains, which include the extracellular ligand-binding domain (LBD), the extracellular amino-terminal domain (ATD), the intracellular carboxyl-terminal domain (CTD) and the transmembrane domain (TMD). The ATD plays a crucial role in receptor oligomerization and receptor trafficking in the membrane. Another important domain in iGluR is LBD which is conserved in different iGluR subunits. All agonists and antagonists are designed by being directed at the structures of the LBDs. The binding of an agonist or antagonist makes conformational changes in the iGluR structure. Structural differences in LBD and TMD underlie the three different iGluRs. The ATDs of GRs have sequence homology and are structurally homologous to the LBD of the mGluR [79–86].

Other than iGluRs, the second type of GR, as mentioned, are the mGluR. They are slow acting GR which act indirectly on membrane-bound ion channels upon binding of glutamate. They are G-protein coupled receptors and, as mentioned, the binding of glutamate to these receptors activates second messenger systems for further downstream signaling.

In my research, I identified different molecular pathways involved in olfactory learning and memory, and I have shown how AMPAR, NMDAR, and mGluR play differential roles in synaptic plasticity. In the following sections, I will discuss in more detail the structure, function and pharmacology of AMPAR, NMDAR, and mGluR.

1.3.1.1.1 AMPAR

Among all the other iGluRs, the AMPAR mediates most of the fast excitatory synaptic transmission. Traditionally, APMAR is known as a non-NMDA-type-receptor. Artificially, AMPAR can be activated by the glutamate neurotransmitter analog AMPA and the receptor's name came from the analog. AMPARs are the most commonly found receptors in different brain areas. The subunit composition of AMPARs has been quantitatively evaluated mostly in the hippocampus [87]. The biophysical properties of AMPAR are dependent on the subunit composition of the AMPAR [88,89].

The architecture of AMPARs is formed by combinations of four subunits: GluA1, GluA2, GluA3, and GluA4 [88,90]. The subunits differ from each other at their CTD, which determines their binding to different scaffolding proteins. All subunits of AMPARs can interacts with PDZ-protein binding domain. Different subunits of AMPARs interact with various other proteins. The major population of AMPARs consists of GluA1-GluA2 combinations [87]. The other major population of AMPARs is a GluA2-GluA3 combination [87] though, this combination is not predominant. One of the striking features of the GluA2 subunit is that it controls Ca^{2+} permeability. The GluR2 lacking AMPARs are permeable to Ca^{2+}. Monyer et al. showed that GluA2 expression is very low during early postnatal development period but the expression increases very quickly during the first postnatal week [91,92].

All AMPAR subunits have an extracellular N terminus, an intracellular C terminus, and four membrane-associated hydrophobic domains and, as reviewed earlier, each subunit consists of four separate domains: the extracellular N-terminal domain also known as ATD, LBD, the membrane-embedded TMD, which forms the ion channel, and the CTD [93].

The CTD of AMPARs is important for subunit specific protein interactions and phosphorylation of the C-tail leads to AMPAR trafficking in the membrane, which is important for synaptic plasticity [94], although, the precise role of C-tail phosphorylation in AMPAR trafficking needs to be clarified in more detail [95–98]. Other than trafficking, phosphorylation at the C-tail can modulate channel conductance [99]. As already described, functional AMPARs are tetrameric in nature. The formation of a tetrameric complex of AMPARs is highly dependent on the TMD [100]., while the LBD determines the opening and closing of the ion channel. AMPARs cannot interact directly with postsynaptic-density protein 95 (PSD-95). The interaction takes place via another protein called stargazin, a type of transmembrane AMPAR regulatory proteins (TARPs). Different TARPs play vital roles in AMPAR trafficking, maturation, and channel function [101–106].

The trafficking of AMPARs in the membrane is crucial for various types of synaptic plasticity. The membrane trafficking of AMPARs is controlled by different interacting proteins and by post-translational modifications including phosphorylation [107]. Protein-kinase-A (PKA) mediated phosphorylation at the S845 residue of GluA1 and calcium/calmodulin-dependent protein kinase II (CaMKII) or protein kinase C (PKC)

mediated phosphorylation at the S831 residue of GluA1 have been extensively studied by Huganir's group [108–110]. Phosphorylation at S845 of Glu1 increases ion conductivity and insertion of AMPARs in the synaptic membrane. Similarly, CaMKII mediated phosphorylation at S831 regulates channel conductance and LTP [111]. S845 and S831 phosphorylation and dephosphorylation are required for several forms of behavior and synaptic plasticity in different brain regions including hippocampal and cortical LTP and LTD [111–113].

Earlier I have discussed about LTP and relation between LTP and AMPAR phosphorylation. But LTD is also one type of synaptic plasticity where weakening of the synapses takes place. This weakening of the synapse requires dephosphorylation of AMPARs. The dephosphorylation of AMPARs is important for LTD like synaptic plasticity. Depending on the site of dephosphorylation, the plasticity of the synapse changes. For example dephosphorylation at the S845 of the PKA induces LTD like changes [111]. The same study also showed that the phosphorylation and dephosphorylation of different sites of AMPARs during LTP and LTD dependent on history of synaptic plasticity [111]. For example, the induction of LTP in naïve synapses increases phosphorylation of S831 [114 115] whereas, previously depotentiated synapses shows increase of phosphorylation at S845 site after LTP induction [115]. Similarly, dephosphorylation of S845 occurs after LTD induction in naïve synapses while, previously potentiated synapses result in dephosphorylation at S831 site [115]..

1.3.1.1.2 NMDAR

NMDA receptors (NMDARs) are one of the major glutamate-gated cation channels with high calcium permeability that play crucial roles in many aspects of the cellular biology of higher organisms. The NMDAR plays a central role in the development and function of the nervous system, including a role in synaptic plasticity, and learning and memory. Apart from its positive effect on the nervous system, it has some neurotoxic effects too. Hyperactivation of NMDARs may lead to cell death, while, hypo-activation can cause cognitive deficits. NMDARs are slow acting channels which require both glutamate and glycine binding and post-synaptic depolarization. The opening of the NMDAR channel depends on a series of events, which includes chemical activation of the NMDAR and removal of the Mg^{2+} block which leads to opening of the channel. Activation of NMDAR requires glutamate and glycine binding, which depend on presynaptic activity. At the same time, postsynaptic depolarization is necessary for removal of the Mg^{2+} block. Removal of the Mg^{2+} block leads to Ca^{2+} permeability through the channel when it is chemically activated. These events have to occur in a simultaneous manner, which makes the NMDAR a coincidence detector in the neuron.

NMDARs are a heteromeric complex formed mainly by three different subunits which include GluN1, GluN2, and GluN3. There are eight different splice variants of GluN1 RNA which produces eight different subunits [116–118]. GluN2 has four different subunits encoded by four genes (GluN2A-D) and GluN3 subunits are encoded by two (GluN3A and B) genes [119]. Functional NMDA receptors consist of two obligatory GluN1

subunits in combination with two GluN2 and/or GluN3 subunits [119,120]. All subunits are structurally similar to each other with a conserved domain organization.

GluN1/GluN2 subunit-containing NMDAR's activation requires two molecules of the co-agonist, glycine [121,122], and two molecules of the agonist, glutamate [123–125]. Whereas NMDA receptors composed of GluN1/GluN3 require only glycine for activation [126,127]. Other than glycine and glutamate, several other molecules can activate NMDA receptors as co-agonists including D-serine, D-alanine, L-alanine, L-serine. Glutamate release from the presynaptic terminal activates synaptic NMDARs by occupying two binding sites localized at the LBD of GluN2 subunits [128]. At the same time, AMPAR mediated postsynaptic depolarization is required to remove the Mg^{2+} blockage, which will allow Ca^{2+} entry that has the ability to activate downstream signaling molecules required for plasticity processes. Calmodulin (CaM) and CaMKII are the two major downstream molecules which get activated by the Ca^{2+} entry through NMDARs. The regulation of these two molecules will be discussed in the molecular mechanisms of synaptic plasticity section. Calcineurin is another downstream molecule which can be activated by NMDAR mediated Ca^{2+} influx.

The functional regulation of NMDARs is mediated by differential phosphorylation at Serine/Threonine (Ser/Thr) sites in NMDARs. There are many Ser/Thr sites that have been identified in NMDARs. Those sites are phosphorylated by different protein kinases, which regulate both channel properties and the intracellular trafficking of NMDARs, leading to changes in synaptic strength. The protein kinases

involved in differential phosphorylation of NMDARs include, CaMKII, PKA, PKC, cyclin-dependent kinase-5 (Cdk5), and protein kinase B (PKB) [129].

Raman et al. showed that PKA activation and PK- mediated phosphorylation of NMDARs increases the amplitude of NMDA receptor-mediated excitatory postsynaptic currents [130]. In another study, Crump et al. showed that the synaptic targeting of NMDARs is increased by PKA activation [131]. Current evidence also revealed that PKA inhibitors reduce the relative fraction of Ca^{2+} influx through NMDARs, which suggests that PKA regulates the calcium permeability of NMDARs [132]. A study by Scott et al. showed that phosphorylation at S897 by PKA and S896 by PKC are both required to increase NMDARs surface expression [133]. Another study from the same group suggests that S896 and S897 site phosphorylation of GluN1 is an important regulator of intracellular trafficking [134]. There are other Ser/Thr sites which get phosphorylated by PKC and CaMKII. Recent studies have shown that phosphorylation at S1416 of the GluN2A subunit by PKC, decreases the binding affinity of CaMKII for the NMDAR, which suggests direct crosstalk between PKC and CaMKII [135] effects. Studies in the hippocampus have shown that CaMKII -mediated phosphorylation of S1303 inhibits receptor-kinase interactions and promotes slow dissociation of preformed CaMKII-GluN2B complexes [136]. This evidence suggests that protein phosphorylation plays a crucial role in the modulation of the function of NMDARs. Even though there is considerable progress in the understanding of NMDA receptor regulation by phosphorylation, many aspects of NMDA receptor phosphorylation related to behavior still remain unsolved.

It has been reported that NMDARs are stably expressed in the postsynaptic membrane, unlike AMPARs. The internalization and trafficking of AMPARs are dependent on dephosphorylation events occurring at different AMPARs sites. On the other hand, NMDAR internalization is a more complex event that needs to be investigated in more detail. Current evidence suggests that the GluN2 subunits interact directly with PSD-95 [137] which is a modular protein highly enriched in the postsynaptic density. This interaction appears to be a major regulator of the NMDAR internalization process [138]. Data suggest that GluN2B extream C-terminal sequence (YEKL) is sufficient to induce internalization [138]. The Roche et al., have also shown that GluN2B-mediated internalization is increased after the deletion of the PDZ-binding domain of GluN2B. In this study, they observed that the GluN2B-mediated internalization is inhibited by the synaptic protein PSD-95. Their data suggest that PSD-95 is involved in NMDARs regulation in the postsynaptic membrane.

1.3.1.1.3 mGluR

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors (GPCRs). Unlike AMPAR and NMDAR, mGluRs are slow acting second messenger systems activated by glutamate. Glutamate binding to mGluRs can modulate excitability and synaptic transmission via second messenger signaling pathways. mGluRs are

extensively present in the central nervous system (CNS), which suggests that these receptors participate in numerous functions throughout the CNS. They are present in both pre and postsynaptic neurons in synapses of the hippocampus, cerebellum, and the cerebral cortex, as well as other parts of the brain and in peripheral tissues. mGluRs have the ability to modulate other glutamate receptors such as the NMDAR, AMPAR, and KAR, and are linked with many cognitive functions including learning and memory, the perception of pain, and anxiety. Researchers have shown that mGluRs are also involved in neurodegenerative diseases [139], which makes mGluR a potential candidate for therapeutic interventions.

mGluRs are one type of GPCR which has seven transmembrane domains like other GPCRs. The resulting change in the conformation of the mGluR induced by glutamate binding activates the protein, which consists of a heterotrimeric complex of α, β, and γ subunits. The activation of the G protein causes the exchange of guanosine 5/-triphosphate (GTP) for guanosine 5/ - diphosphate (GDP) within the α subunit, In the inactive state mGluRs are bound to GDP. mGluRs are class C type GPCRs which are distinguished from class A type GPCRs by their large extracellular N-terminal domain that contains the endogenous ligand-binding site. There are eight subtypes of mGluRs and they are differentially expressed in various cell types. mGluRs are subclassified into three major groups based on sequence homology, ligand selectivity, and G-protein coupling. Group I includes mGluRs 1 and 5, Group II includes mGluRs 2 and 3, and Group III includes mGluRs, 4, 6, 7, and 8. Group II and III are mostly presynaptic while Group I

are postsynaptic. In my research, I focused on Group I mGluR because our findings highlighted postsynaptic activity.

Both mGluR1 and mGluR5, are coupled to phospholipase C via Gq proteins and mediate phosphoinositide hydrolysis. Previous studies have shown that an increase in intracellular calcium concentration can be achieved by the activation of mGluR1 [140]. Primarily, Group-I mGluRs couple to Gq /Gi which activates phospholipase C, resulting in the hydrolysis of phosphoinositides and the generation of diacylglycerol and inositol 1,4,5-trisphosphate (IP3). This classical pathway leads to calcium mobilization from internal storages and can activate different molecular cascades than external calcium?. Depending on the cell type or neuronal population, group I mGluRs activate a range of downstream effectors, including: calcineurin, the transient receptor potential channel, NMDARs, phospholipase D,and other protein kinase pathways such as cyclin-dependent protein kinase 5, casein kinase 1, Jun kinase, the mammalian target of rapamycin (mTOR)/p70 S6 kinase pathway, and components of the mitogen-activated protein kinase/extracellular receptor kinase (MAPK/ERK) pathway [141] [142] [143]. This broad range of signaling cascade activation capacity has made mGluR a key molecule in learning and memory research.

1.3.1.2 Voltage gated L-type Calcium Channel (LTCC)

Other than the NMDAR, the LTCC is another channel that has the ability to produce calcium influx in neuronal cells. The activation of LTCCs is different than that of

NMDARs. The activation of LTCCs is only voltage dependent. The 'L' stands for the long-lasting activity pattern of LTCCs. They are mostly found in cardiac muscle and in brain cells. Due to the high voltage dependency and its Ca^{2+} permeability, LTCCs are also considered key players in synaptic plasticity and learning and memory research.

LTCCs have four subunits which include Cav 1.1, 1.2, 1.3 and 1.4. Among them, Cav 1.2 and Cav 1.3 are most common in the neuron, although Cav 1.4 has been found in retinal cells as well. These subunits are responsible for pore formation of the LTCCs. The pores are formed by the α1 subunit and this is considered as the central building block of the LTCCs. The α subunits form hetero-oligomeric complexes in association with other subunits. The drug binding domain is also present in the α1 subunit of LTCC. Hence, drugs have been designed on the basis of the α1 subunit structure. The β subunit of the LTCCs is present as the cytosolic intracellular part and this subunit regulates the targeting of LTCCs to the plasma membrane. CaM is a molecule which can regulate the channel complex formation. The C terminal region of LTCC α1 subunits are involved in many protein-protein interactions that, in consequence, have the ability to increase or decrease the activity of LTCCs.

Intramolecular and intermolecular protein-protein interactions, posttranslational modifications, and phosphorylation within the Cav1.2 α1 subunits are important for the regulation of the LTCCs. Recent studies have characterized how these modifications and protein-protein interactions change the activity of the LTCCs. A truncated α1 subunit after proteolysis can serve as a transcriptional modulator after entering the nucleus. PKA

mediated phosphorylation at ser-1700 and thr-1704 of the α1 subunit increases the inward current of LTCCs. Involvement of PKA suggests that norepinephrine would have the ability to indirectly increase LTCC activity. As mentioned earlier that CaM also interacts with the LTCC α1 subunit. CaM and calcineurin (CaN) both get activated by Ca^{2+}, which suggests that CaM and CaN may both have the ability to regulate the activity of LTCCs. In fact, CaN-mediated dephosphorylation at the PKA sites is necessary to suppress PKA enhancement of the channel in neurons [144].

1.3.2 Molecular Mechanisms of Synaptic Plasticity

The underlying molecular mechanisms of various forms of synaptic plasticity have been investigated in great detail. Malenka and others have discussed the molecular mechanisms of synaptic plasticity in many reviews [145–148], which provide our current understanding knowledge about the underpinnings of synaptic plasticity. In the following sections, I will discuss LTP, LTD and their molecular mechanisms.

1.3.2.1 Long-term Potentiation (LTP)

LTP refers to the strengthening of a specific synapse. This form of plasticity was most extensively studied in the CA1 region of the hippocampus [149]. Different properties of LTP are similar to those of the memory. Both memory and LTP can be generated rapidly, and strengthened and prolonged by repetition. Other shared properties of memory and LTP are cooperativity, associativity, and input specificity [150]. Cooperativity means

that LTP can be induced by the coincident activation of a critical number of synapses. Associativity is the capacity to potentiate a weak input (activating a small number of synapses) when it is activated in association with a strong input (activating a larger number of synapses). Input specificity indicates that LTP is elicited only at activated synapses and not at adjacent, inactive synapses on the same postsynaptic cell. This feature dramatically increases the storage capacity of individual neurons because different synapses on the same cell can be involved in separate circuits encoding different bits of information.

The bulk of our knowledge about the molecular mechanisms of LTP has been derived from studies of LTP at excitatory synapses on CA1 pyramidal neurons in hippocampal slices. Similar or identical forms of LTP have been observed at excitatory synapses throughout many brain areas. Thus, the conclusions drawn from the study of LTP in the hippocampal CA1 region are often applied to other brain regions.

1.3.2.1.1 Molecular Mechanisms of LTP

The induction of the LTP is mediated by NMDARs and expression of the LTP is mediated by AMPARs [151]. As previously reviewed in the Section 1.3.1.1, AMPARs and NMDARs are the major types of ionotropic glutamate receptors contributing to the postsynaptic response at glutamatergic synapses. The AMPAR has a channel that is permeable to monovalent cations (Na^+ and K^+). Activation of AMPARs provides most of

the inward current that generates the excitatory synaptic potential. This is a fast reacting channel protein. In contrast to AMPARs, the NMDAR exhibits a strong voltage dependence because of the magnesium blockage inside the channel [152]. Therefore, there is little or no contribution of NMDARs during basal synaptic activity to the postsynaptic response. However, after depolarization via AMPARs, magnesium dissociates from its binding site within the NMDAR channel. This event allows ions to enter the cell. More importantly, unlike AMPARs, the NMDAR is permeable to divalent (Ca^{++}) as well as monovalent cations (Na^{+} and K^{+}) ions. The permeability to calcium ions triggers downstream molecules that support various forms of synaptic plasticity. It is well established that the induction of LTP in the CA1 region depends on activation of NMDARs. It has also been shown that the postsynaptic calcium concentration increases during strong postsynaptic depolarization. The coincidence detection property of NMDAR makes it unique and important for LTP. These properties of the NMDARs also explain the basic properties of LTP.

As stated, the calcium signal is essential to activate signal transduction molecules which are necessary to initiate and support LTP [153] [151]. Researchers have suggested some basic criteria for defining the role of a protein as a mediator of LTP induction. The function of individual molecules can be studied either by blocking the activation of the molecule or by activating it during LTP induction. If blocking the molecule blocks LTP induction, or activation of the molecule induces LTP, which occludes further synaptic induction of LTP, this molecule is then considered critical for LTP. Calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) is a key molecule in the molecular

machinery for LTP, which fulfills those requirements. Previous research suggests that CaMKII undergoes autophosphorylation to support LTP induction [114] [154]. In another study, LTP induction was prevented in knockout mice lacking a critical CaMKII subunit [48]. LTP induction was also prevented when a mutation occurred at the autophosphorylation site of CaMKII [155]. Furthermore, inhibition of CaMKII using a peptide blocks LTP [149] [95], whereas an increased concentration of active CaMKII increases synaptic strength [156] [157]. Other than CaMKII, several other kinases have been implicated in the triggering of LTP. It is still a topic of investigation to dissect out the independent molecular pathways involved in triggering LTP.

The expression of LTP is dependent on AMPARs. The increase of AMPARs in the postsynaptic density is a key feature shown in various LTP related studies [158] [99] [147]. This increase of AMPARs is driven by activity-dependent changes in AMPAR trafficking [159] [160]. Phosphorylation of AMPARs at different sites is crucial for the trafficking of AMPARs but detailed molecular functions of these changes remain to be determined. Recent evidence suggests that CaMKII can phosphorylate Ser831 of the GluA1 subunit, which results in a significant increase in single-channel conductance of homomeric GluA1 receptors [99]. Roughly, AMPARs are translocated into the PSD via both exocytosis and lateral movement within the plasma membrane. Most investigators believe that the incorporation of AMPARs into the PSD is the most important change because it appears to be accompanied by structural changes in the dendritic spines and synapses themselves, which is an attractive mechanism for maintaining LTP.

For LTP in the CA1 region of hippocampus, it is clear that the NMDAR plays a vital role in the induction of LTP. On the other hand, AMPARs support the expression of LTP. An increase in NMDAR activation increases the calcium concentration in the dendritic spines; the increased calcium ions activate CaM and CaMKII. CaMKII phosphorylates AMPARs leading to the insertion of AMPARs into the PSD as well as AMPAR trafficking from extrasynaptic sites to synaptic sites. In a parallel process, synapses start exhibiting structural changes such as an increase in the size of the PSD and the dendritic spine.

1.3.2.2 Long-term Depression (LTD)

Synaptic plasticity is bidirectional. Synapses will reach maximum efficacy if they continue to increase in strength. This unidirectional progression could make it difficult for synapses to encode new information. Thus, to make LTP useful, there should be the possibility of a weakening process. One process for the weakening of synaptic strength is called LTD. Establishment of LTD provided support for the idea that memories or experiences were encoded critically by the *distribution* of synaptic strength in neural circuits. There are some computational advantages to having both LTP and LTD in the synapse. Linden hypothesized that saturation of LTP is prevented by the active resetting of already potentiated synapses; of course homeostatic rebalancing of inactive synapses would also have to occur [161]. The resetting mechanism can make synapses more responsive and preserve the dynamic range of responsiveness needed. Other researchers

suggested that LTD of previously potentiated synapses could also serve as a "forgetting" mechanism [162].

1.3.2.2.1 Molecular Mechanisms of LTD

A prolonged repetitive low-frequency stimulation is required to obtain LTD. This prolonged low activity partially relieves Mg2+ blockage in NMDAR and leads to reduced calcium entry into the postsynaptic cells. In a model for the induction of LTP and LTD Malenka et. al, proposed that high-frequency stimulation increases the calcium level to concentrations greater than 5 µM, while, low-frequency stimulation leads to calcium concentrations of less than 5 µM [148]. This suggests that calcium concentration plays a role in both LTP and LTD, mediating the bidirectional effects.

A leading hypothesis for the induction of the LTD signal transduction pathway suggests that LTD involves activation of a calcium-dependent protein phosphatase cascade. The proposed molecular cascade consists of the calcium/calmodulin-dependent phosphatase calcineurin (also known as protein phosphatase 2B) and PP1 [163]. Current evidence supports the hypothesis that postsynaptic inhibition of either PP2B or PP1 prevents LTD induction [164] [165] [166] whereas, direct injection of PP1 into CA1 pyramidal neurons increases LTD [167].

The notion that the NMDAR and calcium concentrations play critical roles in LTD is widely accepted. Walter and colleague showed that blocking NMDARs using

MK-801 prevents LTD induction in both adult and immature mice [168]. In the proposed mechanism, they show that blocking the NMDAR inhibits the activation of p38-MAPK and the dephosphorylation of GluR1 (S831), which prevents LTD. Another proposed mechanism for the support of LTD is dephosphorylation of Ser845 on GluR1 (phosphorylated by PKA). Dephosphorylation of GluR1 at the PKA phosphorylation site leads to LTD [169]. Dephosphorylation of these two sites (S845 and S831) may both contribute to the expression of LTD. Dephosphorylation at the PKA site decreases the AMPAR open-channel probability [170]. Consistent with a critical role for this site, the knock-in alanine substitution of Ser845 on GluR1 prevents NMDAR dependent LTD [111]. A similar outcome is seen if Ser831 (the CaMKII phosphorylation site) is replaced with alanine.

While dephosphorylation mechanisms can alter AMPA receptor conductance to support LTD, as described, the current leading hypothesis in the field of LTD expression is that the expression of NMDAR-dependent LTD is due to activity-dependent endocytosis of synaptic AMPARs [99 145 158 171 160]. The calcium-dependent dephosphorylation of AMPARs leads to their endocytosis and this endocytosis is dependent on phosphatases [172–174]. There is direct evidence from immunohistochemical studies, that endocytic machinery co-localizes with AMPARs and subsequent endocytotic events can be visualized. The endocytotic process is also calcium dependent.

The preferential activation of phosphatase proteins in the dendritic spine takes place only in response to a modest increase in calcium. As a result of phosphatase

activation, AMPARs dissociate from the PSD and move laterally to the endocytotic zone. Finally, these lateralized AMPARs are endocytosed and degraded. LTD also changes the size of the dendritic spine. It appears shrinkage of the dendritic spine is associated with LTD [175,176] and this might relate to the loss of AMPARs from the dendritic spine [177].

1.3.3 Role of Synaptic Plasticity in Learning and Memory

The relation between synaptic plasticity and memory remains a topic of extensive research and debate. It is still not fully understood whether LTP and LTD naturally occur during learning in live animals. The discovery of LTP significantly boosted the research field of learning and memory and the investigation of their relationship to synaptic plasticity mechanisms. Studies on LTP had characterized different key molecules involved in LTP induction. Several studies showed that LTP could be blocked by inhibiting specific protein molecules, or in specific gene knockout animals. Researchers also confirmed that inhibition of these LTP-related molecules could affect various stages of memory (acquisition, consolidation, and retrieval) as reflected in behavior.

1.3.3.1 The Hypothesis of Synaptic Plasticity and Memory

The synaptic plasticity and memory (SPM) hypothesis was developed by several researchers [178]. The key concept of the SPM hypothesis is that synaptic plasticity is a physiological phenomenon whereby specific patterns of neural activity give rise to changes in synaptic efficacy and neural excitability [178]. These changes in synaptic

efficacy induce neural circuit changes that outlast the events that triggered them. This biophysical and biochemical machinery is not only important for neuronal activity but proposed to be critical for memory. After 40 years of research on the properties of LTP, scientists have discovered that LTP processes are required for (a) the acquisition of a memory and the storage of memory traces [179] and (b) the initial phases of memory consolidation (or stabilization) over time [179]. Researchers have concluded that LTP does *not* play a role during memory retrieval [178].

1.3.3.1.1 Criteria for assessing of the SPM Hypothesis

There are four different criteria for assessing the SPM hypothesis [179]. The first criterion is "Detectability" which means that the memories for previous experiences should be associated with changes in synaptic efficacy in the memory circuitry and these changes have to be detectable in the nervous system. The second criterion requires "Mimicry". "Mimicry" refers to the idea that artificial reactivation of the same spatiotemporal pattern of neural circuitry thought to store a particular memory should lead to a display of apparent memory for a previous experience which did not in practice occur. The third concept of SPM hypothesis is identified as "Anterograde Alteration". According to this third criterion, prevention of synaptic plasticity induction during learning events should impair the animal's memory of that experience. The fourth and last criterion for the SPM hypothesis is "Retrograde Alteration". The memory of a previous experience is characterized by changes in specific neural circuitry and altering that circuitry should alter the animal's memory of the experience. Studies providing evidence

that meet the 4 criteria have been conducted in several behavioral models including the olfactory system [180] [181] [182].

1.3.3.1.2 Experimental Strategies Used for Assessing the SPM Hypothesis

Scientists have exploited different experimental strategies to assess the SPM hypothesis. Generally, while the criteria listed above are necessary to demonstrate a causal relationship between synaptic plasticity in neural circuits and behavior, to initially be consistent with the SPM hypothesis there has to be some correlation between the behavioral parameters of learning and memory and some of the properties of synaptic plasticity, which we refer to as "Correlation". The induction of measurable changes in synaptic strength at specific synapses should be also associated with learning ("Induction"). Another testing strategy is "Occlusion", which means that saturation of synaptic strength in a specific network should occlude new memory encoding.

A saturation of synaptic strength refers to a neuronal state in which, at least for a period of time, no further LTP or LTD is possible. For example, a true saturation of LTP prior to behavioral training should prevent new learning in that circuit because no further LTP would be possible [183]. Occlusion has been reported for saturation of LTD as well as LTP [184] "Intervention" is another strategy that has been used extensively in this field of research. A genetic manipulation, pharmacological blockade or enhancement of synaptic plasticity and other recent advanced manipulations (e.g., optogenetic manipulations) should have predictable effects on learning or memory. For example, Morris et al showed that blocking NMDARs blocks hippocampus-dependent spatial learning [185]. Later on,

many researchers have shown that inhibition of NMDARs inhibits learning in a different behavioral paradigms including certain types of olfactory learning [186], contextual fear conditioning [181,187–189], other operant tasks [190]. T-maze alternation [180,191], and delayed reinforcement of low rates of response [181]. Gene knockout and overexpression of one gene have also linked plasticity mechanisms to the mechanisms underlying learning and memory [47].

Izquierdo et al took another approach to investigate the relationship between synaptic plasticity mechanisms and memory. While most of the foregoing strategies focused on memory strengthening and induction protocols, Izquierdo et al have focused on "Erasure". Erasing synaptic plasticity shortly after learning should induce forgetting. Erasure has been achieved by either (a) using the application of drugs or enzyme inhibitors, which prevent the expression of LTP when given shortly after its induction (such as kinase inhibitors,) or (b) using trains that reduced synaptic strength such as those for depotentiation (e.g. low frequency) stimulation [192]. There are various protocols for the induction of LTD, which typically use extended continuous trains of single pulses at 1–5 Hz [182,193,194]. A report also suggests that a few minutes of 5-Hz stimulation can depotentiate recently induced LTP at synapses in the hippocampus CA1 area [182]. However, the LTD induction protocol has not yet been tested in freely behaving animals in-vivo and it is not clear whether LTD protocols induce forgetting.

1.3.4 Metaplasticity

Prior synaptic activity, including activity that does not induce LTP, can have a long-lasting influence on subsequent synaptic plasticity. For example, a short burst stimulus (30 Hz, 150 ms) in hippocampal CA1, which generates short-term potentiation, but not LTP, inhibits future LTP and facilitates future LTD [195]. This phenomenon, first discovered by Abraham and Bear in 1996, was termed metaplasticity. By definition, the plasticity of synaptic plasticity is called metaplasticity. The concept of metaplasticity implies that prior activation of a synapse can determine the later fate of the synapse in response to stimulation and, specifically, modify whether it will be strengthened or weakened by a particular input. The term 'meta' reflects the higher-order nature of the plasticity.

Essentially, metaplasticity refers to changes in the physiological or biochemical state of neurons or synapses that alter their ability to generate synaptic plasticity. In the case of conventional plasticity, it has been shown that the modulation and regulation of the plasticity events overlap in time. But in the case of metaplasticity, prior activation of a synapse can prime the synapse by altering their biochemical and physiological properties and these changes persist at least until the second activation. This unique property distinguishes metaplasticity from more conventional forms of plasticity. There is an extensive range of mechanisms involved in metaplasticity, many of which overlap with

the mechanisms of conventional plasticity [195]. This overlap in mechanism and simultaneous occurrence pose a considerable challenge for metaplasticity research in terms of experimental design and interpretation. In spite of that, there has been a substantial amount of work related to metaplasticity over the past decade. Since our lifetime of experience is a continually evolving one, it may be hypothesized that metaplastic effects are likely to be of particular importance.

1.3.4.1 NMDAR Mediated Metaplasticity

Besides conventional synaptic plasticity, NMDARs also play a role in metaplasticity. Experimenters have used pharmacological or synaptic activation of NMDARs to induce metaplasticity. While NMDAR activation is a key trigger for LTP induction itself, it also affects subsequent LTP induction, which is due to its triggering of metaplastic effects. More importantly, these effects are restricted to the previously activated synapses only. Prior LTP induction prevents subsequent LTP at the same synapse [195]. The inability to induce LTP in the same synapse can be overcome by increasing the stimulus intensity. This result suggests that the priming stimulation does not completely inhibit LTP but instead elevates the threshold for LTP. The LTP inhibition caused by the priming synaptic activity is dependent on activation of NMDARs, p38 mitogen-activated protein kinase (p38 MAPK), adenosine α2 receptors, a calcineurin,

and the protein phosphatases 1A and 2A. While priming stimulation can cause a saturation of the potentiation process, this not the only way of the inhibiting later LTP. Priming inhibition of LTP can be achieved even when the priming stimulation does not cause any detectable change in basal synaptic transmission [195]. The metaplastic effect is not restricted to LTP, it can modulate LTD as well. The activation of NMDAR can also facilitate or depress the subsequent induction of LTD [195] [196].

Researchers have shown that prior LTP induction reduces the postsynaptic voltage threshold for subsequent LTD and facilitates LTD. On the other hand, the same prior activation elevates the threshold for future LTP. However, the mechanisms mediating these effects of metaplasticity are not clear. Researchers suggest that activation of the NMDAR results in LTD of NMDAR (NMDAR LTD) currents themselves. Lower activation of NMDARs or NMDAR LTD results in a reduction in Ca^{2+} entry through the receptor channels subsequently. Previous studies have shown that nitric oxide (NO) and PKC have the ability to mediate NMDAR LTD. The same studies have shown that NMDAR activation increases the production of NO which can suppress NMDAR currents [196] [197]. On the other hand, pharmacological activation of PKC causes trafficking of NMDARs from synaptic sites to extrasynaptic sites [198] [199].

There are other mechanisms also involved in this process. For example, the impaired LTP due to the application of a prior LTP induction is, apparently not mediated by NO and PKC [201,202]. There are other potential molecules present downstream of NMDAR activation, which could also play a role in metaplastic expression. Different

Ca^{2+} dependent kinases and phosphatases are examples. The priming stimulation could alter the intracellular free Ca2+ concentration during plasticity induction, which in turn will alter enzyme activity [195]. Just like synaptic plasticity, in metaplasticity CaMKII also plays a crucial role. The earlier priming affects the phosphorylation state of CaMKII [203,204] which has a critical role in initiating LTP by regulating AMPARs [205]. If the autophosphorylated state of αCaMKII is achieved by mutating Thr286 of αCaMKII to Ala286 (in which its activity becomes Ca2+-independent), this mutation results in the same effects as NMDAR priming stimulation [154]. However, it is not clear, whether a priming stimulation that does not itself cause LTP can be achieved by autophosphorylation of Thr286, which is an important requirement for a purely metaplastic effect. In another study, it was shown that knocking out RC3 (also known as neurogranin) another binding partner of calmodulin, results in a decrease in LTP threshold [206]. Autophosphorylation at Thr305/Thr306 of αCaMKII, might also mediate later inhibition of LTP. Mutation at Thr305/Thr306 to prevent auto-phosphorylation completely abolished the metaplasticity [207].

1.3.4.2 Role of Metaplasticity in Learning and Memory

How metaplasticity is implemented in natural learning has been an active topic for recent neuroscience research. How does learning produce metaplasticity that influences either the current acquisition process or future learning? Enriched environments or stressful stimuli have both been shown to induce metaplasticity and affect synaptic plasticity and learning [208]. However, how learning itself affects future plasticity and

learning capacity remains to be investigated. A recent study showed that learning could induce long-term alterations in after hyperpolarizations (AHPs) which in turn directly affect new learning via altered intrinsic excitability. Learning-induced reductions in the slow AHP and increases in cell firing has been observed up to 5 days in CA1 pyramidal cells in a rabbit eye-blink conditioning experiment [209]. The same study showed that if the conditioned and unconditioned stimulus were given in random orders then these changes were not observed. A persistent decrease in the apamin-sensitive medium AHP was also observed in rat piriform cortical neurons after operant conditioning and olfactory-discrimination training [210,211] and in CA1 pyramidal cells following spatial water maze training [212]. The underlying molecular mechanisms behind the persistent decrease in the slow AHP include a prolonged increase in extracellular-signal-regulated kinase 1 (ERK1) and ERK2 activity, as well as PKC [211]. At the same time, an increase in the GluN2A/GluN2B ratio for piriform NMDARs has been reported [213]. During aging, there is an increase of the slow AH and increased levels of corticosterone,. These changes correlate with impaired learning and memory [214]. Reduction of the slow AHP might also be important for the learning process because it might metaplastically lower the threshold for LTP [214]. This change in the neuronal state could provide a facilitated environment for new learning. This hypothesis is supported by a study where researchers showed a reduction in the slow AHP in rat CA1 pyramidal neurons and found enhanced olfactory discrimination performance with subsequent training [215]. Reduced slow AHP also enhances the ability to learn a different task like spatial navigation in the water maze [215].

Histone acetylation and DNA methylation could be other possible mechanisms through which metaplasticity would affect learning. Pharmacological inhibition of histone deacetylases (HDACs) promotes the formation of long-term memory [216] and the late phase of LTP. Animals that have been trained on memory tasks show increased histone acetylation in the relevant brain regions [217]. In contrast, but related to these epigenetic effects, inhibition of DNA methylation inhibits late phase LTP and blocks memory consolidation [218,219].

1.4 Olfactory System and Olfactory Learning

Rodents rely critically on their sense of smell to navigate the environment and establish social interactions. My research has used an early odor preference learning model in rodents to explore various properties of learning and synaptic plasticity. I particularly focused on two olfactory structures: the olfactory bulb (OB) and the piriform cortex (PC). In this section, I first introduce the organization of the olfactory system (Figure 6.0), then summarize the research on olfactory learning in rodents.

The OB and the olfactory cortex (OC) including the PC are two main areas that are involved in olfactory information processing. Like other sensory systems, olfactory information must be transmitted from peripheral structures (the olfactory epithelium (OE)) to more central structures (OB and OC) but these two regions directly communicate with each other without a thalamic relay, unlike other sensory systems. The

olfactory information is first processed in the OB and OC and then transmitted to other parts of the brain for generating sensory awareness and stimulus-specific behavior.

There is no topographic organization beyond the OB for olfactory stimuli. A topographic organization has been found in the projection from the OE to the OB [220]. But there is no evidence for a topographic organization from the OB to the OC. The spatiotemporal activation patterns to odors occur across large regions of the OC and appear to serve as ensemble representations for odor detection and discrimination.

Olfactory receptor neurons (ORN) have a short lifespan (approximately 30-60 days) and they exhibit significant turnover throughout life. The basal stem cell population in the olfactory epithelium constantly replace ORNs by mitotic division. These are the only neurons that are inserted in the surface epithelium of the body, as a result, they are directly exposed to the environment.

Humans are generally considered as "microsmatic" while rodents belong to "macrosmatic mammals [14]. Microsmatic species have a relatively poorly developed olfactory system compared to macrosmatic species [221]. Certainly, the structural organization of the OB and OC in rodents and carnivores is well defined. However, the human brain also consists of the same olfactory structures which have been shown in rodents and cats. Relatively simple circuitry and direct connections between the OB and

OC have helped scientists to understand the information encoding process in the olfactory pathways.

The OB and piriform cortex (PC) are the two main structures of the olfactory systems which have been most extensively investigated in learning paradigms. Learning studies form our lab have shown molecular and structural changes in these structures following odor experience and/or conditioning. For example, neurotransmitter release patterns are altered in the OB following odor associative learning [222]. A significant enhancement in the activity of PC cells has been observed following odor conditioning [223]. I will discuss the olfactory learning literature in detail in the later sections. Previous studies support the concept that learning-induced synaptic modification occurs both in the OB and the OC and investigations of olfactory learning have broadened our understanding of learning and memory mechanisms in general.

1.4.1 Olfactory Processing and System Anatomy

Odor detection and perception begin with the binding of an odorant molecule to the olfactory receptor (OR) proteins located on olfactory sensory neuron (OSN) dendrites within the nasal epithelium. The binding of the odorant molecules to the OR initiates chemical cascades within the OSN that generate action potentials, which travel to the OB via OSN axons for further processing. Each odorant activates more than one receptor type present in the OSN and odor receptors respond to overlapping sets of odorants [224–228].

Activation of these unique sets of neurons forms the basis of an odor identity code. Later on the encoded odor information transfer to the OB via the projections of the OSNs present in the olfactory epithelium.

Homologous OR gene expressing OSNs project to specific glomeruli in the OB, and form synapses with dendrites of the bulbar output neurons, mitral/tufted cells, as well as with a heterogeneous population of glomerular layer excitatory and inhibitory interneurons. Due to an architecture in which each glomerulus receives input from OSNs expressing the same ORs, glomeruli serve as independent, functional units. As a result, exposure to specific odorants tend to activate a group of glomeruli forming a specific spatial pattern or odotopic map in the olfactory bulb [225,229–233].

The inhibitory circuit within the OB enhances the spatiotemporal features of the OSN glomerular input, which further helps in signal processing. For example, the GABAergic periglomerular (PG) cells within glomerular layer supply both presynaptic and postsynaptic inhibition of OSN input and both intra- and interglomerular postsynaptic inhibition of mitral/tufted cell responses [234–243]. This PG cell population can control lateral inhibition between glomeruli. Interestingly, both PG and granule cell populations are heavily innervated by centrifugal input from several neuromodulatory regions, which in turn play a crucial role in olfactory learning and memory [244].

After initial processing in the OB, the neural code for a given odor is communicated to the

PC via the lateral olfactory tract (LOT) formed by the mitral/tufted cell axons. In the PC, pyramidal neurons form synapses with the mitral/tufted cell axons coming from LOT. Electrophysiological data suggests that individual pyramidal cells at multiple locations are responsive to a given odorant [245]. Unlike the OB, there is no odotopic map present in the PC. In the following sections, I will further discuss these two major olfactory structures, the OB and PC, which are central to my research.

1.4.1.1 The Olfactory Bulb

The OB has a laminar structural organization like other cortical areas. Histological studies by Cajal and colleagues showed that the bulb consists of seven layers [246–250]. These seven layers include the olfactory nerve layer (ONL), glomerular layer (GL),), the external plexiform layer (EPL), the mitral cell layer (MCL) the internal plexiform layer (IPL), and the granule cell layer (GCL) [251–254]. Each OB consists of several thousands of glomeruli. One glomerulus receives thousands of OSN axons and the dendritic branches of approximately 10-70 M/T cells [255–257] which form synaptic connections. These excitatory synapses can be modulated by three types of neurons present in the GL: periglomerular cells (PG), short axon cells (SA), and external tufted cells (ET) [252,253]. The granule cells are another type of cell present deeper in the OB, which influence the fine-tuning of odor representation in the OB via dendrodendritic

GABAergic modulation [258–260]. Volumetrically, the GL and GCL account for the highest percentage of the bulb. Reports suggest that approximately 50% of the bulb is composed of GL and GCL (GL: ~26%; GCL: ~29%).

Each bulb contains various cell types that include principle neurons (M/T cells), interneurons (PG, Granule cells, SA, ET, Van Gehuchten cells, and Blanes cells), and glial cells (astrocytes, olfactory ensheathing cells, NG2, oligodendrocytes, and microglia).

After the processing of odor representations by the aforementioned components, M/T cells relay that information for the higher-order information processing [261 253, 254 257].

1.4.1.2 The Piriform Cortex

The PC is a higher order olfactory processing structure required for multiple cognitive functions related to olfaction. The PC also serves to connect other brain areas with olfactory structures. As mentioned earlier, the LOT is a myelinated axon bundle of M/T cell output that relays odor information from the OB to the PC [264,265]. Price and colleague suggested that the LOT consists of two types of axon bundles: a thinner bundle and a thicker bundle [261,266]. The thinner bundle originates from tufted cells, which projects to the rostral part of the PC and the thicker bundle axons are from the mitral cells and innervate throughout the entire PC [247,263]. The PC serves as the largest recipient of bulbar

projections and is considered the "primary" olfactory cortex [267–270]. Although other primary cortical areas are typically six-layered, the PC reveals a trilaminar organization similar to that of the hippocampus.

The PC is reciprocally and extensively connected to other higher order cortical structures, including the anterior olfactory nucleus, endopiriform nucleus, olfactory tubercle, entorhinal cortex, prefrontal cortex, perirhinal cortex, and cortical amygdala [265,266,268,271–278]. Interestingly, the PC feedback connections have the ability to influence bulbar output by modulating granule cell activity in the OB [264,279–284]. Its distributed bidirectional nature between the periphery and the higher cortical networks that regulate cognition, emotion, memory, and behavior underscores the importance of the PC in regulating many physiological and behavioral events in mammals.

Anatomically, the PC is commonly divided into two segments: the anterior piriform cortex (aPC) and the posterior piriform cortex (pPC) [223,285–295]. Compared to the pPC, the aPC receives relatively more afferent input from the OB and fewer associational inputs from other cortical areas. This suggests that the aPC may encodes odor identity while the pPC is for 'content addressable memory' e.g., odor object identification [296,297 298 273 292 291 299 245 300 301 302 303 277 304]. However, studies using the early odor preference learning model show a developmental change in their roles. Week old pups exhibit more 2-deoxyglucose(2-DG) uptake to odors in the aPC whereas two-week-old pups show more 2-DG uptake in the pPC. This may reflect the maturation of other associational area

input and processing. The PC has multiple advantages for the study of learning and memory effects in a cortical structure: specifically (1) a comparatively simple anatomy with a laminar organization; (2) an accessible anatomical location for physiological and behavioral studies [305 306]; consistent with (3) a lack of thalamic relays from the periphery; while also having (4) a high-level synthetic role in odor perception; and well-delineated (5) afferent, efferent, and auto-associative connectivities.

1.4.2 Olfactory Learning

Olfactory learning is a commonly used model system for investigating learning and memory mechanisms. Olfactory learning research has involved a variety of species including honey bees [65], *Drosophila* [307 308], lobsters [309], moths [310], mice [311 312], humans [313 314], rats [315 316 317 318], zebrafish [319], rabbits [320], and sheep [321]. Olfactory studies in rodents have provided us with detailed descriptions of the underpinnings of infant-mother attachment, associative learning [322 323 324 325 326 327 328], and learning-related pattern separation and completion [329 330].

In the following section, I will describe different types of animal models in olfactory learning research with a focus on the rodent models used in my investigations.

1.4.2.1 Adult Learning Models

Researchers have used both vertebrates and invertebrates to understand the mechanisms of olfaction. Examples of the different species researchers have been using for olfactory research include lobsters [309], moths [310], honey bees [65], Drosophila [299 300], mice [312], turtles [331], rats [307 308 310 320], rabbits [320], sheep [332], zebrafish [319], and humans [313 314 301]. Within these olfactory studies, complex sensory phenomenon like infant-mother attachment learning, associative learning, pattern separation, and pattern completion [271 322 324 325 326 327 328] have been investigated.

One of the adult behavioral models used for investigating olfactory learning and memory is the 'Go-No-Go' paradigm. In this learning model rodents learn to discriminate between odors depending on the valence of the odor (rewarded or non-rewarded odor). Computer-controlled olfactometers generate odor pulses and deliver or withhold rewards facilitating evaluation of a rodent's ability to detect and discriminate odors [333 334 335 336 337 330]. Typically, during training, water-deprived rodents are allowed to either respond positively to reward-associated odor by entering the odor delivery port for a water reward ("Go") or they must refrain from entering the odor delivery port when a non-rewarded odor is delivered ("No Go").

Other than go/no-go tasks, rodents can be trained to go in a left or right direction for reward. Researchers also use food digging behavioral paradigms where odor and food are associated. The positive odor signals digging and food while in the presence of negative odor, animals should not dig. The digging method requires fewer trials, while

the olfactometer odor discrimination paradigms take significantly more trials to reach learning criteria [338], but are automated, thus limiting experimenter oversight requirements.

1.4.2.2 Early Odor Preference Learning Model

Classical conditioning is one of the most widely used associative behavioral paradigms. A feature of the associative learning of temporally distinct events is to requirement to develop and maintain perceptual and cognitive representations [339] [340]. In classical conditioning, the presentation of the conditioned stimulus (CS) is taken as a predictor of a subsequent unconditioned stimulus (UCS) [341]. A neutral stimulus like a bell ring or an odor (ie. CS) will not generate a predictable response unless they are associated with either reward or punishment (ie. UCS). A behavioral response to a previously neutral stimulus occurs when that stimulus, a CS, is associated with a reward or punishment, a UCS, through learning. The learned behavioral response is the conditioned response. An unconditioned response occurs, irrespective of learning, when a stimulus reliably evokes a response, such as avoidance responses for painful stimuli or approach responses to rewarding ones. Early odor preference learning is an example of classical conditioning. In my thesis, I characterized the intracellular molecular pathways involved in early odor preference learning and I use postnatal day (PND) 1- 11 rat or mice pups, a time period of selective plasticity for this form of learning [342] also referred to as a critical period.

Developmentally, a critical period is defined as a stage of life during which the nervous system is especially sensitive to certain environmental stimuli. During the critical

period for early odor preference learning, rat pups cannot see as their eyes open at PND14-15. Hearing thresholds are also higher during this period with responses to loud sounds evident at PND, 8-9 with increasing sensitivity gradually developing from that point[343] . But olfaction and somatosensory inputs are present from birth [344]. Thus, pups rely particularly on olfactory and somatosensory input to help them maintain proximity to their mothers. As might be predicted bilaterally bulbectomized rat pups (at the age of PND1) weigh significantly less than control pups [345]. These data suggest that olfaction plays an important role during the critical period for rat pups. Since neonates use olfaction to locate their mother for food, they can be conditioned to prefer odors with simple experimental manipulations.

The rodent dam provides pups with positive stimuli including licking, access to milk and warmth, but may also provide harsher stimuli such as biting and stepping on pups. In this early period of relative helplessness, it appears that both the positive and even the more negative natural stimuli facilitate neonate-dam attachment. Early odor preference learning, as a type of classical conditioning, consists of a UCS, a stimulus or stimuli associated with maternal care, and a CS, a distinct odor. Early odor preference learning can be supported by multiple UCSs. Rat pups receive a variety of stimuli during maternal care, as mentioned, and, similarly, a range of stimuli can be used as a UCS. Pairing the UCS with a novel odor creates a conditioned approach response to the trained odor. Examples of stimuli used as the UCSs in this paradigm include milk presentation [346] [347] [348], the odor of maternal saliva [349], stroking or tactile stimulation [350] [351] [352] [353] [354] [355],

and mild foot shocks [356] [357] [356] [358] [359], or tail pinches [352] showing that even aversive stimuli can produce odor preferences, rewarding intracranial brain stimulation [360], and the nesting environment [361] [362] are also effective UCSs.

The CS odor can create a variety of conditioned responses depending on the UCS [363] [360] [364]. For example, generally, a rodent shows aversion to peppermint odor. However, Leon and colleagues [365] [366] first showed that rat pups have a behavioral preference for peppermint odor when tested at PND20 if they were exposed to peppermint for the first 19 days of life (3 h/day). Odor preference could be induced with briefer CS-UCS pairings as well. Odor exposure for 10 min/day on PND 1–18, coupled with tactile stimulation, induced an odor preference on PND 19 [352].

The associative nature of early odor preference behavior has been demonstrated by Sullivan and colleagues. Pups trained with CS-only, UCS-only, random CS–UCS presentations, and backward UCS–CS presentations all failed to acquire a preference for the trained odor [357] [358] whereas, pups with synchronous odor and tactile stimulation developed a conditioned approach to the trained odor [357] [358]. Some aversive stimuli like tail pinch and mild foot shock (0.5 mA), also induce an odor associated preference behavior during the critical period [356] [357] [352]. On the other hand, the same UCS induces odor aversive responses in pups during the second and third postnatal weeks [359] [358] [323]. Similarly, stroking loses its effectiveness as a UCS in the second and third postnatal

weeks [369]. Researchers showed that stroking in the presence of odorant does not induce odor preference after PND 10 [369] which suggests a sensitive period for the development of early odor preferences. A single trial 10 min odor+stroking pairing in the sensitive period induces a protein synthesis-dependent odor preference memory which lasts for 24 h [19].

The molecular characterization of early odor preference learning has helped us to understand the events underlying the CS-UCS pairing effects. Earlier work had shown that norepinephrine (NE) plays an important role as the UCS in early odor preference learning [360] and that the locus coeruleus (LC) is the sole source of NE for the olfactory bulb (OB) and the piriform cortex (PC) [244]. Sullivan and colleagues demonstrated that LC-induced NE release is both necessary and sufficient for early olfactory learning [323]. In early odor preference learning, odorant molecules (CS) bind to the olfactory sensory receptors in the olfactory epithelium. This binding event triggers downstream glutamate signaling to the OB and piriform cortex, but this is not sufficient to induce activation of CREB phosphorylation (a key plasticity or memory event). The odorant (CS) primarily activates AMPARs. The concomitant weak activation of NMDARs and LTCCs is not sufficient to result in the phosphorylation of CREB (the transcription factor). On the other hand, the tactile stimulation delivered via paintbrush stroking (the UCS) releases NE by activation of the LC [370]. Pharmacological studies of early odor preference learning suggest β1-adrenoceptors mediate the UCS effect of tactile stimulation (activating LC-mediated NE release in the olfactory bulb) since systemic or intrabulbar β1 antagonists prevent the effect [371] and the UCS itself can be substituted by a β-adrenoceptor agonist.

However, the UCS alone is unable to induce CREB phosphorylation [371] and that requires forward pairing of CS and UCS [371].

1.4.2.2.1 Advantages of the Early Odor Preference Learning Model

In the following section, I will discuss some of the advantages of the early odor preference learning model for studying the substrates of learning and memory.

1.4.2.2.1.1 The memory circuitry is well defined

In early odor preference memory, the circuitry of the CS and UCS pathways are well characterized [352] [372] [316] [373]. The OB, aPC, and LC are the major brain areas involved. Briefly, the CS odor activates AMPARs in the OB (mitral/tufted cells) and aPC (pyramidal neurons). The addition of the UCS recruits NMDAR activation in the same sites. Previous research showed that inhibition of NMDARs in either OB or aPC prevents odor preference memory from forming, but does not hamper odor perception [316]. Blocking of NMDARs prior to early odor preference testing shows that preference memory, once formed, remains intact even though NMDARs was blocked, which suggests that NMDARs plays a crucial role in memory acquisition without affecting odor

perception or memory retrieval[373]. Molecular events critical and necessary for learning have been identified in both the OB and aPC. These include activation of NMDARs [373] [316], adrenergic receptors [323] [373] [374] [316], L-type calcium channels [375], metabotropic glutamatergic receptors [376], and modulation of inhibition (disinhibition) [316]. Previous work also suggests that both the OB and aPC play critical roles in early odor preference learning and memory and both are necessary.

NE is released from axons originating from LC neurons, which project to OB and PC, during stroking. Activation of β-adrenoceptors as a UCS in the OB or the aPC paired with odor can generate early odor preference memories [352]. On the other hand, inhibition of β-adrenoceptors in both structures independently prevents odor preference memory in neonates [373]. Previously, it was shown that the NE released from the LC and together with activation of olfactory bulb β-adrenoceptors are necessary for acquisition of the conditioned behavioral odor approach response [367] [368]. For example, bilateral lesions of the LC [360] or inhibition of β-adrenoceptors in the OB [377] prevent the acquisition of conditioned odor responses. However, the inhibition of β-adrenoceptor after acquisition again does not impair expression of previously learned behaviors [378]. These results suggest that activation of the noradrenergic projection from the LC to the OB is necessary for early olfactory learning. Similarly, β-adrenoceptor inhibition in the aPC during odor+stroking conditioning prevents the development of odor preference memory.

In summary, it is clear that the OB, aPC, and LC play interconnected roles in early odor preference learning. Dissecting the representations of the CS and UCS in these olfactory areas is relatively simple compared to identifying the CS and UCS representations in hippocampal-dependent spatial and contextual memories.

1.4.2.2.1.2 Memory stages are well-defined

Temporally, as described earlier , memories have been divided into stages including, STM, ITM and LTM [27 17 30 379]. These memory stages are not only temporally different, but there are differences in their dependence on protein synthesis as well. STM does not require protein synthesis, whereas ITM depends on translation, but does not require mRNA transcription. LTM requires both translation and transcription [17 26 27 28 6 29 16 31].

These three memory stages have been characterized in the early odor preference learning model [379]. STM lasts up to 3h after training. ITM is currently defined as occurring up to 5h after training and LTM is seen at 24h. ITM can be disrupted by a translational inhibitor (anisomycin) infused into the OB, but not by a transcriptional inhibitor (actinomycin). STM is not affected by translational or transcriptional inhibitors. Since LTM is transcription and translation-dependent, inhibiting either of these processes

prevents LTM [379]. A 1h time window after training is critical for the learning-related protein synthesis necessary for 24h memory. The inhibition of the translational process 3h after training has no effect on 24h memory. The identification of proteins underlying the circuit remodeling required for learning and memory is facilitated by these time windows.

1.4.2.1.3 Memory duration can be manipulated

One of the fundamental questions of learning and memory research is how to prolong our memories. For learning and educational purposes, how we can strengthen our memories? With early odor preference memory, duration can be manipulated with multiple spaced training trials or with local pharmacological strategies. By applying multiple spaced training for four days, early odor preference memory can be extended for at least 48h [369] [380] and early work suggested even lifelong memories could be generated [356]. Previous characterization of the molecular pathways of early odor preference learning has facilitated the development of prolonged memory models created by using pharmacological agents. By manipulating the dephosphorylation of CREB using a PP2B inhibitor, one can prolong the duration of associative intracellular events in the one-trial odor preference training model beyond 24 hr [381]. Other evidence suggests that inhibition of histone deacetylase (HDAC) prior to training extends 24 h odor preference memory up to 5 days and even longer [216]. Additionally, inhibiting the breakdown of the cyclic AMP by a phosphodiesterase type IV inhibitor extends memory [382].

1.4.2.2.1.4 Lateralization of Early Odor Preference Learning

The left and right hemispheres of the brain generally communicate through a series of commissures. These commissural fibers cross the midline of the brain to form reciprocal connections between two hemispheres. Cutting the major commissural connection between the two sides of the brains causes the animal to behave as if they have two separate brainss. If the inflow of sensory information is restricted to one side of the brain then each side of the brain can exhibit relatively independent perception, learning, and memory. Newly born rats have a "natural-split-brain" advantage for early olfactory learning and memory [383]. Pups younger than PND 12 (lacking the development of the anterior commissure during this time period) showed an increased preference for the conditioned odor in a choice task when tested with the spared side (one nostril open during training) open [383]. The same pups showed no preference for the conditioned odor when tested with the occluded side (the side blocked during training) open [383]. A similar observation was made more recently when lateralized odor preference training in rat pups produced an enhanced network response only in the ipsilateral aPC [380]. In contrast, PND 12 pups or older pups showed learned odor preferences with either naris open when they were trained unilaterally. The maturation of specific components of the anterior commissure (the olfactory system's crossed projection pathway) and the bilateral response develops simultaneously [384]. Bilateral olfactory learning in older pups can be blocked by cutting the anterior commissure before unilateral training [385]. The data suggest

that the anterior commissure is crucial for bilateral olfactory learning in older pups. This unique intra-animal control property of neonates reduces the variation seen in between-animal designs (thus reducing the required number of animals in experiments) and allows more precise experimental control for clarifying the molecular pathways of learning and memory. I have utilized these developmental commissural advantages in the experiments to be described in my thesis.

1.4.2.2.1 The Molecular Mechanisms contributing to Early Odor Preference Learning

Our understanding of the molecular processes underpinning learning and memory has accelerated over the past decades. These studies have helped us to understand neural circuits and the basis of their adaptive change within both invertebrate and vertebrate species. The following sections will focus on the cellular and molecular components supporting early odor preference learning in rodents.

1.4.2.2.1.1 LC–Norepinephrine

Approximately, 40 % of noradrenergic fibers of the LC project to the OB [386]. This results in a heightened sensitivity to sensory input during early developmental stages [370]

[387]. Tactile stimulation at early ages promotes NE release from the LC terminals [387]. The release of NE from the LC [388] [389] is required for acquisition of conditioned odor preference. Pharmacological blocking of noradrenergic β-adrenoceptors in both OB and aPC prevents odor learning [314] [357] [367]. Pharmacological activation of β-adrenoceptors in the OB paired with odor can generate 24 h odor preference memory. Odor preference can also be rapidly acquired by pairing odor with direct stimulation of LC [323]. With this pairing, odor preference learning can also occur beyond the critical period [390].

Odor plus tactile stimulation induces a striking increase of NE in the OB in pups during the first postnatal week, which is not seen to odor alone and is greater than that seen to tactile stimulation alone [391]. However, NE levels are lower to combined stimulation by 10 days of age [388]. It is likely that release of NE from LC terminals in the OB and aPC supports odor preference conditioning by engaging the protein molecules discussed below.

1.4.2.2.1.2 β -Adrenoceptors

Systemic blocking of β-adrenoceptors with propranolol in the rat pup blocks early odor preference learning [367]. Later studies also showed that early odor preference learning was prevented when the β-adrenoceptor blocker is restricted and localized to the OB [323]. Systemic injection of the non-selective β-1 and β-2-adrenoceptor agonist isoproterenol

(2mg/kg) produces 24h early odor preference memory when paired with odor. A later study using selective systemic β1 and β2 agonist administration separately showed that odor preference learning induction is specific to β1-adrenoceptor activation [374]. Odor preference can be induced by a moderate dose of isoproterenol (2 mg/kg) paired with a novel odor. However, odor paired with a lower dose (1 mg/kg) or higher doses (4–6 mg/kg) does not [367] [392] [393] create odor preference in neonate rat pups. A similar effect can also be observed with selective β-1 agonist administration [374]. Since isoproterenol infusion directly in the OB paired with a novel odor also produces odor preference learning, it is likely that both LC-mediated NE release and isoproterenol can serve as unconditioned stimuli.

1.4.2.2.1.3 α-Adrenoceptors

The role of β-adrenoreceptors in the early odor preference learning has been the focus in past decades [392] [367] [378] [323] [352] [393]. The function of α-adrenoreceptors in this learning paradigm has only recently received attention. Like β1-adrenoceptor agonists, systemic injection of α1-adrenoceptor agonists paired with odor can generate odor preference learning [374] and again doses exhibit an inverted U-curve relationship to learning. There were no learning effects with a systemic α-2 agonist for the doses explored. However, recent evidence suggests that infusion of 500mM clonidine (α-2 agonist) directly in the OB can also induce early odor preference learning [394]. The likely

mechanism for OB α-2-adrenoceptor action is cAMP independent and appears to be mediated by disinhibition of mitral cells in the OB.

1.4.2.2.1.4 Serotonin

Serotonin is a neurotransmitter widely present in vertebrates, invertebrates, insects, plants, and even in some unicellular organisms. Initially, it was shown that depletion of serotonin locally in the OB on PND 1 [395] prevented the later acquisition of the conditioned odor learning. By increasing the dose of isoproterenol this effect of 5-HT depletion could be rescued [392]. It was reported that 5HT-2 receptors mediate the effect of 5-HT depletion as learning can be prevented by systemic injections of the 5HT 2A/2C antagonist ritanserin [372]. Learning is inducible by stroking in 5HT-depleted pups if there is a prior subcutaneous injection of a 5HT 2A/2C agonist [372]. Unlike α and β-adrenoceptors agonists, it was not possible to induce learning by pairing novel odor and the 5HT 2A/2C agonist alone [396]. These results suggested that serotonin acts to support normal β-adrenoceptor mediation of odor preference learning, but it does not act as UCS. Serotonin release from the raphe nuclei, during CS-UCS pairing, facilitates the induction process.

1.4.2.2.1.5 Cyclic Adenosine Monophosphate

A role for cyclic adenosine monophosphate (cAMP) as a critical intracellular signal in the initiation of associative learning has been demonstrated in a wide variety of species: Drosophila [397 398 399]; Aplysia [400 401]; and rodents [402 393 403].

Since the ground-breaking discoveries in *Aplysia and Drosophila*, a growing body of evidence supports the hypothesis that the cAMP/protein kinase A/cAMP response element binding protein (cAMP/PKA/CREB) cascade might be a universal mechanism underlying learning and memory. Depletion of 5-HT in the OB prevented early odor preference learning with a typically effective UCS (2mg/kg isoproterenol) for which pCREB and cAMP increases were normally observed [393]. A causal role for cAMP increases in odor preference learning was demonstrated by manipulating cAMP levels with the phosphodiesterase IV inhibitor, cilomilast. The inhibitor converts a low learning-ineffective dose of isoproterenol (1 mg/kg) into an effective UCS [404]. In a follow-up experiment, cilomilast restored learning in pups with 5-HT-depleted bulbs when paired with 2 mg/kg isoproterenol, a condition that had previously been shown to be ineffective in producing learning [405]. Interestingly, simply increasing cAMP is not sufficient for early odor preference learning. A high dose of isoproterenol of 4 mg/kg increases cAMP but does not support learning. So, it has been proposed that a specific temporal patterning, as well as a sufficient level of cAMP increases, is required for learning. A cAMP peak was observed at 10 min after training with the normally effective dose of isoproterenol, but then cAMP levels decreased. With a high dose of isoproterenol, there was only a sustained rising increase in cAMP over the 20-min period following training.

Interestingly, the effective dose of isoproterenol alone also produced a similar rising pattern, but when combined with novel odor input, the profile was a 10 min peak elevation in cAMP was followed by a decrease [403]. The natural odor+stroking procedure also produces a 10-min cAMP peak followed by a decrease pattern. These effects are consistent with other reports of calcium modulation of cAMP patterns [406] [407].

1.4.2.2.1.6 Protein Kinase A

The cellular level of cAMP, and likely its pattern, also determines the activity of PKA. PKA is known to play a key role in long-term memory (LTM) formation in both invertebrates [408] and vertebrates [409]. Activation of PKA phosphorylates learning-related downstream substrates such as serine 133 of the transcription factor CREB [410] [411] and serine 845 of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) GluA1 subunit [411] [412]. Since cAMP plays a crucial role in odor preference learning [404] [405], PKA activation is inevitable. As predicted, PKA activation is maximal 10 min following training during the cAMP peak [413]. Blocking of PKA effects by Rp-cAMPs prevents normal odor preference learning. This also inhibits CREB phosphorylation, which is again consistent with a causal role for the cAMP/PKA/CREB cascade in learning.

1.4.2.2.1.7 CREB

As reviewed, β1-Adrenoceptors can mediate the UCS in early odor preference learning. Tactile stimulation (activating LC-mediated NE release) or an agonist of β-adrenoceptor, triggers the cAMP cascade. On the other hand, the odor serves as the CS that adds to the depolarization of postsynaptic cells to result in calcium influx through NMDARs and L-type calcium channels. The pairing of the UCS and CS modifies cAMP profiles and recruits PKA to generate the phosphorylation of CREB [371].

CREB has been referred to in reviews as "the memory gene" (Yin and Tully, 1996). Using western blot analysis, it was shown that phosphorylated CREB (pCREB) levels significantly increase in the OB 10 min following olfactory conditioning (odor+stroking). This increase of pCREB is correlated with 24 h odor preference memorya [393]. . A causal role for OB CREB in early odor learning was demonstrated directly by manipulating the expression of the CREB gene [414]. The literature also suggests that pCREB activation is a temporally focused and restricted event. The effective dose of isoproterenol paired with peppermint increased pCREB expression [371] at 10 min, while, a high dose of isoproterenol failed to produce learning and did not lead to an increase in CREB phosphorylation despite the elevated cAMP levels. Taken together, these results argue that an optimal profile of CREB phosphorylation 10 min post-training is necessary for initiating 24-h memory in the early odor preference learning model.

1.4.2.2.1.8 Phosphatase

Phosphorylation and dephosphorylation of different memory-related proteins serve diverse roles in long-term memory and synaptic plasticity. But unlike kinases, phosphatases have not been extensively studied in memory research. In the early odor preference learning model, dephosphorylation of CREB can be mediated by either protein phosphatase 1(PP1) or calcineurin (PP2B) [393]. Hyper-activation of PKA may relate to a longer inhibition of PP1 and PP2B and a longer duration of CREB phosphorylation. This remains to be investigated.

However, PKA and PP2B were found to be co-localized at postsynaptic densities [415] [416]. Coincidentally, PP2B is also involved in dephosphorylation of CREB, phosphodiesterases and adenylate cyclases [417] [418]. By manipulating these dephosphorylating actions of PP2B we can alter the duration of associative intracellular events in the one-trial odor preference training model. PP2B inhibition in both OB and aPC using FK506 does not prevent normal 24-h odor preference memory [381] but can prolong it.

Unlike isoproterenol, FK506 does not act as a UCS. However, with normal training parameters, blocking the calcium-dependent phosphatase extends odor preference

memory and extends the duration of CREB phosphorylation. This suggests that CREB phosphorylation duration positively modulates memory duration.

Chapter 2

NMDA receptors in mouse anterior piriform cortex initialize early odor preference learning and L-type calcium channels engage for long-term memory (This Chapter is a modified version of Mukherjee and Yuan, Sci Rep. 2016 Oct 14;6:35256. doi: 10.1038/srep35256.)

2.1 Abstract

The interactions of L-type calcium channels (LTCCs) and NMDA receptors (NMDARs) in memories are poorly understood. Here we investigated the specific roles of the anterior piriform cortex (aPC) LTCCs and NMDARs in early odor preference memory in mice.

Using calcium imaging in aPC slices, LTCC activation was shown to be dependent on NMDAR activation. Either D-APV (NMDAR antagonist) or nifedipine (LTCC antagonist) reduced somatic calcium transients in pyramidal cells evoked by lateral olfactory tract stimulation. However, nifedipine did not further reduce calcium in the presence of D-APV. In mice that underwent early odor preference training, blocking NMDARs in the aPC prevented short-term (3 hr) and long-term (24 hr) odor preference memory and both memories were rescued when BayK-8644 (LTCC agonist) was co-infused. However, activating LTCCs in the absence of NMDARs resulted in the loss of discrimination between the conditioned odor and a similar odor mixture at 3 hr. Elevated synaptic AMPAR expression at 3 hr was prevented by D-APV infusion but restored when LTCCs were directly activated, mirroring the behavioral outcomes. Blocking LTCCs prevented 24 hr memory and spared 3 hr memory. These results suggest that NMDARs mediate stimulus-specific encoding of odor memory while LTCCs mediate intracellular signaling leading to long-term memory.

2.2 Introduction

How synaptic signals are transmitted to the nucleus of the neuron to initiate the gene transcription required for long-term memory has been a topic of intense investigations. Calcium as a 2nd messenger initiates cascades of intracellular signaling that are critically involved in synaptic plasticity and learning. Calcium-stimulated activation of cAMP response element binding protein (CREB) and CRE-mediated gene transcription are a universal requirement in memory formation across species[419]. Voltage-gated calcium channels such as the L-type calcium channels (LTCCs) and NMDA receptors (NMDARs) serve as the principal sites for calcium entry at the membrane and are responsible for the activation of altered gene expression [420] [421]. While both channels are involved in synaptic plasticity mechanisms such as long-term potentiation (LTP), a putative cellular mechanism for memory formation, they differ in their roles in LTP induction and intracellular signalling [422–425].

NMDARs have been regarded as co-incident detectors for presynaptic activity and postsynaptic depolarization during LTP induction [426], which permits calcium entry at the synaptic site [427]. LTCCs, which are localized in the somatic membrane and proximal dendrites [375,427–429], have an important role in translating cytosolic calcium increases to gene expression changes [204,430–432]. In the hippocampus, both NMDAR-dependent and LTCC-dependent LTP has been reported in rodents [422–424,433] as well as in humans [425]. Depending on the induction protocols, short-duration LTP (lasting less than 1 hr) can be induced by a single high frequency or theta burst stimulation, which is abolished by

NMDAR blockers and does not require CRE-mediated transcription. Longer-duration LTP (lasting hours) requires stronger induction (e.g. multiple theta bursts or high-frequency trains) and is dependent on LTCC and CRE-mediated transcription [432,434]. These two calcium channels are also involved in LTP in various other structures including the amygdala [435,436] , anterior cingulate gyrus [437], insular cortex [438], superior colliculi [439], and olfactory bulb [440]. Concurrently, both NMDARs and LTCCs are implicated in various learning models such as spatial memory [185,441] , fear conditioning [435,] and associative olfactory learning [375,440].

Early odor preference learning can be induced in neonatal rat [442,443] or mouse [444,445] by pairing a novel odor with a tactile stimulus that signals maternal care (e.g. stroking the body of the pup with a brush). This model has the advantage of being well-defined with respect to the sites of learning and the temporal phases of the memory (short-term memory (STM) vs. long-term memory (LTM)) [329], therefore it is an ideal model to study memory mechanisms. The piriform cortex is critically involved in odor memory encoding. Blocking NMDARs in the anterior piriform cortex (aPC) prevents odor preference learning in pups and LTP induction in vitro [373]. However, whether LTCCs in the aPC are necessary for early odor learning has not been tested. In this study, we first investigated the relationship of the NMDARs and LTCCs in generating somatic calcium transients in aPC pyramidal neurons, and then we studied the interaction of the NMDARs and LTCCs in odor preference learning in week-old neonatal mice.

2.3 Materials and Methods

In the following sections, I will describe my methodology for the study.

2.3.1 Subjects

Postnatal day (PND) 7-9 C57BL/6 mouse pups (Charles River) of both sexes were subjects. Mice were bred on site and housed under a 12 h light/dark cycle with ad libitum dry food and water. Procedures were consistent with the Canadian Council of Animal Care guidelines and approved by the Memorial University Institutional Animal Care Committee.

2.3.2 Fluorescence Immunohistochemistry

PND 8-10 pups were anesthetized with pentobarbital i.p. (150 mg/kg, Rafter 8 Products) and perfused transcardially with saline (0.9%), followed by paraformaldehyde (4%, dissolved in 0.1 M PBS). Brains were collected and placed in 4% paraformaldehyde overnight at 4ºC, and then transferred to a sucrose solution (20%) for an additional 24 h before slicing.

For slicing, 25 µm coronal sections were cut using a cryostat (HM550, Thermo Scientific) and mounted on chrome-gelatin coated slides. Slides were kept at 4ºC for 10 min before being brought to room temperature to dry. An LTCC anti-Cav1.2 antibody

(1:200, Alomone Labs) was applied to the slides. The antibody was dissolved in phosphate buffered saline (PBS) with 2% Triton-X-100, 0.002% sodium azide, and 5% normal goat serum and left on sections overnight at 4°C in a humidified chamber. The following day, the slides were washed with PBS and a goat anti-rabbit Alexa 488 2nd antibody (1:200, Molecular Probe) was applied to the slices for 1 hr. Slides were then washed 3x10 min in PBS and coverslipped with anti-fade mounting medium (Vectashield, Vector). Images were taken with a Fluoview FV1000 confocal microscope (Olympus) and processed in Corel Photo-Paint X4 software.

2.3.3 Calcium Imaging

Pups were decapitated under halothane anesthesia. Sagittal PC slices (300 μm) were cut in an ice-cold sucrose cutting solution (in mM: 83 NaCl, 2.5 KCl, 3.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, 72 sucrose, 0.5 $CaCl_2$) equilibrated with 95% O_2/5% CO_2[36,37]. Slices were then incubated in the same sucrose solution containing Oregon Green BAPTA-1 AM (10-20 μM, with 0.02% Pluronic F-127, Molecular Probes) at 34˚C for 30-60 min, before washed and left in no-dye solution at room temperature. The recording was conducted in an open bath chamber where slices were perfused with artificial cerebrospinal fluid (aCSF in mM: 110 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, 2.5 $CaCl_2$) at 30.0°C. Slices were visualized with an Olympus BX51WI upright microscope.

In vitro population, calcium imaging followed established procedures[36]. Image acquisition (488 nm excitation, 2x2 binning, 15 Hz) was carried out with a cooled-CCD camera system (Andor Clara, T.I.L.L. Photonics). A concentric bipolar stimulating electrode (FHC) was placed in the lateral olfactory tract (LOT) of the aPC. LOT was stimulated (25-30 µA) by an ISO-Flex stimulator (AMPI) with a 200 µsec pulse. Sagittal slice cutting and recording configurations are demonstrated in Supplementary Figure 2.1. Drugs used included APV (50 µM, Sigma Aldrich), nifedipine (10 µM, Tocris), BayK 8644 (20 µM, Tocris) and NBQX (40 µM; Tocris).

Image processing and analysis were performed with ImageJ (NIH) and Excel. Activity maps of cell ensembles were constructed by averaging 4-6 frames of evoked calcium responses from 5 stimulus trials, background subtracted and median filtered for illustration only (examples in Figure 1A1, 1B1, 2A1, 2B1, 3A1 and 3B1). Changes of somatic [Ca^{2+}] were expressed as relative fluorescence changes ($\Delta F/F$, where F is the baseline fluorescence before a stimulus and ΔF is the evoked change in fluorescence). $\Delta F/F$ was measured in areas of interest in the soma of layer II/III pyramidal cells. The average of the three peak frames immediately following the stimulation was used to indicate the size of the calcium transient. Fifteen to thirty-five cells were randomly selected in each slice image.

2.3.4 Behavioral Studies

Behavioral experiments were carried out in a temperature-controlled room (27°C) and followed previously established protocols[32,35] as described below.

2.3.5 Drug infusion

Intracerebral drug infusions were carried out on PND7 pups following cannula surgeries. Pups were anesthetized via hypothermia (under ice) and placed skull flat in a stereotaxic apparatus. An incision of the skin was made to expose the skull where two small holes were drilled. Two infusion cannulas (Vita Needle, MA) were inserted into the brain at specific coordinates for aPC (1.8 mm anterior and 2 mm bilateral, 3.5 mm ventral with respect to bregma). The aPC coordinates were verified with 4% methylene blue dye or fluorescence bead (example see Supplementary Figure 2.2) in pilot experiments (n = 6). Drugs or vehicle were infused directly via the cannulas. Half µl of the desired solution was infused bilaterally at a rate of 0.25 µl/min using a Hamilton syringe operated by a precision pump (Fusion 400, Chemyx Inc). The infusion tubing and cannula was left for another min before being withdrawn gently from the brain and the skin was sutured. The pups were allowed to recover on the warm bedding for 30 min before odor training. Pharmacological agents used included D-APV (500 µM, dissolved in saline), nifedipine (100 µM, dissolved in 1% ethanol+saline), and a cocktail of D-APV (500 µM) and BayK 8644 (200 µM, dissolved in 1% ethanol+saline). The vehicle used was 1% ethanol+saline.

2.3.6 Odor preference training

After drug or vehicle infusion, pups were subjected to an odor plus stroking (O/S^+) or an odor only (O/S^-) condition. Pups in the O/S^+ groups were placed on peppermint-scented bedding (0.3 ml peppermint extract in 500 ml bedding) and stroked with a paintbrush at 30 sec interval for 10 min, thus comprising 10 trials of 30 sec stroking, interleaved by 30 sec rests. Pups in the O/S^- groups were placed in peppermint-scented bedding for 10 min without being stroked. All pups were returned to the dam after training.

2.3.7 Odor preference testing

Three or twenty-four hours following the odor training, pups were tested for odor preference memory in a testing apparatus. The apparatus contained a stainless steel box (30 x 20 x 18 cm) placed over two training boxes separated by 2 cm. One box contained peppermint-scented bedding and the other contained unscented bedding, or vanillin-scented bedding (0.3 ml vanillin in 500 ml bedding; dissimilar odor test), or peppermint (70%) + vanillin (30%) mixture bedding (similar odor test). During testing, pups were placed in the 2 cm central zone. Times that pups spent over peppermint-scented versus other bedding were recorded in five one-minute trials. Pups were allowed 1 min rest between the trials in a clean cage. The percentage of the time spent over peppermint bedding over total time spent over either bedding was calculated for each pup. To

evaluate stimulus-specific memory, rats were tested alternately with dissimilar odors or similar odors first, followed with the other odor pairs 10 min later.

2.3.8 Synaptic AMPAR measurement

In separate cohorts, 3 hr following odor training, pups were decapitated, and aPCs were collected and flash frozen on dry ice [446]. The aPC is located posterior to the olfactory bulbs but anterior to the termination of the LOT. The LOT is visible on the ventral surface of the brain and the aPC lies dorsal and lateral to it. Tissue was collected in a triangular shape from the ventral surface of the brain at and lateral to the LOT, posterior to the olfactory bulbs, and anterior to the termination of the LOT. Samples were stored at -80°C until further processing.

2.3.9 Synaptic membrane isolation

Extraction of synaptic membrane followed previously published procedures [446]. Tissue samples were homogenized in sucrose buffer (300 µl) on ice containing (in mM): 320 sucrose, 10 Tris (pH7.4), 1 EDTA, 1 EGTA, 1X complete protease inhibitor mixture and phosphatase inhibitor mixture (Roche). The homogenized tissue was centrifuged at 1000 rpm for 10 min. The supernatant was taken and centrifuged at 10,000 rpm for 30 min to obtain a pellet, which was subsequently re-suspended in 120 µl sucrose buffer using a pestle mixing/grinding rod (Thomas Scientific) directly in the microfuge tube.

Eight volumes of Triton X-100 buffer (in mM: 10 Triton-100, 1 EDTA, 1 EGTA, 1X protease and phosphatase inhibitors pH7.4) were added for detergent extraction (final 0.5% v/v). This suspension was incubated at 4 °C for 35 min with gentle rotation and then centrifuged at 28,000 rpm for 30 min. The pellet containing postsynaptic densities and synaptic junctions that are insoluble in Triton X-100[39] was re-suspended in 100 µl of TE buffer (in mM: 100 Tris, 10 EDTA, 1% SDS, 1X protease and phosphatase inhibitors), sonicated, boiled for 5 min and stored at -80ºC until use. Protein concentrations for each sample were determined by a BCA protein assay kit (Pierce). The volume of lysate required to make 35 µg of protein for each sample was calculated.

2.3.10 Western blotting

A total of 100 µl lysate solution, sample buffer (0.3 M Tris-HCl, 10% SDS, 50% glycerol, 0.25% bromophenol blue, 0.5 M dithiothreitol), and dH2O were prepared and boiled for 2 min at 100°C. Samples and a protein ladder (Thermo Scientific) were loaded into a 7.5% SDS-PAGE gel. Sample separation by SDS-PAGE was followed by transference to a nitrocellulose membrane (Millipore). Membranes were cut horizontally at the 72 kDa level, and the upper portion was probed with a rabbit antibody for GluA1 subunits (1:7000, Cell Signalling) [447], and the lower portion was probed for β-actin (1:5000, Cell Signalling). Membranes were incubated in primary antibody overnight at 4°C on a shaker. Next day membranes were washed 3x5 min with 1X TBST. HRP-bounded 2nd antibodies were applied (1:10,000, anti-rabbit; Pierce) for 1 hr. Membranes

were then washed 3x10 min in TBST and enhanced chemiluminescence Western blotting substrate (Pierce) was applied. Blots were then developed on x-ray film (AGFA). Films were scanned using an image scanner (CanoScan LiDE 200), and the optical density (OD) of each band was measured using ImageJ software. Each sample was normalized to the corresponding β-actin band that was run on the same gel.

2.3.11 Statistical Analyses

Statistical analyses were performed using OriginPro software (Originlab, MA). Data were presented as Mean ± S.E.M. One-way ANOVAs were used for behavioral tests with Fischer LSD post-hoc comparisons to evaluate differences between behavioral groups. One-way ANOVAs with Fisher LSD post-hoc tests or paired t-tests were used for Western blotting and calcium imaging data.

2.4 Results

In the following sections, I will describe my results from the first Chapter.

2.4.1 LTCC activation is dependent on NMDAR activation in aPC pyramidal cells

We first looked at the LTCC Cav1.2 expression in the aPC using immunohistochemistry. LTCCs were expressed in the membrane of the soma and the base of the apical dendrites of pyramidal cells in layer II/III (Figure 2.1a, n = 3), similar

to the expression pattern in the hippocampus as reported previously [429]. In contrast, in neocortex such as the motor cortex, LTCCs were also expressed in the shaft of apical dendrites (Figure 2.1b). In neither area did we observe LTCC expression in distal dendritic arbors as is normally observed for NMDARs [448,449]. However, we cannot exclude the possibility that LTCCs are expressed in distal dendrites in low density that is beyond the detection threshold in our method.

We then studied the effects of LTCC or NMDAR blockade on somatic calcium transient evoked by LOT stimulation. Action potentials in dye-loaded cells elicit somatic calcium transients such that cells recruited by LOT stimulation can be identified [450,451]. Cells with somatic transients were largely confined to the pyramidal cell layers. Similar to evoked EPSCs, somatic calcium transients correlates positively with LOT stimulation intensities (Supplementary Figure 2.3). The somatic calcium transient was reduced in the presence of APV and abolished when NBQX was added (Figure 2.2a1-2.3), suggesting calcium transients seen here were post-synaptic responses evoked by the LOT stimulation. With the moderate stimulation intensities used (25-30 μA), single LOT stimulation evoked ~2-10% somatic calcium increase in individual cells (e.g. Figure 2.2a2, 2.2b2, 2.3a2, and 2.3b2). On average, APV reduced single LOT stimulation evoked calcium transient to 54.2 ± 2.7% of the baseline, while the residual calcium was almost abolished (4.2 ± 3.1% of the baseline) in the presence of NBQX ($n = 80$ cells from 4 slices, $t = 12.38$, $p < 0.001$ compared to APV; Figure 2.2a3). Adding nifedipine to aCSF reduced calcium transients to 86.3 ± 0.8% of the baseline, which was reversed following

30 min wash (101.6 ± 0.02%, n = 110 cells from 4 slices, t = 10.61, p < 0.001 compared to nifedipine, Figure 2.2b1-3).

We next studied the interaction of the LTCC and NMDAR in eliciting somatic calcium transients. In the presence of APV, nifedipine failed to further reduce the somatic calcium transient (50.7 ± 0.02% of the baseline in APV vs. 50.0 ± 0.02% in APV+nifedipine, t = 0.68, p = 0.49, n = 55 cells from 3 slices; Figure 2.3a1-3). This suggests that LTCC activation was subsequent to the NMDAR activation. We then tested whether a stronger stimulus could recruit LTCCs directly as may happen during theta burst or high-frequency stimulation. When 4 LOT stimulations at 100Hz were used, nifedipine still failed to further reduce calcium transients (70.8 ± 0.01% in APV vs. 70.0 ± 0.01% in APV+nifedipine, t = 1.13, p = 0.26, n = 70 cells from 3 slices; Figure 2.3a4-5). It is noted that the calcium transient evoked with the train stimulation has a smaller NMDAR component (30%) compared to that in the single stimulus (50%), suggesting additional recruitment of other voltage-gated calcium channels or mGluRs under the stronger stimulation.

If LTCCs act downstream of the NMDARs, then direct activation of the LTCCs in the presence of NMDAR blockade should allow additional calcium influx to the cells. This is indeed the case when BayK 8644, an LTCC agonist was added in the presence of APV. Single LOT stimulation was used. BayK 8644 increased the calcium transient to 76.0 ± 4.4% compared to 46.9 ± 2.9% in APV only (t = 11.53, p < 0.001, n = 125 cells from 5 slices; Figure 2.3b1-3).

2.4.2 Differential roles of the NMDAR and LTCC in early odor preference learning

We then pursued behavioral experiments to test the roles of the NMDAR and LTCC in early odor preference learning in neonate mice. We tested the effects of NMDAR or LTCC blockade on short-term (3 hr) and long-term (24 hr) memory [452] (Figure 2.4a) and tested whether BayK 8644 could rescue the learning from NMDAR blockade at the two-time points.

NMDAR blockade prevented 3 hr memory while blocking LTCC had no effect on this short-term memory. One-way ANOVA shows significant differences in treatment conditions ($F_{4, 20} = 53.66$, $p < 0.001$; Figure 2.4b). Post-hoc Fisher test shows a significant difference between the O/S^++vehicle (65.18 ± 1.78) and the O/S^-+vehicle (33.09 ± 2.06) groups (n=5, $t = 9.70$, $p < 0.001$). D-APV infusion prevented odor preference memory (36.83 ± 2.68%, n=5, $t = 8.57$, $p < 0.001$) while the nifedipine group showed comparable odor preference (65.11 ± 1.56%) compared to the O/S^++vehicle group ($t = 0.02$, $p > 0.05$). Activating LTCCs with BayK-8644 rescued the 3 hr odor preference memory from the NMDAR blockade (67.92 ± 3.22, n=5, $t = 9.40$, $p < 0.01$ compared to the D-APV only group).

For the 24 hr memory, both NMDAR and LTCC blockade prevented it. One-way ANOVA shows significant group effects ($F_{4, 30} = 8.69$, $p < 0.001$; Figure 2.4c). Post-hoc Fischer test shows a significant difference between the O/S^++vehicle (62.87 ± 3.92, n = 7)

and the O/S^{-}+vehicle (35.08 ± 2.93) groups (n = 7, t = 4.29, p < 0.01). Both nifedipine (35.61 ± 5.31, n = 6) and APV (32.56 ± 5.32, n = 7) prevented 24 hr preference memory compared to the O/S^{+}+vehicle pups (p < 0.001). However, adding BayK 8644 to the APV rescued the learning at 24 hr (53.54 ± 4.95, n = 8, t = 3.34, p < 0.01) compared to the D-APV only group.

2.4.3 NMDAR blockade impairs stimulus-specific discrimination of the conditioned odor

We were intrigued that either isolated NMDAR activation (in the presence of nifedipine) or isolated activation of LTCCs (D-APV+BayK 8644) was able to induce 3 hr memory. AMPAR synaptic insertion is implicated in both short-term and long-term odor preference memory [447,453]. We tested the amount of AMPAR synaptic membrane expressions in these conditions (Figure 2.5a&b; Full length blots are presented in Supplementary Figure 2.4). AMPAR synaptic expression at 3 hr in the aPC mirrored the behavioral outputs ($F_{4, 24} = 3.21$, p < 0.05; Figure 2.5b). The nifedipine group showed higher AMPAR expression (1.31 ± 0.10, n = 6) compared to the O/O+vehicle group (1.04 ± 0.04, n = 5, t = 2.12, p = 0.04). D-APV only prevented the AMPAR increase (1.00 ± 0.09, n = 6, t = 0.30, p = 0.77). Co-infusion of BayK-8644 increased AMPAR (1.34 ± 0.10, n = 6, t = 3.24, p < 0.01 compared to D-APV only group).

To understand whether and how the memories formed through either LTCCs or NMDARs differ, we performed experiments to test stimulus specificity of the 3 hr

memory using odor discrimination between the conditioned odor peppermint, and a dissimilar odor vanillin, or an odor mixture (70% peppermint + 30% vanillin) (Figure 2.5a). Dissimilar odor testing yielded peppermint preference patterns similar to that in the 3 hr peppermint vs. normal bedding test ($F_{3, 25} = 47.53$, $p < 0.001$; Figure 2.5c1). Both nifedipine (70.97 ± 3.83, n = 7) and D-APV+BayK 8644 (74.11 ± 2.45, n = 8) groups showed significantly higher time spent over peppermint bedding compared to the O/S^- +vehicle group (32.67 ± 2.50, n = 7, $p < 0.01$). It has been reported previously that rat pups form a generalized avoidance or approach to other novel odors when olfactory bulb GABAa receptor [454] or CaMKII [447] is blocked. To test whether aPC LTCC activation alone without NMDARs results in generalized approach response, we tested vanillin preference in a cohort of mouse pups. D-APV+BayK 8644 pups trained with peppermint did not show any preference to the novel odor vanillin (Supplementary Figure 2.5).

However, when peppermint was tested against a similar odor mixture ($F_{3, 25} = 35.93$, $p < 0.001$; Figure 2.5c2), the D-APV+BayK 8644 group showed no preference for the peppermint bedding (43.35 ± 2.94, n = 7) compared to the O/S^-+vehicle group (38.23 ± 2.99, n = 7, t = 1.22, p = 0.23), while the nifedipine group still showed a clear preference (67.26 ± 1.69, n = 7, t = 6.73, $p < 0.01$). The fact that in the D-APV+BayK 8644 group, the same pups showed preference for peppermint in the dissimilar odor test but no preference for peppermint in the similar odor test suggests that activating LTCCs alone in the absence of NMDAR activation results in loss of stimulus specificity of the odor memory – the memory is extended to other similar stimulus that has a large component overlapping with the conditioned stimulus.

2.5 Discussion

NMDARs and LTCCs demonstrated differential roles in early odor preference learning in mouse pups. Previous work defined three temporal phases for early odor preference memory: a short-term memory (up to 3 hr) which is independent of transcription and translation, an intermediate memory (5 hr) which requires transcription but not a translation, and a long-term memory (24 hr) which is dependent on both transcription and translation [379]. Blocking NMDARs during learning prevented both short-term (3 hr) and long-term (24 hr) memories. However, LTCC blockade prevented 24 hr memory but did not interrupt short-term memory. It is striking that LTCC blockade itself did not affect 3 hr memory and associated AMPAR increase, but activating LTCCs when the NMDARs were blocked nevertheless induced 3 hr memory and AMPAR increases. These results suggest that NMDARs, but not LTCCs, are normally required for 3 hr memory. However, when LTCCs are overdriven, it could compensate NMDAR loss to promote the AMPAR insertions needed for short-term memory.

The NMDAR and LTCC are involved in different forms of LTP in hippocampus [432,434] , however, whether they engage in different phases of memory has not been tested. In the amygdala, both NMDARs and LTCCs are required in LTPs with distinct induction protocols. Spike-timing-dependent LTP generated by associating pre- and postsynaptic activities requires LTCCs but not NMDARs, while a tetanic stimulation engages NMDARs exclusively [435]. Interestingly, similar to what we observed in this study, NMDAR blockade prevents both short and long-term fear memory while blocking

LTCCs exclusively affects only long-term memory [435]. However, how these two channels are differentially engaged in the signaling pathways that lead to either short-term or long-term memory is not known. Both long-term fear memory [455] and odor preference memory [373,443,456] require CREB signaling. Short-term memory may only require local AMPAR insertion into the synaptic membrane mediated by calcium-activated CaMKII signalling [447].

LTCCs are expressed in the somatic membrane and at the base of the apical dendrites of pyramidal cells in the aPC. This subcellular distribution of LTCCs in pyramidal cells in the aPC is consistent with that in other structures [375,427–429] and implies a differential role of LTCCs in intracellular signaling from NMDARs. Early experiments in striatal neurons demonstrated sequential activation of AMPARs, NMDARs, and LTCCs [457]. A model was put forward to suggest that LTCC activation is dependent on NMDARs due to their longer opening kinetics than those of AMPARs. Once activated, LTCC allows Ca^{2+} influx and activation of a kinase pathway to translocate to the nucleus to phosphorylate CREB at Ser133 [457]. Our calcium imaging data showing NMDAR-dependent activation of the LTCC is consistent with this model.

Detailed downstream signaling from these calcium channels is best characterized in the hippocampal neurons. In the hippocampus, although both the NMDAR and LTCC are driven by physiologically relevant synaptic inputs to engage CREB signaling [204], the LTCC appears to be particularly important in coupling synaptic signaling to the nucleus [204,458,459]. α_{1C}-comprised LTCC contains a calmodulin (CaM) binding domain and

calcium influx through LTCC activates CaM, leading to phosphorylation of CREB [459]. Various routes are suggested to mediate CaM signaling from the cytosol to the nucleus [458]. Either CaM translocates to the nucleus to activate calmodulin kinase IV (CaMKIV) [431,457,460] , or it activates other kinases (e.g. mitogen-activated protein kinase, MAPK), which in turn translocate to the nucleus to phosphorylate CREB [459].

Another intriguing finding is that LTCC activation in the absence of NMDAR activation results in impaired discrimination of the conditioned odor from a similar odor mixture. This suggests a critical role of NMDAR in mediating the stimulus specificity in early odor preference learning. NMDAR hypofunction has been linked to impaired pattern separation in the hippocampal dentate gyrus [461]. We propose that synaptic NMDARs associate odor-induced glutamate input with stroking/norepinephrine-induced excitation of pyramidal cells to initiate memory encoding and ensure input-specificity of the learning by activating CaMKII signaling and CaMKII- mediated AMPAR insertion [447]. Meanwhile, the activation of the NMDAR leads to prolonged depolarization of the pyramidal cells and subsequently engages LTCCs. Calcium influx through LTCCs initiates CaM-mediated protein kinase translocation into the nucleus to activate CREB transcription. Direct activation of LTCCs without NMDARs may lead to AMPA insertion that affects a broader range of synapses.

Interestingly, cognitive decline during aging has been associated with increased LTCC activities in the hippocampus [462–464]. There is a shift from NMDAR-dependent LTP to LTCC-dependent LTP in the aging hippocampus [465,466]. Abnormal activity of

hippocampal neurons is correlated with impaired pattern separation ability both in aged humans [467] and in aged animals [468]. Olfaction dysfunction is also common in aging populations and is one of the earliest signs indicating Alzheimer's disease (AD) development [469,470]. However, it is not known whether altered expressions and functions of LTCCs in the aPC underlie the olfactory deficiency in AD patients.

In summary, our results highlight the importance of balanced NMDAR and LTCC functions in encoding input-specific long-term memory.

2.6 Figures of Chapter 2

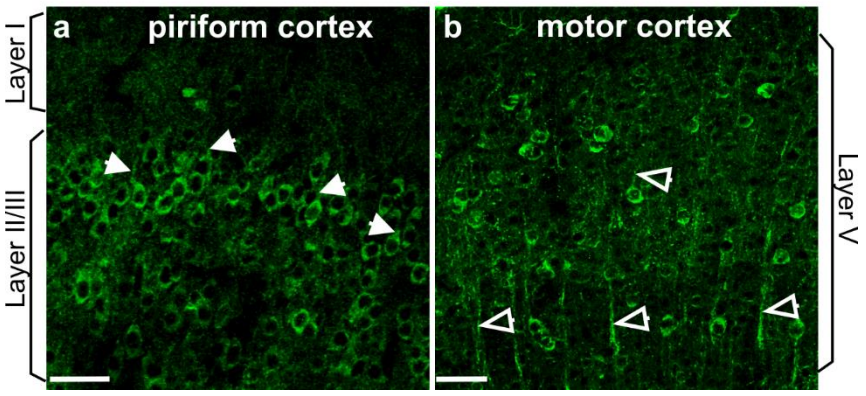


Figure 2.1. L-type calcium channels (LTCCs) are expressed in the piriform cortex pyramidal cells

(a) LTCC expression in the anterior piriform cortex layer II/III pyramidal cells using an antibody against Cav1.2 channels. Solid arrows indicate the LTCC staining at the base of the apical dendrites. (b) LTCC expression in the motor cortex layer V pyramidal cells. Note that apical dendritic shafts (arrows) are stained.

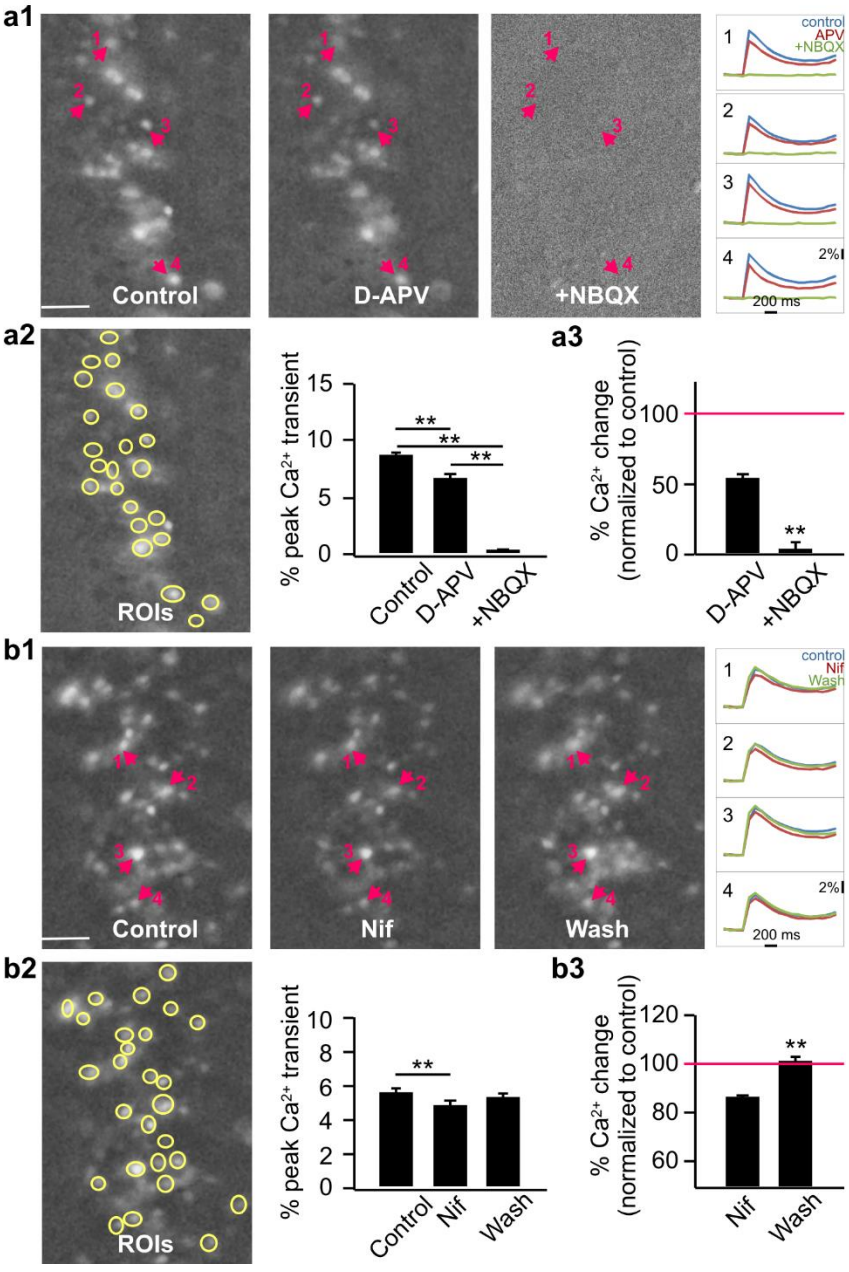


Figure 2.2. Lateral olfactory tract (LOT) stimulation activates LTCCs
(a1-a3) Somatic calcium transients in the anterior piriform pyramidal cells by LOT stimulations are dependent on postsynaptic AMPARs and NMDARs. (a1) Example images of population calcium imaging evoked by a single LOT stimulation in control, D-

APV and D-APV+NBQX conditions. Images were constructed by averaging 4-6 frames of evoked calcium responses (ΔF/F) from 5 stimulus trials. Example calcium transient traces from 4 cells are shown on the right. (a2) Peak calcium transients (ΔF/F%) averaged from a population of cells (yellow circles, regions of interest) on the same slice. (a3). Normalized calcium changes (to control) during D-APV and D-APV+NBQX applications from 4 slices (n = 80 cells). (b1-b3) Blockade of LTCCs reduces somatic calcium transients in pyramidal cells. (b1) Example images of population calcium imaging in control, nifedipine (Nif) and Nif washout conditions. (b2). Peak calcium transients averaged from a population of cells on the same slice. (b3) Normalized calcium changes during Nif and Nif washout from 4 slices (n = 110 cells). **$p < 0.01$.

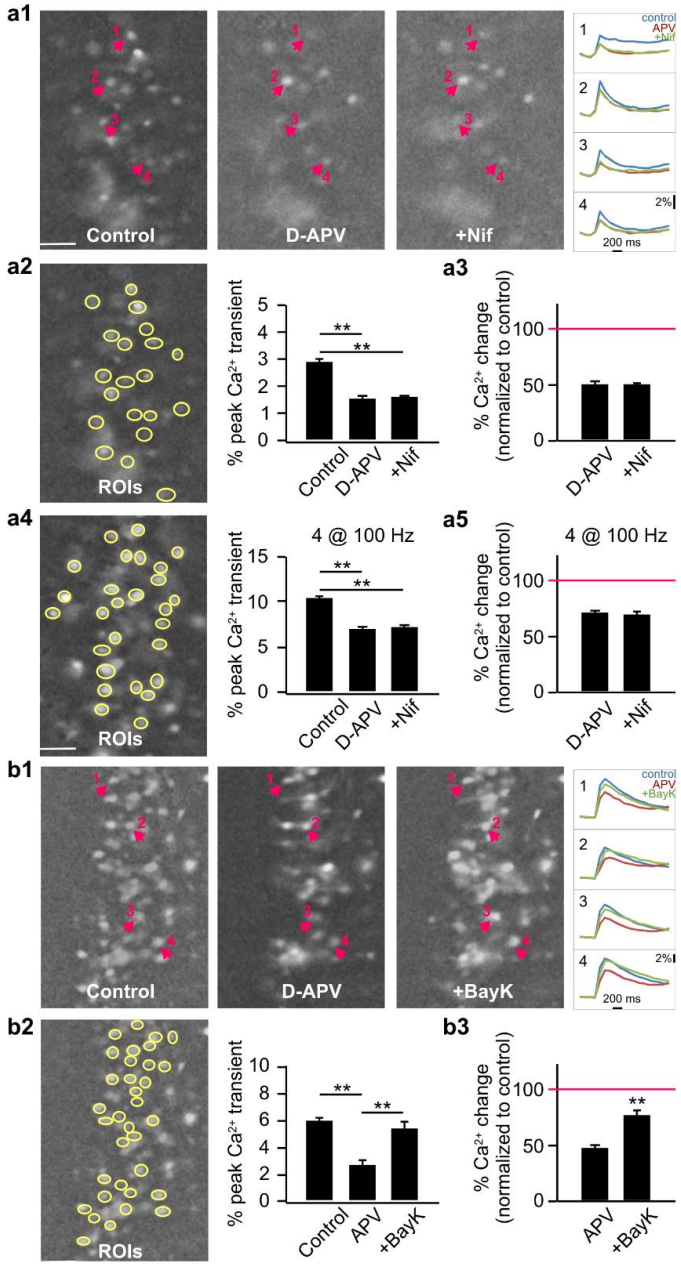


Figure 2.3. LTCC activation is subsequent to NMDAR activation

(a1-a5) Nifedipine does not further reduce calcium transients in the presence of NMDAR blockade by D-APV. (a1) Example images of population calcium imaging in control, D-APV and D-APV+Nif conditions. Example calcium transient traces from 4 cells are

shown on the right. (a2) Peak calcium transients (ΔF/F%) to a single LOT stimulation, averaged from a population of cells (yellow circles) on the same slice. (a3) Normalized calcium changes (to control) to a single LOT stimulation during D-APV and D-APV+Nif applications from 3 slices (n = 55 cells). (a4) Peak calcium transients to 4 LOT stimulations at 100 Hz. (a5) Normalized calcium changes to 4 LOT stimulations from 3 slices (n = 70 cells). (b1-b3) Application of BayK-8644 increased somatic calcium transients in the presence of D-APV. (b1) Example images of population calcium imaging in control, D-APV, and D-APV+BayK-8644 conditions. (b2) Peak calcium transients averaged from a population of cells on the same slice. (b3) Normalized calcium changes during D-APV and D-APV+BayK-8644 from 5 slices (n = 125 cells). **$p < 0.01$.

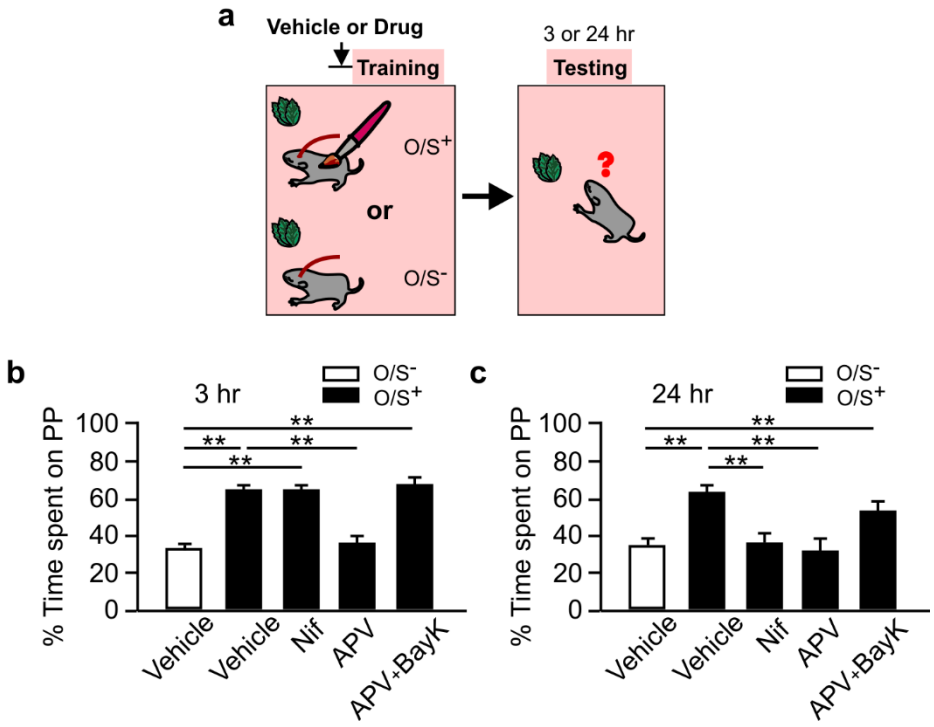


Figure 2.4. Differential roles of NMDARs and LTCCs in early odor preference learning

(a) Schematics of the odor preference training and testing paradigm. (b) Percentage of time spent over peppermint (PP)-scented bedding at 3 hr testing. (c) Percentage of time spent over PP-scented bedding at 24 hr testing. **p < 0.01.

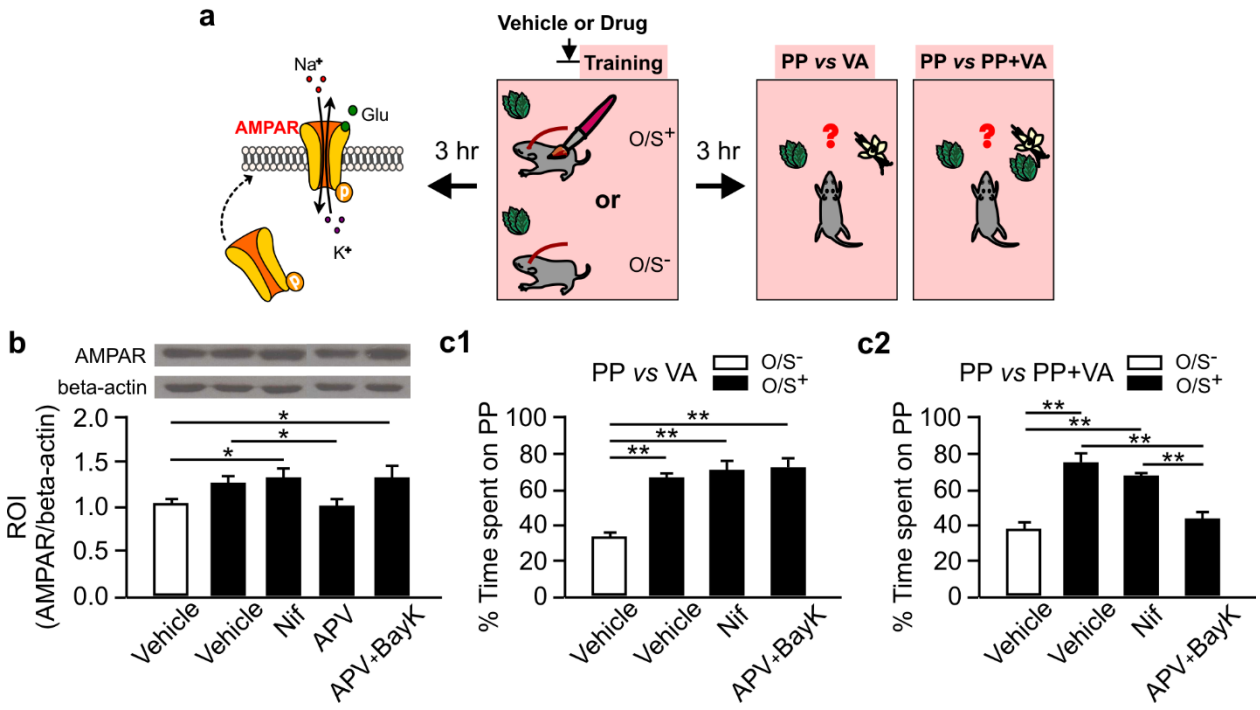


Figure 2.5. NMDARs but not LTCCs mediates input-specific discrimination of the conditioned odor

(a) Schematics of the odor preference training and testing paradigm. (b) Relative optical densities (ROIs) of AMPAR expression (normalized to beta-actin) in various groups at 3 hr post-training. Full-length blots are presented in Supplementary Figure 2. (c1) Percentage of time spent over peppermint (PP)-scented bedding when tested with two dissimilar odors. (c2) Percentage of time spent over PP-scented bedding when tested with PP *vs.* PP+VA (vanillin) mixture. *$p < 0.05$, **$p < 0.01$.

Chapter 3

Unlearning: NMDA receptor-mediated metaplasticity in the anterior piriform cortex following early odor preference training in rats. (This Chapter is a modified version of Mukherjee et al. J Neurosci. 2014 Apr 9;34(15):5143-51. doi: 10.1523/JNEUROSCI.0128-14.2014.)

3.1 Abstract

Here we demonstrate metaplastic change in NMDA receptor (NMDAR) number in the anterior piriform cortex (aPC) in rat induced by, a 10 min pairing of peppermint odor+stroking, which significantly modifies later learning and memory. Using isolated synaptoneurosomes, we found GluN1 receptor down-regulation 3 hr after training and up-regulation at 24 hr. Consistent with the GluN1 pattern, the NMDAR-mediated EPSP was smaller at 3 hr and larger at 24 hr. Subunit composition was unchanged. While long-term

potentiation (LTP) was reduced at both times by training, long-term depression (LTD) was facilitated only at 3 hr.

Behaviorally, pups, given a pairing of peppermint+stroking 3 hr after an initial peppermint+stroking training, lose the normally acquired peppermint preference 24 hr later. To probe the pathway specificity of this unlearning effect, pups were trained first with peppermint and then, at 3 hr, given a second training with peppermint or vanillin. Pups given peppermint training at both times lost the learned peppermint preference. Pups given vanillin re-training had normal peppermint preference when trained 3 hr later. Down-regulating GluN1 with siRNA prevented odor preference learning. Finally, the NMDAR antagonist, MK-801, blocked the LTD facilitation seen 3 hr post-training and giving MK-801 prior to the second peppermint training trial eliminated the loss of peppermint odor preference. A training-associated reduction in NMDARs facilitates LTD 3 hr later; while training at the time of LTD facilitation reverses an LTP-dependent odor preference. Experience-dependent, pathway-specific metaplastic effects in a cortical structure have broad implications for the optimal spacing of learning experiences**.**

3.2 Introduction

Early odor preference learning in the week-old rat pup is a well-studied model of Pavlovian associative conditioning [271]. As rat pups have neither normal visual or auditory

input, they are dependent for survival on the dam; thus orienting to dam-associated odors is essential [471]. Odor associative learning also occurs in newborn human infants [472,473]. In the rat pup model, a single 10 min pairing of odor (conditioned stimulus or CS) and maternal care signals such as dorsal whole body stroking (unconditioned stimulus or US) induces a protein-synthesis dependent 24 hr odor preference memory [379].

In addition to neural changes in the olfactory bulb, which have been extensively studied [414,474], neural changes in the anterior piriform cortex (aPC) are also necessary, and sufficient, for the expression of early odor preference learning [373]. A CS, typically peppermint odor, paired with a β-adrenoceptor agonist infusion in aPC, induces odor preference learning, while either a locally infused β-adrenoceptor antagonist, or a locally infused NMDA receptor (NMDAR) antagonist, prevents odor preference learning.

A strong feature of the rat pup odor-conditioning model is that the anterior commissure is not yet developed [383] and odor input can be restricted to a single hemisphere using temporary unilateral naris occlusion during training [451,475]. The reduction in response variability permitted by this within-subject control revealed, in ex vivo slices, that an increase in the AMPA receptor (AMPAR)-mediated synaptic response to lateral olfactory tract (LOT) input in the aPC parallels odor preference memory [373,451,475]. Calcium imaging in the same preparation reveals an increase in the activation of

pyramidal cells in aPC following training [451], implying a stronger network representation for the trained odor.

The NMDAR plays a critical role as a co-incidence detector for mediating AMPAR plasticity in associative learning (Malenka and Bear, 2004), including early odor preference learning [373,476]. Research in the past two decades has provided evidence that the NMDAR itself is dynamic and undergoes plastic changes, including changes in the number of receptors and in subunit composition [477]. Long-term plasticity of NMDAR-mediated synaptic transmission such as LTP and LTD has been extensively characterized in vitro, using acute brain slices and neuronal cultures [195,478]. Activity-dependent NMDAR plasticity in vivo has been reported in the visual cortex [479,480] and the olfactory system [213,476,481]. However, its functional significance in learning is not well understood. Here, we examine odor training-induced modulation of the NMDAR and its associated plasticity effects in the aPC. We find metaplastic, pathway–specific changes that modulate the rat pup response to subsequent associative odor training.

3.3 Materials and Methods

In the following sections I will describe all the methods I have used for my experiment in my second project.

3.3.1 Animals and Ethics Statement

All experimental procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland with adherence to the guidelines set by the Canadian Council on Animal Care. Sprague Dawley rat pups of either sex (Charles River) were used in this study. Animals were bred and pups were born on-site at the research facility. Litters were culled to 12 pups with equal numbers of males and females on postnatal day 1 (PND1; day of birth is designated PND0). Dams were maintained with ad libitum access to food and water.

3.3.2 Behavioral Studies

Behavioral experiments were carried out in a temperature controlled room at approximately 28°C and followed the standard protocol previously established for early odor preference learning [443,482] as described below. One-way ANOVAs and post hoc Fisher tests were used to determine statistical significance throughout the experiments.

3.3.3 Odor preference training and testing

On PND 6 or 7, pups were assigned to an odor plus stroking (O/S^+) or an odor only (O/S^-) condition. Pups were removed from the nest and placed on normal bedding for 10 min. After this habituation period, pups receiving conditioning training (O/S^+)

were placed on scented bedding (peppermint or vanillin; 0.3 mL odorant extract in 500 mL bedding) and vigorously stroked with a paintbrush for 30 sec, followed by a 30 sec rest, for a total of 10 min. Pups in the non-learning condition (O/S^-) were placed in peppermint-scented bedding for 10 min without stroking, following the habituation period. Pups were returned to the dam after training.

For O/S^+ pups that were re-trained at 3 hr or 24 hr following the first training, they were exposed to peppermint or vanillin-scented bedding while being stroked, with the same procedure as in the first training. A control group was initially trained with peppermint+stroking, and then re-trained with peppermint odor only at 24 hr. A subset of peppermint+stroking re-trained pups underwent systemic intraperitoneal (i.p.) injection of either MK-801, an NMDAR antagonist (0.1 mg/kg in 50 µl saline, Tocris), or saline, 40 min before the onset of the re-training.

Twenty-four hrs after a given training or retraining episode (e.g. 48 h after initial training for pups receiving a 24 h retraining event), pups were tested for odor preference memory using a two-choice odor preference procedure. The testing apparatus was a stainless steel box (30 x 20 x 18 cm) placed over two training boxes. One box contained peppermint-scented bedding and the other contained normal, unscented bedding. Testing boxes were separated by a 2 cm neutral zone. For testing, pups were removed from the dam and placed in the neutral zone. Times that pups spent over scented versus normal

bedding were recorded in five one-minute trials, each separated by a one-min rest in a clean cage. The average time spent over peppermint bedding was calculated for each pup.

3.3.4 Reversible naris occlusion for ex vivo experiments

Nose plugs were constructed using polyethylene 20 (PE 20) tubing and silk surgical thread as per procedures described previously [373,483]. A small dab of a sterile jelly of local anesthetic, 2% Xylocaine (AstraZeneca), was applied to the left naris of the pup and let rest for ~3 min before the plug was gently inserted in the left naris. The pup was then placed on unscented bedding to habituate to the nose plug, followed by appropriate odor conditioning training with peppermint-scented bedding. The nose plug was removed immediately following training and pups returned to dams. Some control pups were subjected to naris occlusions only for the same amount of time without undergoing odor training. Pups that underwent lateralized odor training were killed at various time points for tissue collection for either Western blot or electrophysiological recording.

3.3.5 Intracerebral infusion of SiRNA

SiRNA for the NMDA GluN1 receptor was infused into the aPC on PND 3, followed by behavioral training on PND 7. PND 3 pups were anesthetized via hypothermia and placed in a stereotaxic apparatus in a skull flat position. A horizontal incision was made to expose the skull and two small holes were drilled. One μl of 1% GluN1 SiRNA mixed in RNase free vehicle (Dharmacon ACCELL SiRNA delivery media, Thermo Scientific) or 1% control non-targeting SiRNA was injected into the aPC (2 mm anterior to bregma, 3 mm bilateral, 5 mm below the surface) using a Hamilton syringe. In some pups, SiRNA or control was injected into the olfactory bulbs. The injection was carried out over 4 min and the syringe was left in site for another 6 min before being gently withdrawn from the brain. The skin was sutured and the pups recovered in a heated cage. Thirty min following recovery from the surgery, pups were returned to the dam.

On PND 7, pups with bilateral GluN1 SiRNA or non-targeting control infusions underwent behavioral training and on the following day were tested with peppermint-scented bedding. Pups with unilateral GluN1 SiRNA infusion and non-targeting control SiRNA injected into the opposite aPC or olfactory bulb were killed for tissue collection for Western blots.

3.3.6 Western blots of synaptoneurosomes

Three or 24 hr following lateralized odor training, or on PND 7 following unilateral GluN1 SiRNA injections (conducted on PND 3), pups were decapitated, and aPCs or olfactory bulbs were collected and flash frozen on dry ice. Samples were stored at -80°C until further processing.

3.3.7 Synaptoneurosome isolation

All samples were kept on ice during the synaptoneurosome extraction in order to minimize proteolysis. Brain samples were homogenized in oxygenated HEPES buffer (in mM: 50 HEPES, 124 NaCl, 26 $NaHCO_2$, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 3.2 KCl, 1.06 KH_2PO_4, 10 glucose, 1 EDTA, 1 PMSF, complete protease inhibitor cocktail (Roche), complete phosphatase inhibitor cocktail (Roche), saturated with 95%O_2/5% CO_2, pH 7.4), using Teflon-glass tissue homogenizers (Thomas Scientific). Lysed samples were kept on ice for 10 min for incubation in the buffer and were then passed through 3 pre-wetted filters using a 13 mm diameter syringe filter holder (Millipore). The first two filters were 100 µm nylon filters (Small Parts Inc.) and the last filter was a 5 µm pore hydrophilic filter (Millipore). Filtrates were centrifuged at 1000 x g for 20 min at 4°C, following which the pellets were resuspended in HEPES buffer. These pellets represent the synaptoneurosome fraction. Protein concentrations for each sample were determined using a BCA protein assay kit (Pierce). The volume of lysate required to make 40 µg of protein for each sample was calculated.

3.3.8 Western Blotting

Sixty µL total volume solutions of lysate (volume required for 40 µg protein), sample buffer (0.3 M TRIS-HCl, 10% SDS, 50% glycerol, 0.25% bromophenol blue, 0.5 M dithiothreitol) and dH_2O were prepared and boiled for 5 min at 100ºC. Samples were then loaded into lanes of a 7.5% SDS-PAGE gel, along with a protein ladder (Thermo Scientific) and loading buffer for empty lanes. Sample separation occurred through SDS-PAGE, followed by transference to a nitrocellulose membrane (Amersham). Membranes were cut horizontally at the 72 kDa level and the upper portion was probed with a rabbit antibody for GluN1 (1:2000, blocked in BSA; Cell Signalling) subunits, and the lower portion was probed for β-actin (1:5000, blocked in 4% m.f. milk; Cedarlane). Membranes were incubated in primary antibody and agitated overnight at 4ºC. Secondary antibodies bound to horseradish peroxidase were applied the following day (1:20,000, anti-rabbit; Pierce) and membranes were then washed in Enhanced chemiluminescence Western blotting substrate (Pierce). Finally, blots were developed on X-ray film (AGFA).

Films were scanned onto a computer using an image scanner (CanoScan LiDE 200) and the optic density (OD) of each band was measured using ImageJ software. Each

sample was normalized to the corresponding β-actin band that was run on the same gel. In pups that underwent lateralized odor training, each spared hemisphere was normalized to its naris-occluded counterpart. In pups with SiRNA infusion, the GluN1 SiRNA infused hemisphere was normalized to its non-targeting control counterpart. Experimental values are reported as mean ± SEM of the relative expression of the normalized GluN1 subunit. A one-way ANOVA was used to evaluate differences in the mean OD.

3.3.9 Electrophysiology: Tissue Preparation and Extracellular Recording

Pups were anesthetized by halothane or isoflurane inhalation and quickly decapitated. Brain tissue was extracted and placed in a high glucose artificial cerebral spinal fluid (aCSF; in mM, 83 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 3.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, & 72 sucrose, equilibrated with 95% O_2 and 5% CO_2) for approximately 10 minutes. Sagittal slices (400μm) of the piriform cortex were cut using a vibrating blade (Vibratome 1000P, Leica Microsystems) and incubated at 34°C in the aforementioned solution for 30 minutes then returned to room temperature. Tissue slices were recorded in aCSF (in mM, 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, equilibrated with 95% O_2 and 5% CO_2) at 30-32°C and viewed with an upright microscope (Olympus BX51). Extracellular field potentials were recorded with glass pipettes filled with aCSF and placed in layer Ia of the aPC. A concentric bipolar stimulating pipette (FHC) was lowered into the LOT and delivered single test pulses, ranging from 10-60 μA.

Electrophysiological data were recorded with Multiclamp 700B (Molecular Devices), filtered at 2 kHz and digitized at 10 kHz. Data acquisition and analysis were performed with pClamp10 and ClampFit 2.10 (Molecular Devices) and Igor Pro 6.10A (WaveMetrics). Student's t-tests were used to determine statistical significance.

3.3.10 Ex vivo electrophysiology

PND 7-10 pups undergoing ex vivo electrophysiological recording were subjected to odor conditioning with one naris occluded as described earlier. Following a 10 min habituation period, pups underwent O/S^+ or O/S^- training on peppermint-scented bedding. Upon completion, the plugs were removed and pups were returned to the dam. Pups were sacrificed 3 or 24 hr following training and brain slices were prepared. The hemispheres of the brain were kept separately within the incubation chamber in order to achieve intra-animal control.

The NMDAR component of the field EPSP (fEPSP) was isolated using NBQX, an AMPAR antagonist (5 μM, Tocris) and low Mg^{2+} (100 μM) aCSF [475,481]. The NMDAR input/output (I/O) relationship was measured as the ratio of the size of the presynaptic fiber volley (FV) to the slope of the fEPSP, and compared between the spared and occluded slices. The AMPAR component of the fEPSP was measured as the initial slope of the fEPSP in normal aCSF [373,451]. The ratio of AMPAR to NMDAR was measured as

the slope of fEPSP recorded in normal aCSF to that recorded in NBQX and low Mg^{2+} at the same stimulation intensity. Ifenprodil (3 μM; a GluN2B antagonist, Tocris) was used to isolate the non-GluN2B NMDAR synaptic potential and bath applied to the slice for 30 min. The GluN2B mediated fEPSP was measured as the fraction of NMDAR fEPSP that was blocked by ifenprodil.

For LTP recording, a baseline of fEPSPs evoked by single pulse test stimulation was recorded at 0.05 Hz until the last 10 min were consistent and was then followed by eight theta burst stimulations (TBS; 10 times 5 Hz trains, each train contains 5 pulses at 100 Hz) separated by 30 sec. The stimulation intensity for recording and stimulation was determined as that at which 50% of the maximum response was evoked. For LTD recording, the same procedure was used except the induction was initiated by 900 low frequency stimulations at 1 Hz. The stimulation intensity was set to provoke 60-70% of the maximal fEPSP response. In a subset of LTD experiments, MK-801 (40 μM) was bath applied to the slice for 10 min before induction and washed out after induction. The recordings were analyzed to determine the amount of LTP or LTD (compared to the baseline) at the LOT to aPC synapses. Data were binned to demonstrate the fEPSP value per min.

3.4 Results

In following sections I will describe my results from the second Chapter.

3.4.1 Plasticity of NMDARs in the aPC 3 hr and 24 hr following early odor preference learning

Morrison et al (2013) showed that early odor preference training results in increased AMPAR responses at the LOT synapses in the aPC at both 3 hr and 24 hr post-training. NMDARs in the aPC mediate early odor preference learning and likely mediate the AMPAR LTP observed ex vivo following learning. The present study tests whether the NMDAR itself undergoes plasticity following early odor preference learning.

Rat pups underwent early odor preference learning with single naris occluded during training. Western blot analyses of synaptoneurosome extracts from the aPC of occluded and spared hemispheres were compared at 3 hr or 24 hr time points post training. A one-way ANOVA revealed significant differences among groups ($F_{(5,41)}$ = 3.17, $p < 0.05$; Figure 3.1). At 3 hr following O/S^+ learning, there was a significant down-regulation in GluN1 (0.67 ± 0.12, n = 9) that was not found 3 hr following O/S^- training (1.40 ± 0.19, n = 9). At 24 hr, there was a significant up-regulation in GluN1 in O/S^+ (1.59 ± 0.15, n = 7) animals, and not O/S^- animals (1.02 ± 0.18, n = 8). Animals that did not receive any training and were subjected to naris occlusions only did not show different GluN1 levels from two hemispheres at either time point. Post hoc Fisher LSD tests showed that O/S^+ 3 hr pups expressed significantly less GluN1 compared to both no odor 3 hr animals ($p < 0.05$) and O/S^- 3 hr animals ($p < 0.01$). O/S^+ 24 hr animals yielded

significantly higher GluN1 expression compared to O/S^+ 3 h ($p < 0.05$) and O/S^- 24 h animals ($p < 0.05$). Together, these results suggest that conditioning leads to NMDAR GluN1 subunit expression down-regulation 3 hr and an up-regulation 24 hr following O/S^+ training.

Next we examined the expression of NMDAR plasticity in the aPC. We focused on the LOT afferent input synapses where AMPAR LTP was previously observed following early odor preference learning [373,451]. Ex vivo electrophysiology was conducted 3 hr or 24 hr following lateralized odor training (O/S^+). Lateralized training allows a within-animal control at this age [373,451,475]. We measured the NMDAR fEPSP I/O relationship in the presence of NBQX. The I/O relationship measuring the ratio of fEPSP slope to the presynaptic FV of the LOT allows us to directly compare the relative number of functional NMDARs at LOT synapses from spared and occluded hemispheres of the same animals [475,481]. A paired t-test showed that at 3 hr post training, there was a decrease in the NMDAR I/O in the spared (0.021 ± 0.004, $n = 6$) hemisphere compared to the occluded hemisphere (0.049 ± 0.007; $n = 6$, $t = 5.75$, $p < 0.01$; Figure 3.2A1), indicating less NMDAR response for a given input. However, at 24 hr post O/S^+ training, there was an increase in NMDAR I/O in the spared (0.060 ± 0.008, $n = 7$) versus the occluded hemisphere (0.034 ± 0.004, $n = 7$, $t = 2.71$, $p < 0.05$; Figure 3.2A2).

The decrease of NMDAR fEPSPs at 3 hr, and the increase at 24 hr post odor training are consistent with the changes of GluN1 subunit expression levels observed with Western blotting, suggesting plasticity of GluN1 occurs at the LOT synapses. To test whether there is also an NMDAR composition change such as a switch from GluN2B to GluN2A subunits as observed following odor associative learning in adult rats [213], we measured the percentage of the GluN2B response relative to the total NMDAR fEPSP. At both 3 hr (42.4 ± 10.27% occluded, vs. 37.24 ± 8.99 spared, n = 5, t = 0.29, p > 0.05; Figure 3.2B1), and 24 hr (45.92 ± 9.61% occluded, vs. 43.48 ± 12.20 spared, n = 7, t = 0.19, p > 0.05; Figure 3.2B2), there was no difference in the fraction of GluN2B responses. Together, these results suggest NMDAR plasticity at the LOT synapse following early odor preference learning, is due to changes in the numbers of GluN1 essential subunits. The fractions of GluN2B subunits are similar in both hemispheres suggesting that GluN2B levels parallel GluN1 levels and there are no subunit composition changes.

3.4.2 GluN1 down-regulation at 3 hr coincides with decreased LTP and increased LTD at the LOT synapse

What is the consequence of the changes in NMDARs for the synaptic state and plasticity? We first looked at the ratio of AMPAR to NMDAR response at the LOT synapse following learning. We found that there was an increase in the AMPAR/NMDAR ratio at 3 hr (28.17 ± 3.47 occluded, vs. 66.56 ± 6.47 spared, n = 6, t =

4.46, $p < 0.01$; Figure 3.2C1). This is consistent with both an increase in AMPAR response as observed previously (Morrison et al., 2013) and a decrease in the NMDAR response as observed in this study. However, at 24 hr post-training, there was no difference between the occluded hemisphere (37.15 ± 4.03, $n = 7$) and the spared hemisphere (40.45 ± 9.62, $n = 7$, $t = 0.36$, $p > 0.05$; Figure 3.2C2). The finding of no change in the AMPAR/NMDAR ratio at 24 hr is likely due to proportional increases in both AMPAR [373] and NMDAR responses. Developmentally, an increased AMPAR/NMDAR ratio coincides with decreased AMPAR LTP in the olfactory cortex [481].

We then explored how changes in NMDAR expression and response following early odor learning shape synaptic plasticity at the LOT synapse. It has been shown that LTP at the LOT synapse is excluded in the trained hemisphere at 24 hr following early odor preference learning [373]. Consistent with this result, we observed a learning-induced LTP exclusion effect also at 3 hr post-training. In the untrained hemispheres, on average the fEPSP slope size increased to $115.5 \pm 2.9\%$ at 30 min following TBS induction ($n = 6$), whereas in the trained hemisphere, no LTP of the fEPSP was observed ($95.9 \pm 4.2\%$, $n = 6$). Accordingly, there was a significant difference between the two groups at 30 min post-induction ($t = 3.80$, $p < 0.01$, Figure 3.3A). We next tested LTD inductions 3 hr following odor training ex vivo in naris occluded versus spared hemispheres. A low frequency induction protocol (900 pulses at 1 Hz) [484] resulted in LTD of the fEPSP in the spared hemisphere ($80.5 \pm 9.4\%$ of the baseline, $n = 6$, $t = 2.08$, $p < 0.05$), but not in the

occluded hemisphere (105.7 ± 18.3% of the baseline, n = 6, t = 0.31, p > 0.05; Figure 3.3B). Interestingly, at 24 hr following odor training, there was no LTD induction and no difference in the two hemisphere groups (96.8 ± 6.4% occluded, vs. 93.0 ± 11.9% spared, n = 7, t = 0.29, p > 0.05, Figure 3.3C). Together with the previous report [373], we show that at 3 hr post-training, there is decreased LTP induction at the LOT synapse, in parallel with an increase in LTD induction. However, at 24 hr post-training, there is only decreased LTP induction, without a parallel and opposite change in LTD induction. While an increased AMPAR response at both 3 hr and 24 hr [373] may result in a ceiling effect of synaptic LTP and account for LTP exclusion at both time points, a decrease in NMDARs at 3 hr may also account for less LTP and appears to be solely responsible for a lower threshold for LTD induction. Down-regulation of NMDAR induces metaplasticity that alters the plastic state of the previously active synapses.

3.4.3 GluN1 down-regulation at 3 hr interferes with learning at the same synapse

What is the functional significance of NMDAR down-regulation following early odor learning? Particularly, how would down-regulation of NMDARs at 3 hr following the first training influence another episode of learning at the same synapse? To answer this question, we designed experiments in which animals were trained in two trials separated by a 3 hr interval. During the first training session, pups were assigned to one of the two bedding conditions and underwent O/S^+ training: peppermint bedding, or vanillin bedding. The vanillin bedding group was re-trained with peppermint bedding 3 hr later.

Pups in the peppermint bedding group during the first training were divided into five groups: one group was re-trained with peppermint bedding at 3 hr, one group was re-trained with vanillin bedding at 3 hr, one group was re-trained with peppermint bedding at 24 hr, one group was exposed to peppermint bedding only at 24 hr, and one group was not re-trained. An O/S^- group trained once was also included as a negative control. Odor preference testing showed significant group effects ($F_{(6,56)} = 14.70$, $p < 0.01$; Figure 3.4). Interestingly, pups trained two times in peppermint bedding with a 3 hr interval showed no preference learning for the peppermint (42.98 ± 3.88%, n = 10) 24 hr later, compared to the O/S^+ one-time training group (61.30 ± 4.78%, n = 10, $p < 0.01$). The vanillin bedding group showed normal 24 hr learning to peppermint when re-trained with peppermint bedding 3 hr following the first training (66.03 ±4.30%, n = 10), compared to the O/S^- group (31.92 ± 3.62%, n = 10, $p < 0.01$). Re-training with vanillin bedding did not impair the odor preference memory to the first training odor peppermint (71.69 ± 4.84%, n = 7) compared to the O/S^- group ($p < 0.01$). Pups trained two times in peppermint only bedding but separated by a 24 hr interval did not show any learning deficiency (66.77 ± 4.41, n = 9), this is in comparison with the group that was re-exposed to peppermint only at 24 hr, which showed no preference memory to peppermint (25.43 ± 7.45%, n = 7, $p < 0.01$). This suggests any effect of re-training with peppermint+stroking cannot be explained by peppermint exposure itself and confirms the limited duration of one trial peppermint preference memory.

Our results suggest that re-training at 3 hr interferes with the expression of odor preference induced by initial exposure. This learning interference coincides with NMDAR down-regulation, reduced LTP and increased LTD induction at LOT synapses. To establish the causal relationship between NMDAR down-regulation and learning deficiency, we infused SiRNA that specifically down-regulates the NMDA GluN1 subunit bilaterally into the aPCs. Western blots showed that four days following the initial injection of SiRNA into the brain, the GluN1 subunit was significantly down-regulated compared to the control hemisphere, no matter whether it was in the olfactory bulb (51.4 ± 12.3% of the control hemisphere, $n = 5$, $t = 3.95$, $p < 0.05$; Figure 3.5A) or in the aPC (33.7 ± 4.9% of the control counterpart, $n = 6$, $t = 13.62$, $p < 0.01$; Figure 3.5B). Behavioral experiments ($F_{(3,30)} = 3.66$, $p < 0.05$; Figure 3.5C) demonstrated that SiRNA bilateral infusion into the aPCs prevented early odor preference learning (41.8 ± 6.3%, $n = 10$) compared with the controls (67.0 ± 6.1%, $n = 8$, $p < 0.01$). This result suggests that GluN1 down-regulation globally in the aPC results in odor learning deficiency in rat pups. Therefore it is likely that GluN1 down-regulation 3 hr following the first training accounts for impaired learning during re-training.

3.4.4 Metaplasticity at 3 hr following early odor training is NMDAR-dependent

How could re-training involving the same synapses that supported learning impair subsequent learning or memory expression? NMDAR down-regulation at 3 hr following the first training coincides with enhanced LTD induction at the LOT synapse. NMDAR-

dependent Ca^{2+} influx is critical for both LTP and LTD. A high rise of intracellular Ca^{2+} through NMDAR triggers LTP and a slower, lower rise of Ca^{2+} favors LTD induction [145,485]. We tested the hypothesis that fewer NMDARs at the same synapse during re-training lowers the amount of Ca^{2+} influx and favors LTD induction. We first asked, by using ex vivo slices 3 hr post O/S^+ training, whether LTD induction at the LOT synapse is affected by NMDAR blockage. When MK-801, an NMDA open channel blocker was bath applied to the slices, LTD induction was abolished (104.7 ± 10.9% of the baseline at 30 min post-induction, $n = 7$, $t = 0.43$, $p > 0.05$, Figure 3.6). This result suggests that the NMDAR and likely calcium influx through NMDAR, mediates LTD induction 3 hr following O/S^+ training.

We next examined whether NMDAR blockage during re-training would allow early odor preference memory to be expressed. We compared three groups: 3 hr re-training with saline injection, 3 hr re-training with a prior MK-801 injection, and an MK-801 injection only at 3 hr without re-training. One way ANOVA shows significant group effects ($F_{(2,27)} = 16.67$, $p < 0.01$; Figure 3.7). Re-training at 3 hr with a saline injection, prevented the expression of odor preference memory tested at 24 hr (29.38 ± 4.32%, $n = 10$), consistent with the previous result (Figure 3.4). However, when MK-801 was administered to the pups at the time of the 3 hr re-training session, the odor preference memory at 24 hr was intact (71.39 ± 3.51%, $n = 10$, $p < 0.01$ compared to the saline re-training group). MK-801 injection itself 3 hr following the O/S^+ training did not interfere with the expression of the odor preference memory (58.28 ± 7.22%, $n = 10$, $p < 0.01$

compared to the saline re-training group). Together, these results suggest NMDAR blockage abolishes metaplasticity occurring at 3 hr following the first O/S^+ training, but does not affect the expression of already formed memory from the first O/S^+ training.

The key results in this study are summarized in Table 1.

3.5 Discussion

3.5.1 Bi-phasic NMDAR Plasticity Following Early Odor Preference Learning in Rats

Here we report bi-phasic changes in NMDAR synaptic transmission and in absolute amount of the essential subunit GluN1 in the aPC following early odor preference learning in rats. NMDAR LTD was inducible at the LOT to pyramidal cell synapse 3 hr post odor training. Contrary to what was seen with odor rule learning in adult rats [213], in which a switch from the GluN2B to GluN2A subunit occurred following learning, we observed no change in the GluN2B-mediated synaptic potential relative to the total NMDAR response in the learning hemisphere compared to control. This suggests down-regulation of the GluN1 subunit at the LOT synapse is responsible for the reduced NMDAR synaptic transmission 3 hr post-training.

As reported previously in nucleus accumbens [486], NMDAR LTD 3 hr posttraining is concurrent with AMPAR LTP [373]. It is likely that both the AMPAR LTP and NMDAR LTD are mediated through Ca^{2+} influx through NMDARs [477] and implies that the same postsynaptic Ca^{2+} concentration induces opposite effects on AMPAR- and NMDAR-mediated synaptic responses [486].

At 24 hr post-training we observed LTP of the same NMDAR-mediated synaptic responses, likely due to GluN1 up-regulation at the LOT synapse. This delayed NMDAR LTP accompanies AMPAR LTP at the same synapse [373], which restores the AMPAR to NMDAR ratio to control levels.

Despite extensive characterization of NMDAR plasticity in vitro [195,478], activity-dependent changes of NMDARs following behavioral conditioning have been less commonly reported [478]. Changes of NMDAR subunit composition such as the switch between GluN2B and GluN2A subunits underlie developmental changes [479], sensory deprivation/enrichment effects [480], and associative learning [213]. The differential activation of NMDAR subunits translates into different dynamics for NMDAR ion conductance and charge transfer, which influence NMDAR-mediated synaptic integration and plasticity [477].

Induction mechanisms for the bi-phasic NMDAR changes following early odor preference learning require further investigation. In vitro work shows that, similar to

AMPAR plasticity, both LTP and LTD of NMDAR require either NMDAR or mGluR activation, and a postsynaptic Ca^{2+} rise [478]. Postsynaptic Ca^{2+} influx activates the enzymatic signaling required for plasticity, including protein kinases PKA [487], PKC [488,489], and Src [489] for LTP induction, and phosphatases such as protein phosphatase 1 (PP1)/PP2A for LTD induction [490].

Expression mechanisms involve NMDAR trafficking into, and out of, the synaptic membrane through endo/exocytosis as well as lateral movement (Hunt and Castillo, 2012). NMDAR LTD can be mediated by Ca^{2+}-dependent actin depolymerization, which promotes lateral diffusion of NMDARs from synaptic membrane to extrasynaptic sites [490,491], or dynamin-dependent endocytosis [492]. LTP of the NMDAR can be expressed by exocytosis of NMDARs via SNARE-dependent processes [493] or recruitment of NMDARs from extrasynaptic sites to the synaptic membrane [494].

It is unclear how the bi-phasic changes in NMDARs, seen here following a single odor preference training session, occur. The initial depression may involve only transient receptor trafficking out of the synaptic membrane independent of new protein synthesis, or only local synaptic proteins and mRNA may be involved, while the later potentiation may result from homeostatic regulation to match AMPAR number [495] and require protein synthesis. If this were true, separate signaling would be involved in the early LTD and late LTP of NMDARs following early odor preference training.

3.5.2 Down-regulation of NMDAR Leads to Synaptic and Behavioral Metaplasticity

Metaplasticity refers to the change of capacity for synaptic plasticity due to prior synaptic activity [195]. Given the role of NMDARs in mediating AMPAR synaptic plasticity, one consequence of NMDAR plasticity is a change in the ability to induce NMDAR-dependent plasticity of AMPAR-mediated synaptic transmission [478]. Studies in visual cortex demonstrate changes in LTP or LTD capability following periods of altered visual experience [480]. Our results provide direct evidence in support of NMDAR plasticity as a mechanism for metaplasticity in natural learning.

We show that down-regulation of the GluN1 subunit and LTD of NMDAR-mediated synaptic responses 3 hr following odor preference learning coincides with decreased AMPAR LTP (de-potentiation) and increased AMPAR LTD. This shift favoring LTD induction is not solely due to synaptic ceiling effects on AMPARs since at 24 hr, LTD of the AMPAR is no longer inducible, while LTP of the AMPAR persists [373]. Together, GluN1 down-regulation and NMDAR LTD are most likely to account for the threshold shifts for AMPAR LTP and LTD induction.

Behavioral metaplasticity has been used to refer to the ability of stress experiences to alter behavioral learning due to system wide changes [496]. Here behavioral

metaplasticity occurs at specific synapses. A shift in the threshold of AMPAR synaptic plasticity induced by training has consequences for learning using the same synapse. Three hrs following initial training, re-training with the same odor+stroking protocol, not only did not consolidate initial learning, as is seen with repeated training at 24 h, but impaired odor learning. Initial training induced LTD of NMDARs, leading to metaplasticity of AMPAR-mediated synaptic responses. Re-training at the same synapse likely caused de-potentiation of AMPAR LTP, or caused AMPAR LTD, and either way, abolished odor preference learning. The metaplasticity here is pathway-specific as training with two different odors with the same 3 hr interval did not interfere with preference learning for peppermint odor. Input specificity of behavioral metaplasticity is consistent with in vitro work demonstrating synapse-specific metaplasticity in hippocampus [497–499].

Metaplasticity at the LOT synapse following early odor preference learning not only results from changes in NMDAR number, but as seen in vitro [497–499], depends on the NMDAR itself for its expression. NMDAR blockade abolished the AMPAR LTD induced 3 hr following odor training. Furthermore, blocking NMDARs before re-training permitted odor preference memory expression. It requires further investigation to know how re-training at 3 hr impairs odor preference memory. However, one likely explanation is that NMDAR down-regulation at 3 hr results in less Ca^{2+} influx through NMDARs during re-training and favors AMPAR LTD or AMPAR LTP de-potentiation.

3.5.3 Functional significance of NMDAR metaplasticity

How metaplasticity occurs in vivo in a way that is relevant to cognitive function is not well understood [500]. Behavioral metaplasticity is proposed to alter learning and memory, and the likely mechanism is altered capacity for synaptic plasticity [496]. Studies on stress-induced metaplasticity support this view. Behavioral stress impairs LTP induction and hippocampal-dependent learning and involves NMDAR activation [501]. Stress, or high-levels of corticosterone, may also impair recollection of previously acquired memories.

Behavioral stress appears to retard plasticity at a global level involving multiple brain structures [496], but the present study provides some of the first evidence that learning itself affects future learning capacity at the same synapses. A recent study of amygdala-dependent fear conditioning showed that a weak training trial that does not produce fear memory, primes future learning such that another trial delivered 60 min to 3 days later results in long-lasting and robust fear memory [502]. Together, these studies suggest behavioral metaplasticity occurs, and the strength of the priming event may determine the nature and time course of the synaptic changes and the character of the behavioral modulation.

There are two ways to view the present results. Down-regulation of the NMDA receptor is physiologically necessary following activation and the behavioral effect is adventitious. Or down-regulation supports a behaviorally relevant homeostatic regulation of learning. Substantial increases in circuit strength, and consequent dominant sensory control over approach behavior may be limited, particularly in the case of sustained odors, by a waxing and waning mediated by the metaplasticity change described. Odor preference must be sufficiently weak to permit pups to leave the dam as they become more mobile. Consistent with such variation in odor preference learning is evidence that preference for peppermint constantly present in the maternal cage fades rapidly after exposure ends, while peppermint on the dam, providing a more variable stimulus over the same period, produces an enduring preference [503].

Correspondingly, multiple spaced presentations of odor/stroking at 24 h intervals produces an enduring preference compared to a single association, as might be predicted from the present NMDAR changes [451,504]. Understanding behavioral metaplasticity, both globally, and through pathway-specific changes, has significant implications for enhancing stimulus-specific adaptive learning and for diminishing, or ameliorating, the behavioral impact of traumatic learning.

3.6 Figures of Chapter 3

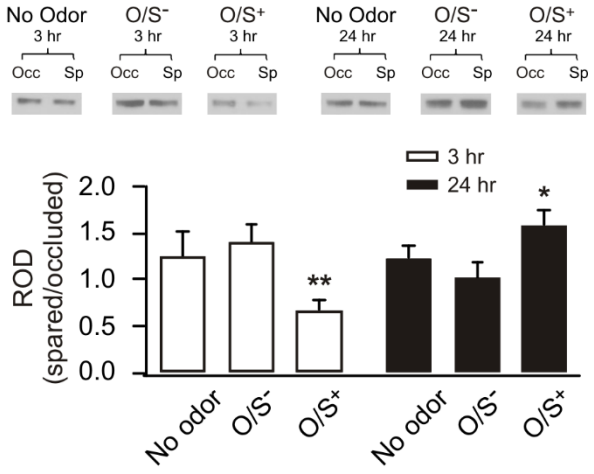
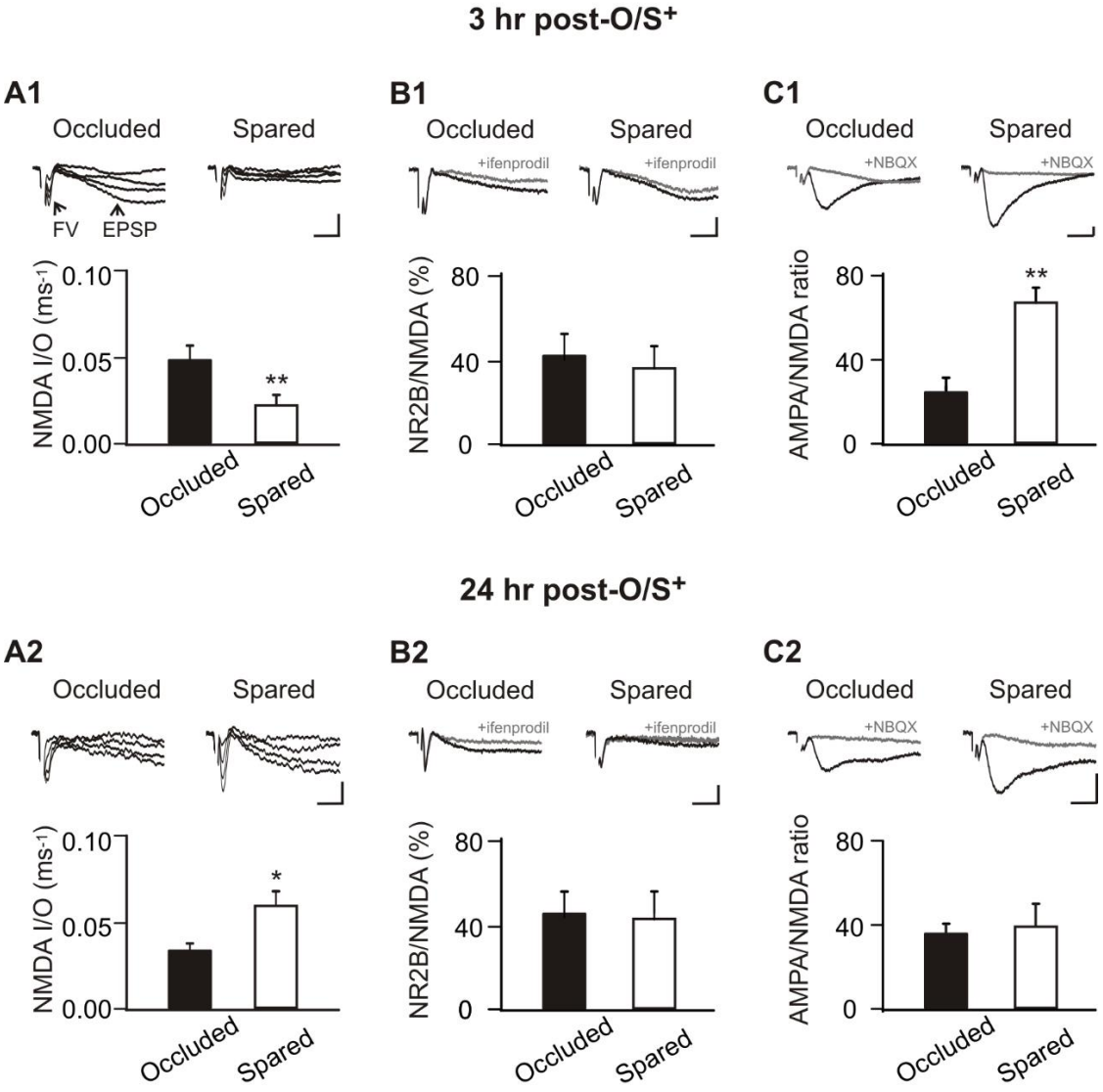


Figure 3.1. NMDA GLUN1 plasticity in the anterior piriform cortex (aPC) at 3 hr and 24 hr following early odor preference learning

Western blots of synaptoneurosome extracts of aPCs from occluded and spared hemispheres of the same animals. Upper panel, example Western blot bands of GLUN1 in different groups. O/S^+, odor plus stroking; O/S^-, odor only without stroking; Occ, occluded; Sp, spared. Lower panel, relative optical density (ROD) of GLUN1 expressions in various groups. The values are normalized GLUN1 expressions in spared aPCs to the occluded counterparts. *$p < 0.05$. **$p < 0.01$. Error bars, mean ± SEM.


3 hr post-O/S+
A1
Occluded
Spared
FV
EPSP
NMDA I/O (ms-1)
0.10
0.05
0.00
**
Occluded
Spared
B1
Occluded
Spared
+ifenprodil
+ifenprodil
NR2B/NMDA (%)
80
40
0
Occluded
Spared
C1
Occluded
Spared
+NBQX
+NBQX
AMPA/NMDA ratio
80
40
0
**
Occluded
Spared
24 hr post-O/S+
A2
Occluded
Spared
NMDA I/O (ms-1)
0.10
0.05
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*
Occluded
Spared
B2
Occluded
Spared
+ifenprodil
+ifenprodil
NR2B/NMDA (%)
80
40
0
Occluded
Spared
C2
Occluded
Spared
+NBQX
+NBQX
AMPA/NMDA ratio
80
40
0
Occluded
Spared

Figure 3.2. NMDAR down-regulation at 3 hr and up-regulation at 24 hr occurs at the LOT synapse

A1-A2. NMDAR input/output (I/O) relationship recorded at 3 hr (A1) and 24 hr (A2) *ex vivo* following early odor preference learning. NMDAR fEPSP was isolated by NBQX (5 μM) in low Mg^{2+} aCSF. FV, fiber volley. **B1-B2.** Fractions of GLUN2B to total NMDAR fEPSPs recorded at 3 hr (B1) and 24 hr (B2) post-training. GLUN2B component was isolated by ifenprodil (3 μM), an GLUN2B antagonist. **C1-C2.** AMPAR/NMDAR ratio recorded at 3 hr (C1) and 24 hr (C2) post-training. Scale bars, 0.2 mV and 5 msec. *$p < 0.05$. **$p < 0.01$. Error bars, mean ± SEM.

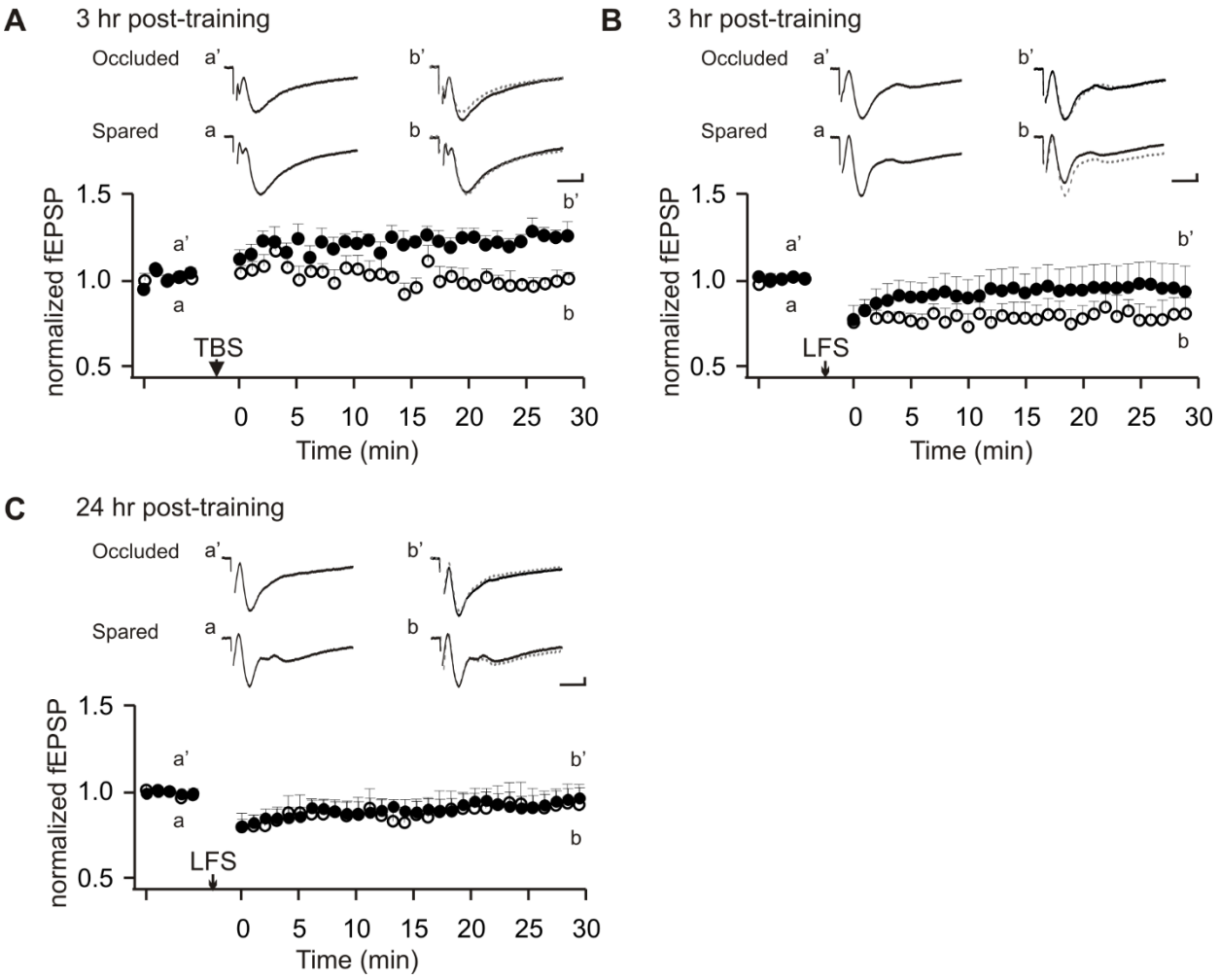

Figure 3.3. NMDAR down-regulation at 3 hr coincides with decreased LTP and inducibility of LTD at the LOT synapse

A. *Ex vivo* LTP induction 3 hr post-training in occluded and spared aPCs from the same animals. TBS, theta burst stimulation. **B.** *Ex vivo* LTD induction 3 hr post-training. LFS, low frequency stimulation. **C.** *Ex vivo* LTD induction 24 hr post-training. LFS, low frequency stimulation. Solid circles representing data from occluded aPC. Open circles representing data from spared aPC. Scale bars, 0.2 mV and 5 msec. Error bars, mean ± SEM.

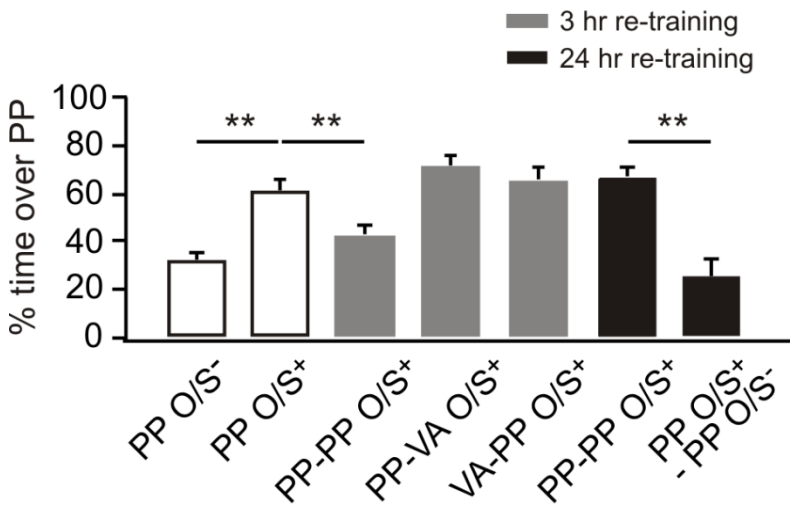

Figure 3.4. O/S+ re-training at 3 hr impairs learning at the same synapse
Percentage of time spent over peppermint-scented bedding in a two choice test. O/S^{+}, odor plus stroking. O/S^{-}, odor only. PP, peppermint. VA, vanillin. PP-PP, both first and re-training with peppermint-scented bedding. PP-VA, first training with peppermint-scented bedding, re-training with vanillin scented-bedding. VA-PP, first training with vanillin scented-bedding, re-training with peppermint scented-bedding. **$p < 0.01$. Error bars, mean ± SEM.

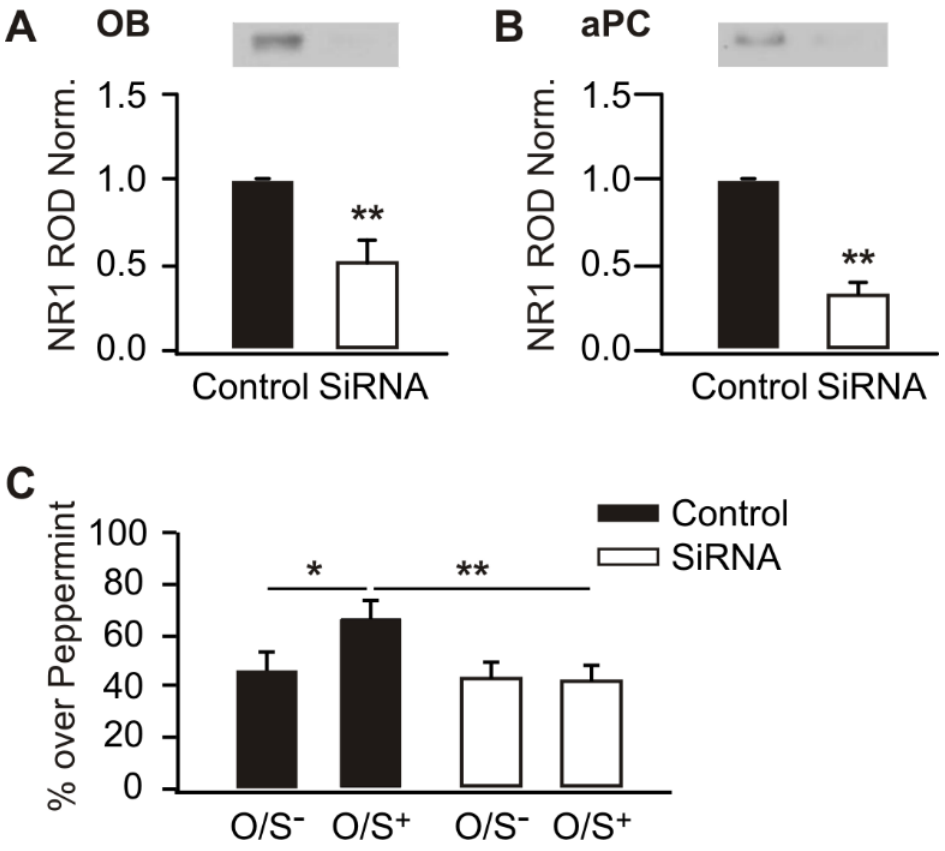

Figure 3.5. GLUN1 down-regulation by SiRNA impairs early odor preference learning **A.** Relative optical density (ROD) of GLUN1 in SiRNA infused olfactory bulb (OB), normalized to vehicle infused counterpart. **B.** ROD of GLUN1 in SiRNA infused anterior piriform cortex (aPC), normalized to vehicle infused counterpart. **C.** Percentage of time spent over peppermint-scented bedding in a two choice test. *$p < 0.05$, **$p < 0.01$. Error bars, mean ± SEM.

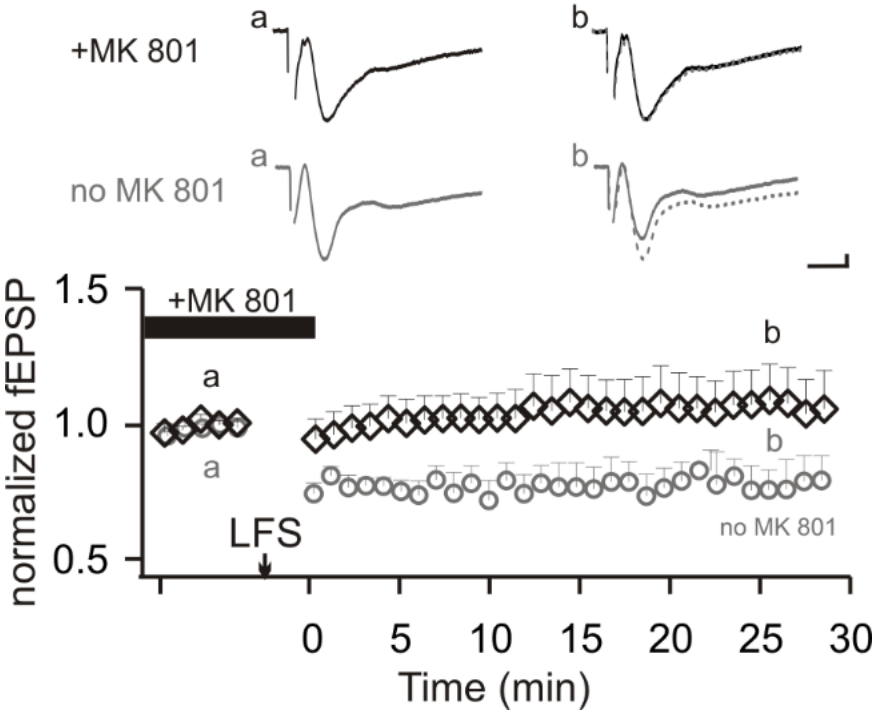

Figure 3.6. Ex vivo NMDAR blockage 3 hr following odor training blocks LTD at the LOT synapse

Ex vivo LTD induction 3 hr post O/S^+ training in the presence of NMDAR antagonist MK 801. LFS, low frequency stimulation. Squares representing data from MK 801 experiments. Gray circles are taken from Figure 3B, recording in spared slices following O/S^+ training without MK 801. Scale bars, 0.2 mV and 5 msec. Error bars, mean ± SEM.

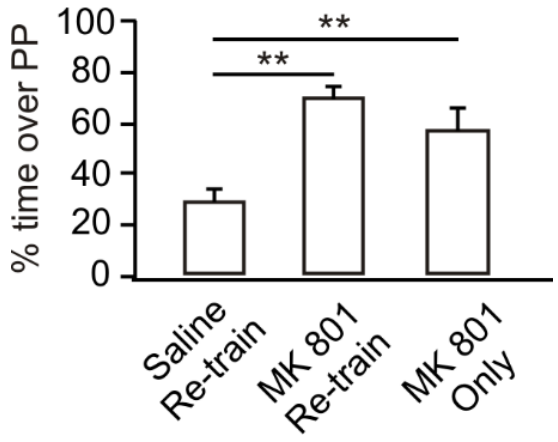

Figure 3.7. NMDAR blockage during re-training allows odor preference memory to be expressed

Percentage of time spent over peppermint-scented bedding in a two choice test. Saline or MK 801 was injected to pups before 3 hr re-training. MK 801 only group refers to MK 801 injection at 3 hr without re-training. **$p < 0.01$. Error bars, mean ± SEM.

Chapter 4

Title: Learning-induced metaplasticity? Associative training for early odor preference learning down regulates synapse-specific NMDA receptors via mGluR and calcineurin activation (This Chapter is modified version of Mukherjee el al Cereb Cortex. 2017 Jan 1;27(1):616-624. doi: 10.1093/cercor/bhv256.)

4.1 Abstract

Rat pups readily form a 24 h associative odor preference after a single trial of odor paired with intermittent stroking. Recent evidence shows that this training trial, which normally increases AMPA receptor responses in the anterior piriform cortex both 3 h and 24 h following training, induces a down-regulation of NMDA receptors 3 h later followed by NMDA receptor up-regulation at 24 h.

When retrained with the same odor at 3 h, rat pups unlearn the original odor preference. Unlearning can be prevented by blocking NMDA receptors during retraining. Here the mechanisms that initiate NMDA receptor down-regulation are assessed. Blocking mGluR receptors or calcineurin during training prevents down-regulation of NMDA receptors 3 h following training. Blocking NMDA receptors during training does not affect NMDA receptor down-regulation. Thus down-regulation can be engaged

separately from associative learning. When unlearning occurs, AMPA and NMDA receptor levels at 24 h are reset to control levels. Calcineurin blockade during retraining prevents unlearning consistent with the role of NMDA receptor down-regulation. The relationship of these events to the metaplasticity and plasticity mechanisms of long-term depression and depotentiation is discussed. We suggest a possible functional role of NMDA receptor down-regulation in offline stabilization of learned odor representations.

4.2 Introduction

In rat pup odor preference learning, a single 10 min exposure to a novel odor paired with intermittent stroking produces a 24 h protein-synthesis dependent odor preference[379], which is associated with enhanced AMPA receptor (AMPAR) currents in olfactory inputs to both the olfactory bulb (OB)[475] and the anterior piriform cortex (aPC)[373,451]. AMPAR phosphorylation and increased AMPAR membrane insertion occur concomitantly with olfactory learning and memory in this model[453], consistent with a prediction of enhanced network representations with training. The neural changes and behavioural memory endure over multiple days with repeated spaced trials[451], while the odor representational network becomes more stable following spaced training in both the OB and aPC[338].

In both OB and aPC, the NMDA GluN1 receptor levels are decreased 3 h following a single training trial [476,505] but then increase beyond control levels at 24 h[505]. This contrasts with increases in AMPAR responses (likely due to increased membrane

expression) at both time points[373]. In the aPC in vitro, long-term depression (LTD), normally difficult to induce in rat pup slices, is readily induced from slices taken at 3 h post odor preference training when NMDA receptors (NMDARs) are down regulated[505]. Behaviorally, 'unlearning 'occurs when a second odor preference training is given 3 h following the initial training[505]. Unlearning is specific to the trained odor since pairing a different odor with stroking at 3 h does not affect the learning of the new odor assessed 24 h later, and does not disrupt memory for the originally trained preference[505]. The critical role of the reduced NMDAR expression in unlearning is demonstrated by blocking NMDARs 3 h prior to the 2nd training event, while this prevents the original associative odor learning if given before the first training trial[373], NMDAR blockade before the 2nd trial at 3 h permits the original 24 h odor preference to be maintained rather than 'unlearned'[505].

The present experiments test the potential mechanisms for NMDAR down regulation recruited during the original training and implicated by earlier studies in metaplasticity, a process by which prior events temporally alter subsequent plasticity susceptibility[208,478]. The outcomes reveal that activation of metabotropic glutamate receptors and calcineurin are required for NMDAR down regulation. A model is presented supporting a functional role for NMDAR down regulation in establishing stable memory networks.

4.3 Materials and Methods

In the following sections I will describe my methodologies for Chapter 3.

4.3.1 Animals and Ethics Statement

Sprague Dawley rat pups of either sex (Charles River) were used in this study. Animals were bred and pups were born on-site at the animal care facility. Litters were culled to 12 pups on postnatal day 1 (PND1; day of birth is designated PND0). Dams were maintained with ad libitum access to food and water. All procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland adherent to the guidelines by the Canadian Council on Animal Care.

4.3.2 Behavioral Studies

Behavioral experiments were carried out in a temperature controlled room at approximately 27°C and followed the previously established protocols [451,505] as described below. One-way ANOVAs and post hoc Fisher tests, or two-sample t-test were used to determine statistical significance throughout the experiments.

4.3.3 Odor preference training

PND6 rat pups were assigned to an odor plus stroking (O/S^+) or an odor only (O/S^-) condition. Pups were removed from the nest and placed on normal bedding for 10 min habituation. Pups receiving conditioning O/S^+ were placed on peppermint-scented bedding (0.3 mL peppermint extract in 500 mL bedding) and vigorously stroked with a paintbrush for 30 sec, followed by a 30 sec rest, repeated for 10 min. Pups in the control condition O/S^- were placed in peppermint-scented bedding for 10 min without being stroked. All pups were returned to the dam after training. A subset of O/S^+ pups were re-trained at 3 hr after the first training, following the same procedure as in the first training.

Three types of experiments were employed. First, O/S^+ pups were trained with single naris occluded, followed by brain tissue collection at 3 h for NMDAR GluN1 measurement (Figure 4.1). Another group was re-trained at 3 h and killed at 24 h for both GluN1 and GluA1 measurement (Figure 4.7). Second, pups underwent aPC drug or vehicle infusions right before a single O/S^+ training. These pups were either killed at 3 h for GluN1 measurement (Figures 4.2, 4.3, 4.4) or tested for odor preference the next day (Figure 4.5). Third, pups underwent aPC drug or vehicle infusions before the re-training at 3 h following the first training. These pups were then tested for odor preference the next day (Figure 4.6).

4.3.4 Odor preference testing

Twenty-four hours following the initial training, pups were tested for odor preference memory using a two-choice odor preference procedure. The testing apparatus

was a stainless steel box (30 x 20 x 18 cm) placed over two training boxes. One box contained peppermint-scented bedding and the other contained normal, unscented bedding, separated by a 2 cm neutral zone. During testing, pups were removed from the dam and placed in the neutral zone. Times that pups spent over scented versus normal bedding were recorded in five one-minute trials, each separated by a one-min rest in a clean cage. The percentage of the time spent over peppermint bedding over total time spent over either bedding was calculated for each pup.

4.3.5 Reversible naris occlusion

Nose plugs were constructed using polyethylene 20 (PE 20) tubing and silk surgical thread as described previously[451,505]. A small dab of 2% Xylocaine gel (AstraZeneca) was applied to the left naris of the pup and the pup was let rest for ~3-5 min before the plug was gently inserted in the left naris. After 10 min habituation, pups were assigned appropriate odor training. The nose plug was removed immediately following training and pups returned to dams.

4.3.6 Cannula implantation and intracerebral infusion

Cannula implantation was carried out on PND5. Pups were anesthetized via hypothermia and placed in a stereotaxic apparatus in a skull flat position. A horizontal incision was made to expose the skull where two small holes were drilled. Two guide cannulas (Vita Needle, MA) with insect pins were inserted into the brain in specific

coordinates for aPC (from Bregma: Anterior posterior: +2.5, Mediolateral: +3.5 and Depth: -5.5;[373]), and cemented with dental acrylic to the skull. The skin was then sutured around the cannulas. The pups were recovered on warm bedding before returned to the dams.

All drugs were infused into the aPC on PND6 either 20 min before the first training or before re-training. One µl of a drug was injected bilaterally into the aPCs for behavioral experiments using a Hamilton syringe. In pups for quantitative immunoblotting, drugs were infused in one aPC and vehicles were infused in the contralateral hemisphere. The injection was over 4 min, and the syringe was left in site for another min before being gently withdrawn from the brain. The pups were returned to the dams for ~5 min before habituation and training. Pharmacological agents used include a NMDAR antagonist D-AP5 (5 mM and 500 µM, dissolved in saline; Sigma Aldrich), an NMDAR agonist NMDA (5 mM, dissolved in saline; Tocris), an mGluR1 antagonist AIDA (5 mM and 500 µM; dissolved in a small amount of 1 M NaOH and further diluted with saline, the same ratio of NaOH and saline was used as vehicle; Tocris), an mGluR5 antagonist MPEP (5 mM; 10% DMSO in saline; Tocris), an mGluR group I/II blocker MCPG (100 mM, dissolved in saline; Tocris), a calcineurin (phosphatase 2B) inhibitor FK-506 (5 mM, 10% DMSO in saline; Tocris) and a phosphatase 1/2A inhibitor okadaic acid (500 µM, 10% DMSO in saline; Calbiochem). All drug concentrations used are comparable with published results using in vivo brain infusions[381,476,506,507]. The cannula locations were verified to be within the aPC during brain extractions. The spread of infusion was tested with 4% methylene blue dye in pilot experiments (<2 mm^3; n=6). We

have also validated in previous studies [373,505] that the drug spread using the same infusion parameters and techniques was confined to the aPC.

4.3.7 Immunoblotting

Three hours following odor training, pups were decapitated, and aPCs were collected and flash frozen on dry ice. For pCREB measurement, brains were taken 10 min following O/S^+ training. Samples were stored at -80°C until further processing.

4.3.8 Synaptic membrane isolation

Purification of synaptic membrane followed previously published procedures[508]. Tissue samples were homogenized using a Teflon glass tissue homogenizer (Thomas Scientific) in ice-cold sucrose buffer (300 µl) containing (in mM): 320 sucrose, 10 Tris (pH7.4), 1 EDTA, 1 EGTA, 1X complete protease inhibitor mixture and phosphatase inhibitor mixture (Roche). The homogenized samples were centrifuged at 1000 rpm for 10 min. The supernatant was spun at 10,000 rpm for 25 min to obtain a pallet, which was subsequently re-suspended in 120 µl sucrose buffer using a pestle mixing/grinding rod (Thomas Scientific) directly in the microfuge tube. Then 8 volumes of a non-ionic detergent Triton X-100 buffer (final 0.5% v/v) were added for detergent extraction. The Triton X-100 buffer contained (in mM) 10 Tris (pH 7.4), 1 EDTA, 1 EGTA, 1X protease and phosphatase inhibitors. This suspension was incubated at 4 °C for 35 min with gentle rotation. Then the suspension was centrifuged at 28,000 rpm for 30 min. The pellet

(postsynaptic densities and synaptic junctions that are insoluble in Triton X-100,[479]) was re-suspended in 100 µl of TE buffer containing 100 mM Tris (pH 7.4), 10 mM EDTA, 1% SDS, 1X protease and phosphatase inhibitors, sonicated, boiled for 5 min and stored at -80ºC until use. Protein concentrations for each sample were determined by using a BCA protein assay kit (Pierce). The volume of lysate required to make 35 µg of protein for each sample was calculated.

4.3.9 Tissue isolation for phosphorylated CREB (pCREB) measurement

APC tissue was placed in microcentrifuge tubes and homogenized with a manual motor pastel in 100 µL of lysis buffer containing 0.1% SDS, 1% NP-40, 20 mM PMSF, 10% glycine, and 1.37 mM sodium chloride with 1 µL/mL leupeptin, 2 mM PMSF, 8.9 U/mL aprotinin, and 1 mM sodium orthovanadate. The homogenate was centrifuged at 13,500 rpm for 15 min at 4°C. After determining the protein concentration, the clear lysate supernatant was stored at -80°C.

4.3.10 Western Blotting

A total of 100 µl lysate solution, sample buffer (0.3 M Tris-HCl, 10% SDS, 50% glycerol, 0.25% bromophenol blue, 0.5 M dithiothreitol), and dH2O were prepared and boiled for 2 min at 100°C. Samples were then loaded into lanes of a 7.5% SDS-PAGE gel, along with a protein ladder (Thermo Scientific) and irrelevant samples in empty lanes. Sample separation occurred through SDS-PAGE, followed by transference to a nitrocellulose membrane (Millipore). Membranes were cut horizontally at the 72 kDa

level, and the upper portion was probed with a rabbit antibody for GluN1 (1:2000, blocked in 5% Milk; Cell Signaling Technology) subunits, and the lower portion was probed for β-actin (1:5000, blocked in 5% skim milk; Cedarlane). A pCREB antibody (1:5000, Cell Signalling) and a control GAPDH antibody (1:7000, Cell Signalling) were used to measure pCREB levels. A GluA1 antibody (1:10000, Cell Signalling) was used to probe AMPAR membrane levels. Membranes were incubated in primary antibody overnight at 4°C in continuous shaking condition. Next day membranes were washed three times for 5 min each with 1X TBST. Secondary antibodies bound to HRP were applied after the wash (1:10,000, anti-rabbit; Pierce) for 1 h, and membranes were then washed again with 1X TBST three times for 10 min each. Then blots are washed in enhanced chemiluminescence Western blotting substrate (Pierce). Finally, blots were developed on x-ray film (AGFA). Films were scanned onto a computer using an image scanner (CanoScan LiDE 200), and the optic density (OD) of each band was measured using ImageJ software.

Each sample was normalized to the corresponding β -actin or GAPDH band that was run on the same gel. In pups that underwent lateralized odor training, each spared hemisphere was compared with its naris-occluded counterpart. In pups with drug infusions, the drug infused hemisphere was compared with its vehicle infused counterpart. Experimental values are reported as mean ± SEM of normalized optical densities. A paired t-test was used to evaluate differences in the mean optical densities between the two groups.

4.4 Results

In the following sections I will describe my results of Chapter 3 project.

4.4.1 Synaptic GluN1 down-regulation following early odor preference learning is mGluR-dependent

Previously, using a synaptoneurosome preparation, we have shown that GluN1 subunits are down regulated 3 h following O/S$^+$ training in rat pups[505]. Synaptoneurosomes are composite structures enriched in synaptic proteins [509] and have been used to measure activity-dependent changes in AMPARs and NMDARs in the olfactory system[213,453,476,505]. Here we employed a protocol to further separate synaptic from extrasynaptic membrane compartment [508] using a subcellular fraction approach followed by extraction with Triton X-100 as postsynaptic densities and synaptic junctions are shown to be insoluble in Triton X-100[510]. We validated this method by showing PSD-95 was particularly abundant in the synaptic fraction compared to either the extra-synaptic or cytosolic fractions (supplementary Figure 4.1). Our analysis revealed that 3 h following O/S$^+$ training, the synaptic GluN1 subunit was significantly lower in the spared aPC (normalized OD: 0.69 ± 0.12) than in the occluded counterpart (1.14 ± 0.10, n = 5, t = 3.13, p = 0.04; Figure 4.1). This confirms that GluN1 down-regulation occurs at synaptic membrane.

We next investigated whether synaptic GluN1 down-regulation is dependent on NMDARs. D-APV was infused in one aPC before the O/S^+ training, while vehicle was infused into the other aPC. Two concentrations (500 µM and 5 mM) were tested. Neither 500 µM (vehicle: 0.87 ± 0.14 vs. D-APV: 1.0 ± 0.13, n = 6, t = 0.97, p = 0.37) nor 5 mM (vehicle: 0.92 ± 0.18 vs. D-APV: 0.81 ± 0.14, n = 6, t = 1.37, p = 0.23; Figure 4.2A&B) dose altered GluN1 expression, suggesting GluN1 down-regulation is not NMDAR dependent. To validate the drug effect in aPC, we measured pCREB expressions following D-APV infusion and O/S^+ training. The level of pCREB was lower in the D-APV infused aPC (1.08 ± 0.27) than the control vehicle side (2.16 ± 0.53, n = 8, t = 2.61, p = 0.03, Figure 4.2C), consistent with NMDAR-dependent CREB phosphorylation and odor preference learning[373,476]. This result confirms the effectiveness of the drug infusion protocol.

We then tested the potential involvement of mGluRs. When MCPG, an mGluR group I/II antagonist was infused, the GluN1 levels were significantly higher in the MCPG infused aPC (1.12 ± 0.17) than the control side (0.63 ± 0.18, n = 8, t = 2.18, p = 0.03; Figure 4.3A&B). This results suggest MCPG prevents GluN1 down-regulation following O/S^+ training. To test the specific subtype of mGluRs involved, an mGluR5 specific antagonist MPEP (5 mM) was infused into the aPC. MPEP prevented down-regulation of GluN1 (MPEP: 1.59 ± 0.19 vs. vehicle: 1.00 ± 1.82, n = 8, t = 3.86, p = 0.006; Figure 4.3C). However, an mGluR1 specific antagonist AIDA (500 µM and 5 mM) was ineffective. Neither the 500 µM (vehicle: 0.72 ± 0.16 vs. AIDA: 0.76 ± 0.20, n = 6, t = 0.48, p = 0.65) nor the 5 mM (vehicle: 0.93 ± 0.17 vs. AIDA: 0.82 ± 0.20, n = 6, t

= 0.80, p = 0.46; Figure 4.3D) dose altered GluN1 expression, suggesting GluN1 down-regulation is mediated by mGluR5, but not mGluR1.

4.4.2 Calcineurin signalling is involved in GluN1 plasticity

Activation of mGluRs during early odor preference learning may trigger intracellular Ca^{2+} release, which would activate phosphatase pathways and lead to NMDAR dephosphorylation and internalization. We infused two phosphatase inhibitors - a calcineurin inhibitor, FK-506 (5 mM), and a phosphatase 1/2A inhibitor, okadaic acid (500 µM). FK-506 infusion prevented GluN1 down-regulation (vehicle: 0.62 ± 0.16 vs. FK-506: 1.39 ± 0.39, n = 8, t = 2.58, p = 0.04), while okadaic acid had no effect (vehicle: 0.93 ± 0.26 vs. FK-506: 0.88 ± 0.56, n = 6, t = 0.16, p = 0.88; Figure 4.4).

As described GluN1 down-regulation following O/S^+ training induces unlearning upon re-training at 3 h[505]. If GluN1 down-regulation is blocked, what would happen in the animals that are re-trained at 3 hr following the initial O/S^+ training? We infused either FK-506 or MCPG bilaterally into aPCs before the first O/S^+ training and tested the effects on odor preference memory 24 h later, with or without 3 h re-training. When infused without re-training, neither drug affected odor preference learning compared to the O/S^- control. A one-way ANOVA revealed significant differences among groups ($F_{(3, 15)}$ = 11.02, p < 0.001; Figure 4.5A). FK-506 infused group spent significantly more time on peppermint scented bedding (60.80 ± 5.45%, n = 4) than the vehicle infused O/S^- control group (37.47 ± 2.88%, n = 6, t = 4.59, p < 0.001), and not different from vehicle infused

O/S^+ learning group (55.58 ± 2.70%, n = 5, t = 0.99, p = 0.34), suggesting calcineurin inhibition does not interfere with early odor preference learning. Similarly, MCPG infused pups also learned (62.12 ± 3.87, n = 4, t = 4.85, $p < 0.001$ compared to the vehicle O/S^- group). However, when re-training occurred at 3 h after the first training, either drug abolished unlearning. A one-way ANOVA revealed significant differences among groups ($F_{(2, 10)} = 54.57$, $p < 0.001$; Figure 4.5B). FK-506 infusion prevented unlearning (vehicle O/S^+-O/S^+: 33.06 ± 3.46%, n = 5, vs. FK-506 O/S^+-O/S^+: 78.49 ± 2.77%, n = 4, t = 10.13, $p < 0.001$). MCPG infusion also prevented unlearning in the re-trained group (MCPG O/S^+-O/S^+: 63.61 ± 3.11%, n = 4, t = 6.81, $p < 0.001$ compared to the vehicle O/S^+-O/S^+ group).

4.4.3 Calcineurin signalling mediates unlearning

We have previously shown that unlearning with 3 h re-training is NMDAR-dependent[505]. To test whether a phosphatase mediated depotentiation or LTD pathway mediates unlearning, we infused FK-506 into aPCs at 3 h with or without re-training. Without re-training, FK-506 infusion did not affect odor preference learning. One-way ANOVA showed significant group effects ($F_{(2, 11)} = 4.52$, $p < 0.05$; Figure 4.6A). FK-506 infusion at 3 h without re-training (65.01 ± 7.39, n = 5) induced odor preference learning compared to the vehicle O/S^- group (40.2 ± 7.64, n = 4, t = 2.70, p = 0.02). However, FK-506 infusion prevented unlearning when animals were re-trained at 3 h (vehicle O/S^+-O/S^+: 43.58 ± 6.38%, n = 6, vs. FK-506 O/S^+-O/S^+: 74.18 ± 3.71%, n = 4, t = 4.14, $p < 0.01$; Figure 4.6B).

4.4.4 Unlearning resets AMPARs and NMDARs to the baseline non-learning levels

AMPARs and NMDARs are both up-regulated at 24 hr following a single O/S^+ training[373,505]. Here we measured how AMPARs and NMDARs changed at 24 h following the initial O/S^+ training, with a 3 h re-training episode. Re-trained pups showed no difference of AMPAR GluA1 subunit expressions in the occluded (1.33 ± 0.18) and spared aPC (1.33 ± 0.17, n = 8, t = 0.32, p = 0.76; Figure 4.7A&B). Similarly, NMDAR GluN1 subunits in the spared aPC (1.08 ± 0.13) were not different from the occluded hemisphere (1.00 ± 0.21, n = 8, t = 0.49, p = 0.64; Figure 4.7A&C). These results suggest re-training at 3 hr resets the levels of AMPAR and NMDAR to the baseline condition through metaplasticity.

4.5 Discussion

In the following sections I will discuss Chapter 4 in more detail.

4.5.1 NMDAR plasticity following early odor preference learning

In the last decade, studies have begun to examine the activity-dependent regulation of NMDAR trafficking in in vitro preparations[511,512]. However, the molecular basis of activity-dependent NMDAR plasticity in intact, physiological conditions remains to be determined[478]. Our studies have provided among the first evidence that NMDAR

activity is regulated by learning - early odor preference learning in rat pups induces an early transient and reversible down-regulation of NMDAR and a delayed up-regulation ([505]and present results). Both rapid down-regulation and delayed up-regulation [478] of NMDA receptors have been reported in in vitro models.

AMPAR function is up-regulated in aPC at both the 3 h and 24 h time points[373], thus there is a dissociation of GluN1 and GluA1 changes at the 3 h time point. While not typically reported, LTP of AMPAR- and LTD of NMDAR-mediated opposite changes has previously been described in nucleus accumbens neurons[486].

4.5.2Molecular Basis of Early GluN1 Down Regulation with Single Trial Training

GluN1 down-regulation was not affected by NMDAR blockage through D-APV infusion. MCPG, a general group I and II mGluR antagonist, blocked learning induced GluN1 down-regulation. Further testing with mGluR5 antagonist, MPEP, also blocked GluN1 down-regulation. However, an mGluR1 specific inhibitor, AIDA, was not effective. This suggests that mGluR5 mediates the down-regulation of GluN1s. These receptors are strongly expressed in neocortical pyramidal neurons in rat pups of this age[513].

GluN1 down-regulation was also prevented by calcineurin inhibitor infusion, but not by inhibition of phosphatase 1/2A. Activation of mGluRs during learning may activate calcineurin, which could dephosphorylate and enhance the activity of the tyrosine

phosphatase STEP, which in turn dephosphorylates NMDAR GluN2B receptors. Dephosphorylation of GluN2B subunit promotes NMDAR internalization via the clathrin-mediated pathway as shown in other systems[514]. Our previous result demonstrating GluN1 down-regulation in a synaptoneurosome preparation (including both synaptic and extra-synaptic membranes) [505]also favors an enhanced NMDAR internalization model, compared to increased lateral diffusion of the receptors. (See [492] for a discussion of the generality and power of NMDAR endocytosis as a plasticity mechanism.)

Since early odor preference learning and AMPAR LTP at the synapses are critically dependent on NMDARs[373], it was unexpected that an NMDAR antagonist that prevented pCREB expression after training was unable to prevent GluN1 down-regulation at 3 h. That is to say successful associative plasticity itself was not required to induce GluN1 down-regulation. This demonstrates that the mGluR5 pathway and the NMDAR pathways are engaged in parallel by associative training and can function independently when recruited by such training. The blockade of NMDARs prevents odor preference memory, but does not prevent the 'silent' down-regulation of GluN1 receptors induced by training.

In contrast, blockade of calcineurin, which prevents GluN1 down-regulation driven by mGluR activation in this paradigm, typically enhances learning and memory[381]. In the odor preference model, the calcineurin inhibitor FK506 in the OB before training extends the duration of single trial memory and renders suboptimal unconditioned

stimulus optimal. In the present study, rat pups that had received FK506 before the initial training showed a strong odor preference memory following retraining at 3 h, rather than unlearning. It remains to be assessed whether prevention of GluN1 down-regulation contributes to learning enhancement.

Calcineurin also appears to have a role in the NMDAR-mediated unlearning event itself. Blockade of calcineurin immediately before retraining at 3 h, despite normal GluN1 down-regulation at this time, renders the second training trial effective. This is likely due to calcineurin's role as a negative regulator of NMDA plasticity, preventing the depotentiating or depressing NMDAR effect normally recruited by fewer receptors during the retraining.

4.5.3 Metaplasticity: Long-term depression or depotentiation?

Unlearning mediated by changes at the aPC synapse (decreased AMPAR LTP and inducible LTD) following early odor preference learning not only results from changes in NMDAR number, but also depends on the NMDAR itself for its expression[505]. Blocking NMDARs during the retraining abolishes unlearning and permits the expression of odor preference memory[505].

NMDARs have a role in both depotentiation (returning potentiated synapses to baseline) and long-term depression (reduced NMDAR currents promote LTD). Either of these mechanisms may underlie the unlearning effect observed here. Both are synapse-

specific. Consistent with recruitment of LTD by retraining is the ability of a low frequency protocol to recruit LTD in trained slices, but not untrained slices, when examined 3 h, but not 24 h, post training[505]. On the other hand, resetting both NMDAR and AMPAR to control levels at 24 h by the 'unlearning' event, as seen here, is consistent with the reset function of depotentiation. Depotentiation is less likely to occur for strong LTP protocols [515] and stronger odor preference training paradigms appear not to be associated with GluN1 down regulation [216]. Single trial odor preference learning is by definition a weak protocol since it only produces 24 h memory, but it is a long-term protein transcription-dependent memory[379], so these experiments are exploring the manipulation of mechanisms underlying the durations of long-term memories themselves.

Depotentiation is reported to depend on the GluN2A subunit whereas long-term depression is associated with the GluN2B subunit[516]. The known role of calcineurin in promoting endocytosis of the NMDAR through the GluN2B subunit [517] suggests an LTD mechanism may be more likely here (but see[518]).

These distinctions are of interest since metaplasticity is thought to recruit long-term depression, but not depotentiation[208]. On the other hand since both metaplasticity and plasticity mechanisms are likely to be recruited with natural learning [208] it may not be possible to disentangle their contributions in odor preference learning. Most importantly, the synaptic changes induced by associative training lead to a temporally evolving and complex modulation of plasticity predispositions in the engaged circuitry. Selective

manipulations of GluN2B and GluN2A subunits during the second training event might clarify the nature of the 'unlearning' mechanism.

4.5.4 Functional role of synapse-specific GluN1 receptor regulation with associative training

The up-regulation of GluN1 receptors at 24 h can readily be envisaged to provide a mechanistic basis for the memory strengthening effects of spaced learning (see [519] for review of spaced learning effects). In the odor preference learning model longer term memories and longer maintenance of AMPAR enhancement are associated with odor preference training spaced at 24 h[451]. Imprinting in chicks also induces delayed GluN1 up-regulation in structures specifically associated with memory[519]. Interestingly, the strength of the imprinting memory is positively correlated with the degree of delayed NMDAR up-regulation[520].

We suggest early down-regulation of the GluN1 receptor may be related to the removal of weak synapses in the learned odor representation during off line reactivation. Such a function has been argued in several models[521,522]. Spontaneous network activity in conjunction with NMDAR-mediated LTD is thought to eliminate weaker synaptic connections[522]. In one study[523], mGluR5s, but not NMDARs, were shown to be critically involved in the rewiring of neocortical microcircuitry through the selective elimination of weaker connections.

Recently Arc^+ cells have also been shown to express LTD mediated by mGluR group I (1/5) activation[524]. In the rat pup odor learning model, we have shown that odors recruit Arc activation and that the odor representation by Arc^+ cells following multiple spaced training is altered to have a larger proportion of stable or strong synaptic connections[338]. Thus it is possible that one training effect of mGluR5 activation is to help shape important odor representations by reducing unstable connections during subsequent off line activation as described for NMDA circuits in hippocampus[521,522]. GluN1 down regulation would mediate unstable synapse elimination in odor representations following training.

4.6 Conclusions

In this study we characterize the molecular mechanisms of associative learning-induced NMDAR plasticity. GluN1 down-regulation was initiated by mGluR-mediated calcineurin signalling and inferred dephosphorylation and internalization of NMDARs. Blocking synapse-specific GluN1 down-regulation signalling prevents unlearning induced by a re-training episode. Unlearning during GluN1 down-regulation was shown to be mediated by an NMDAR-dependent calcineurin pathway inducing both AMPAR and NMDAR internalization. Blocking GluN1 down-regulation signalling prevents unlearning induced by the re-training episode (see Figure 8 for an illustration of the molecular mechanisms involved).

We suggest learning-induced GluN1 down-regulation contributes to the increased stability of learned odor representations, while delayed GluN1 up-regulation supports the benefits of spaced training on memory duration.

4.7 Figures of Chapter 4

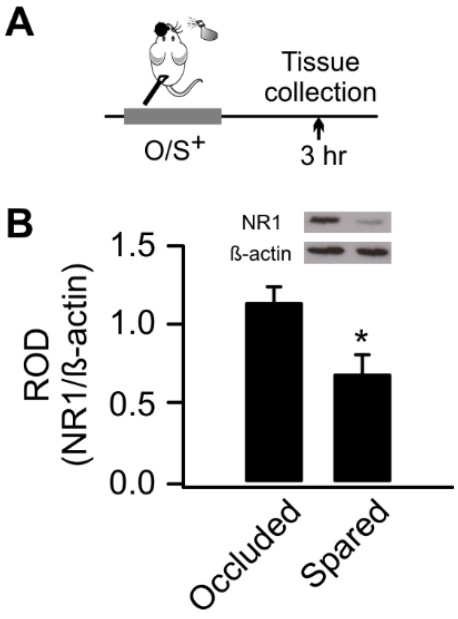

Figure 4.1. Early odor preference learning in rat pups down-regulates synaptic GluN1 receptors in the anterior piriform cortex (aPC).

A. Schematics of the odor training and tissue collection paradigm. O/S^{+}: odor paired with stroking.

B. Relative optical density (ROD) of GluN1 expression (normalized to β-actin) in occluded and spared aPCs.

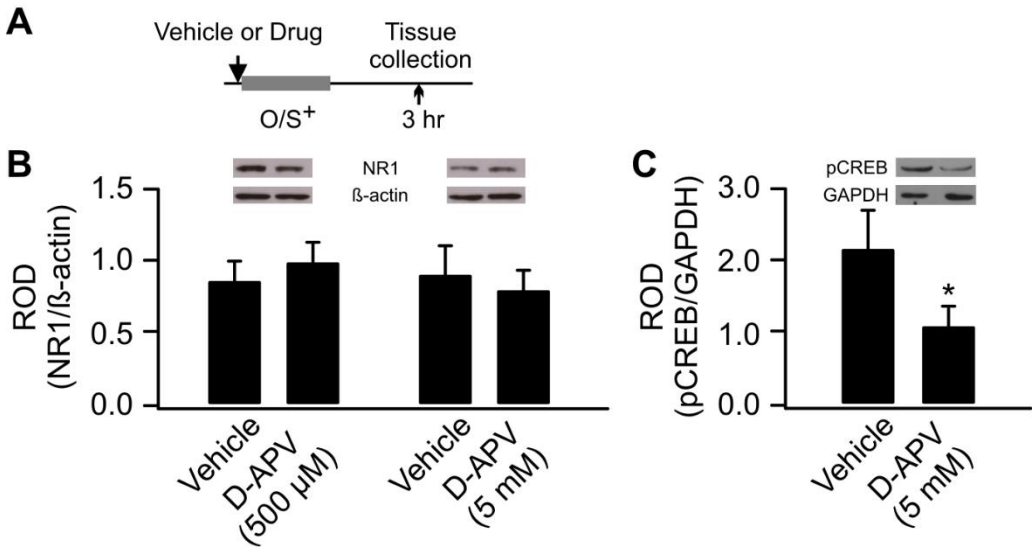

Figure 4.2. GluN1 down-regulation is not dependent on NMDAR activation during early odor preference learning.

A. Schematics of the odor training and tissue collection paradigm. O/S^{+}: odor paired with stroking.

B. Relative optical density (ROD) of GluN1 expression (normalized to β-actin) in vehicle and D-APV infused aPCs.

C. ROD of pCREB expression (normalized to GAPDH) in vehicle and D-APV infused aPCs.

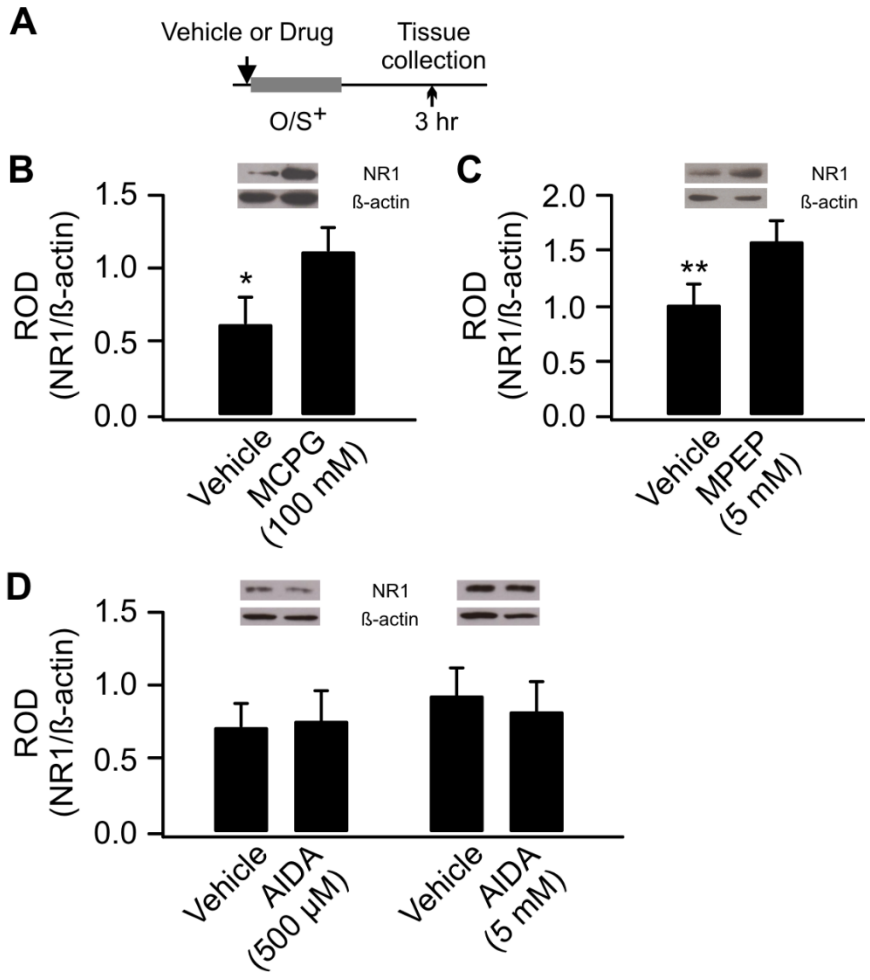

Figure 4.3. GluN1 down-regulation is dependent on mGluR activation.

A. Schematics of the odor training and tissue collection paradigm. O/S^{+}: odor paired with stroking.

B. Relative optical density (ROD) of GluN1 expression (normalized to β-actin) in vehicle and MCPG infused aPCs.

C. ROD of GluN1 expression in vehicle and MPEP infused aPCs.

D. ROD of GluN1 expression in vehicle and AIDA infused aPCs.

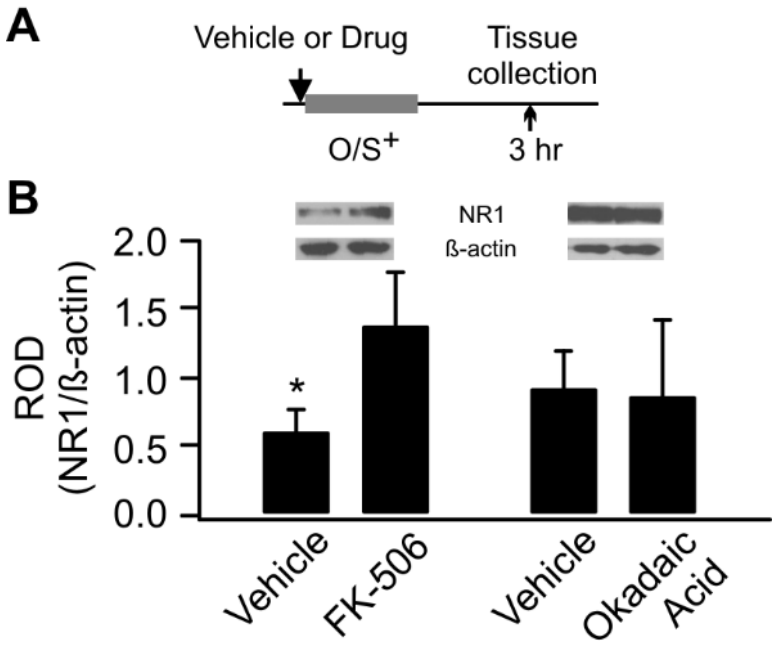

Figure 4.4. GluN1 down-regulation is dependent on calcineurin signalling.

A. Schematics of the odor training and tissue collection paradigm. O/S^{+}: odor paired with stroking.

B. Relative optical density (ROD) of GluN1 expression in vehicle, FK-506 or okadaic acid infused aPCs.

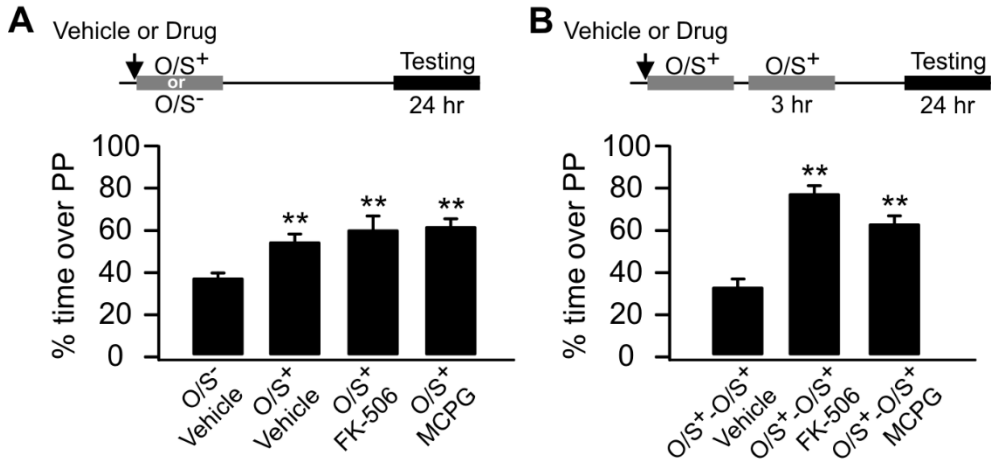

Figure 4.5. Inhibition of group I mGluR or calcineurin before first O/S+ training rescues early odor preference memory from re-training induced unlearning.

A. Percentage of time spent over peppermint (PP)-scented bedding without re-training.

   O/S^+: odor paired with stroking. O/S^-: odor only without stroking.

B. Percentage of time spent over PP-scented bedding with re-training.

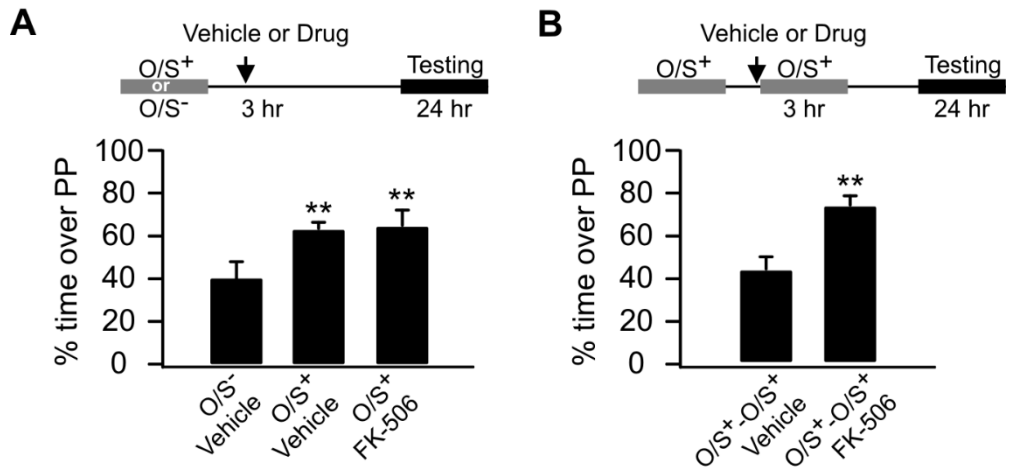

Figure 4.6. Inhibition of calcineurin before re-training rescues early odor preference memory.

A. Percentage of time spent over peppermint (PP)-scented bedding without re-training.

   O/S^+: odor paired with stroking. O/S^-: odor only without stroking.

B. Percentage of time spent over PP-scented bedding with re-training.

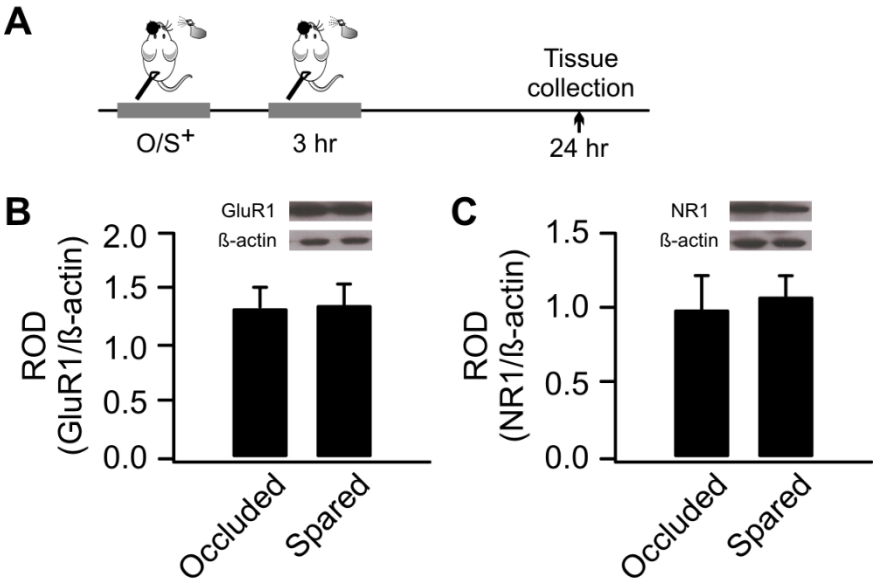

Figure 4.7. Re-training resets AMPAR and NMDAR to the baseline level.

A. Schematics of the odor training and tissue collection paradigm. O/S^{+}: odor paired with stroking.

B. Relative optical density (ROD) of GluA1 expression (normalized to β-actin) in occluded and spared aPCs.

C. ROD of GluN1 expression in occluded and spared aPCs.

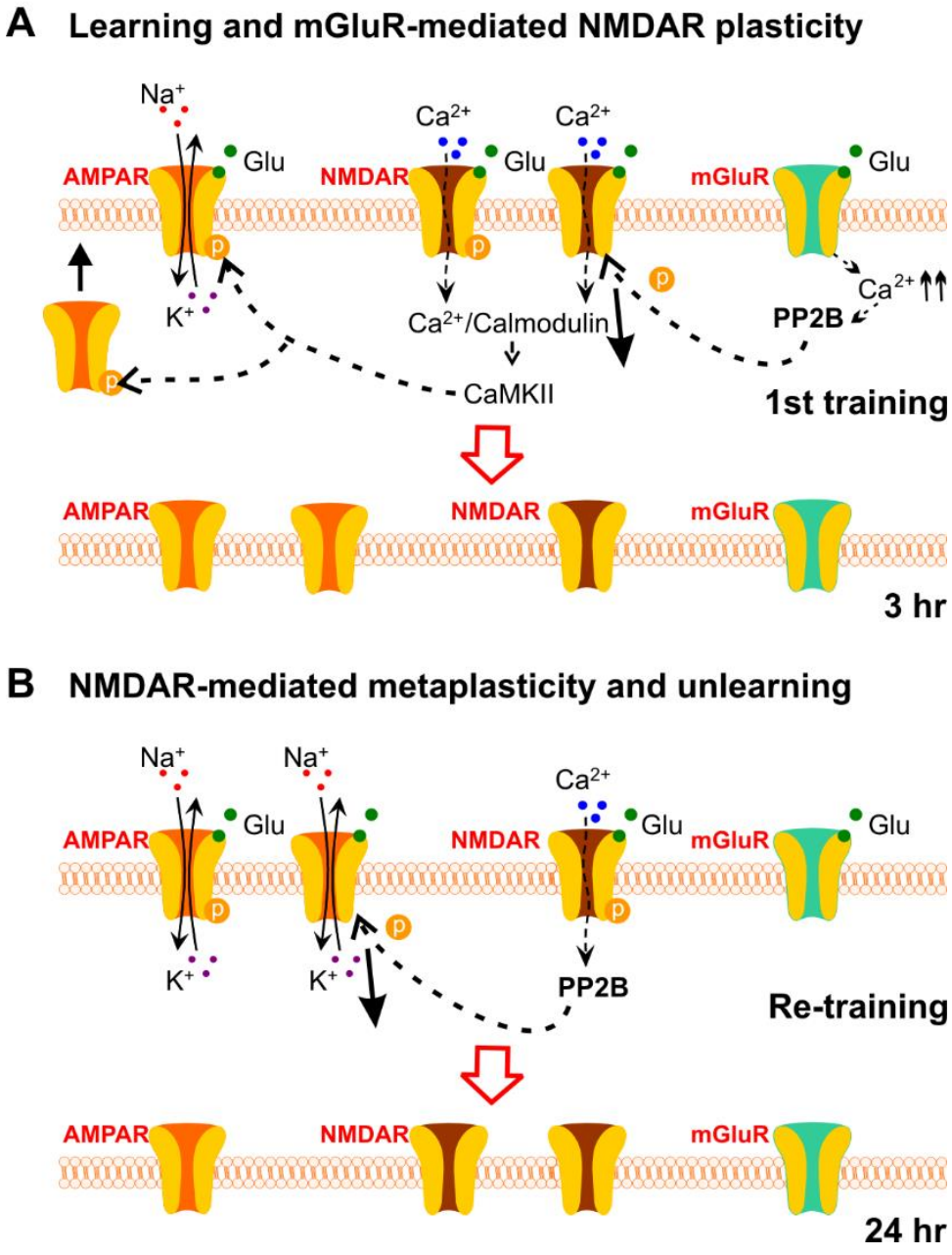
A
Learning and mGluR-mediated NMDAR plasticity
Na+
Ca2+
Ca2+
AMPAR
Glu
NMDAR
Glu
mGluR
Glu
K+
Ca2+/Calmodulin
Ca2+
PP2B
CaMKII
1st training
AMPAR
NMDAR
mGluR
3 hr
B
NMDAR-mediated metaplasticity and unlearning
Na+
Na+
Ca2+
AMPAR
Glu
NMDAR
Glu
mGluR
Glu
K+
K+
PP2B
Re-training
AMPAR
NMDAR
mGluR
24 hr

Figure 4.8. Summary of pathways involved in NMDAR plasticity and metaplasticity in early odor preference learning in rats.

A. Learning and mGluR-mediated NMDAR plasticity. During the 1st training, Ca^{2+} influx through NMDARs activates CaMKII, which phosphorylates AMPARs and facilitates AMPAR insertion into the synaptic membrane. Meanwhile, mGluR activation leads to Ca^{2+} release intracellularly and activates PP2B pathway and dephosphorylates NMDARs. The latter results in NMDAR endocytosis and down-regulation at 3 hr. Glu: glutamate; PP2B: phosphatase 2B (calcineurin).

B. NMDAR-mediated metaplasticity and unlearning. If re-training occurs at 3 hr, reduced Ca^{2+} influx through fewer NMDARs results in PP2B mediated AMPAR dephosphorylation and endocytosis. This re-sets the level of AMPARs to the original state before the 1st training.

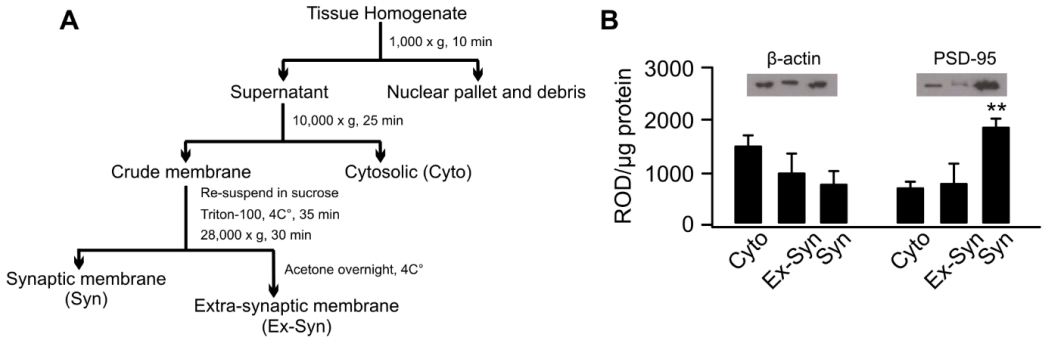

Figure S1. Synaptic fraction is rich in PSD-95.

A. Schematics of the tissue extraction protocol (also see Goebel-Goody et al 2009).

B. Normalized relative optical density (ROD) of PSD-95 (1:8000, Abcam) and β-actin expressions (per µg protein) in cytosolic, extra-synaptic and synaptic membrane fractions. ** $p < 0.01$

Chapter 5

Revisit metaplasticity: the roles of calcineurin and histone deacetylation in unlearning odor preference memory in rat pups (This Chapter is a modified version of Battacharya, Mukherjee el al. Neurobiol Learn Mem. 2018 Feb 5. pii: S1074-7427(18)30016-9. doi: 10.1016/j.nlm.2018.02.003.)

5.1 Abstract

Previous work has shown that 24 h duration odor preference learning, induced by one-trial training, generates a down-regulation of the GluN1 receptor in anterior piriform cortex at 3 h, and results in metaplastic unlearning if a second training trial is given at 3 h. The GluN1 receptor upregulates at 24 h so 24 h spaced training is highly effective in extending memory duration. The present study replicates the piriform cortex unlearning result in the olfactory bulb circuit and further studies the relationship between the initial training strength and its associated metaplastic effect. Intrabulbar infusions that block calcineurin or inhibit histone deacetylation normally produce extended days-long memory. If given during training, they are not associated with GluN1 downregulation at 3 h and do not recruit an unlearning process at that time. The two memory strengthening protocols do not appear to interact, but are also not synergistic. These outcomes argue that it is critical to understand the metaplastic effects of training in order to optimize training protocols in the service of either memory strengthening or of memory weakening.

5.2 Introduction

Real life learning is not an isolated, stand-alone experience. Each learning event builds on previous experience and hence is unique in each individual. If learning is an accumulative, built-up process, then our memories cannot be accounted for by a single synaptic plasticity event during one experience. Metaplasticity describes the phenomenon by which the capacity for synaptic plasticity is altered by prior synaptic activity[195]. Thus metaplasticity is likely critically involved in complex learning and directly influences behavioral outcomes. However, how metaplasticity occurs in vivo in a way that is relevant to cognitive function is not well understood.

Previously we have demonstrated that metaplasticity occurs in a natural learning model – early odor preference learning in rat pups[446 505]. Week-old rat pups form a preference to an odor that is paired with a tactile stimulus signaling maternal care (e.g. stroking using a brush)[329,442]. One trial, 10-min training can lead to a preference memory for the conditioned odor lasting up to 24 hs. Increasing the training strength by multi-trial, 24-h spaced training extends the memory to days[451]. However, two trials of training separated by 3 h actually prevent the odor preference memory[505].

Interestingly, synaptic NMDA receptors (NMDARs) in the anterior piriform cortex (aPC) decrease at 3 h, while they increase at 24 h following one trial training[505]. The altered plasticity at 3 h post the initial training is likely induced by occurring at the time of NMDAR down-regulation. Reduced Ca^{2+} entry via decreased numbers of NMDARs during the 2nd associative training at 3 h results in depotentiation (or LTD) of the aPC synapses and unlearning of the previous experience[505]. Indeed, blocking NMDARs during the 2nd training prevents the unlearning[505]. Further work shows that NMDAR down-regulation is mediated by both mGluR5 and calcineurin signaling[446]. Blocking either aPC mGluR5 or calcineurin during the initial training also prevents the unlearning induced by the 2nd training, 3 h later [446]. The timing between the prior and subsequent training appears to be critical, as re-training at 24 h, when NMDA receptors are increased [505], enhances memory [451].

Here we study the relationship between the strength of the initial learning and its metaplastic effect on subsequent training. We explore whether a stronger induction that produces "stronger" memory has the same metaplastic effect on 3 h re-training. Two protocols have been established previously that induce stronger odor preference memories (i.e. memories with prolonged durations). Blocking calcineurin with FK-506 [381] or blocking histone deacetylation with class I/II histone deacetylase (HDAC) inhibitor trichostatin A (TSA) [216] in the olfactory bulb (OB) extends one-trial odor preference memory for days. We have established that both the OB and aPC are plastic sites that are critical for early odor preference learning. NMDAR blockade in either site prevents odor

preference memory formation[373,476]. OB NMDARs are also down-regulated at 3 h following training[476]. Since both calcineurin [446,514] and histone deacetylation [525] down-regulate NMDAR GluN1 subunit in other brain structures, here we first examine the effects of FK-506 or TSA OB infusion during single trial training on GluN1 expression and subsequent learning at 3 h. After establishing their effects on NMDAR regulation and unlearning, we examined possible cross-talk between calcineurin and histone acetylation in the OB.

5.3 Materials and Methods

In the following sections I will describe the methodologies I have used for my last project.

5.3.1 Animals

Sprague Dawley (Charles River, Canada) rat pups of both sexes were used in this study. The day of birth was considered postnatal day (PND) 0. Litters were culled to 12 rat pups on PND1. Animals were kept in temperature-controlled rooms (20-25^{o}C) on reverse 12 h light/dark cycles. All experimental procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland following the guidelines set by the Canadian Council on Animal Care.

5.3.2 Cannula implantation and olfactory bulb infusion

On PND 5, rat pups were anaesthetized by hypothermia and placed in a stereotaxic apparatus. The skull was exposed and two small holes were drilled over the central region of each OB. The cannulae were implanted into the OB and cemented to the skull. The skin was sutured together and pups were allowed to recover on warm bedding before being returned to the dam. All drugs were infused into the OB on PND 6 at 20 min before the first training. 1.0 µl of a drug was injected bilaterally into the OB for behavioral experiments using a 10 µl micro-syringe. In pups for quantitative immunoblotting, drugs were infused either bilaterally into the OB (Fig. 5.4) or in one side into the OB with vehicle infused into the contralateral bulb (Fig. 5.2,5.3,5.5,5.6 and 5.7).

5.3.3 Drug Preparation

Pharmacological agents used include TSA (working concentration 0.05 µg/µl/OB as described earlier (Bhattacharya et al., 2017) (dissolved in 10% DMSO; Cedarlane, Canada; Cat. No. T-1052), a calcineurin (phosphatase 2B) inhibitor FK-506 (5 mM, dissolved in 10% DMSO; Tocris; Cat. No. 3631) and a phosphatase 1/2A inhibitor okadaic acid (500 µM, dissolved in 10% DMSO; Calbiochem; Cat. No. 459620). The

working concentrations for FK-506 and okadaic acid used produced the published results in our previous work[446].

5.3.4 Training

A single 10 min training session was performed on PND 6 rat pups in temperature controlled (28^{o}C) behaviour rooms. After the drug infusion, pups were placed on peppermint-scented bedding for 10 min and stroked with a paint brush for 30 sec every other 30 sec. Pups in the non-learning condition were placed on the peppermint-scented bedding for 10 min without stroking. Peppermint-scented bedding was prepared by adding 0.3 ml of peppermint extract (G.E. Barbour Inc., Canada) to 500 ml of regular unscented woodchip bedding. Pups were returned to the dam immediately after training until re-training, testing or sacrifice. For the re-training behaviour experiment, pups were re-trained at 3 h after the first training. Pups were re-exposed to peppermint-scented bedding while being stroked using the same procedure as in the first training. Pups were returned to the dam after re-training.

For Figure 1 experiment, unilateral naris occlusion was performed before the odor+stroking training as described previously[446,451,505]. Nose plugs were constructed using polyethylene 20 tubing and silk surgical thread. 2% Xylocaine gel (AstraZeneca) was applied to the left naris of the pup. After 3-5 min rest, the plug was gently inserted in

the left naris of the pup. After 10-min habituation, the pup underwent training. The nose plug was removed immediately following training and the pup returned to dams.

5.3.5 Testing

Twenty- four hours following the initial training, pups were tested for odor preference memory by using a two-choice odor preference test. Testing was carried out in a stainless steel test box placed over two training boxes. For all of the tests, one training box contained peppermint-scented bedding, and the other contained normal, unscented bedding. Training boxes were separated by a 2 cm neutral zone. During testing, each rat pup, one at a time, was separated from the dam and transferred to a no bedding holding cage in the testing room to prevent odor contamination. To start the testing, the pup was placed in the neutral zone of the test box. The percentage of time spent over peppermint-scented bedding or normal bedding for each pup was recorded during each of five 1 min trials. Pups were given 30 sec of resting time in a clean holding cage between each of the five 1 min trials.

5.3.6 Immuno-Blotting

After training, pups were decapitated and OBs were quickly removed and flash-frozen on dry ice at one of three different time points (5 min., 10 min. and 3 h). Samples were stored at -80^{0}C until further processing.

5.3.7 Synaptic and Extra-synaptic Protein Extraction Protocol

Tissues were homogenized in 100 µl sucrose buffer (in (mM): 320 sucrose, 10 Tris (pH 7.4), 1 sodium dodecyl sulfate tris-ethylenediaminetetraacetic acid (EDTA), 1 ethylene glycol tetraacetic acid (EGTA), 1× complete protease inhibitor mixture and phosphatase inhibitor mixture (phosSTOP, Roche) at 5500 xg for 20 sec in a homogenizer (Precellys® 24). The homogenate was centrifuged at 1000 xg at 4^{0}C for 15 min to remove the nuclear fraction and incompletely homogenized material (P1). The supernatant (S1) was collected and centrifuged at 10,000 xg at 4^{0}C for 20 min to obtain the membrane fraction (P2) and remove the cytosolic fraction (S2). The pellet (P2) was re-suspended in 80 µl 1X STE (sodium dodecyl sulfate tris-EDTA): 100 mM Tris (pH 7.4), 10 mM EDTA, 1% SDS, 1× protease and phosphatase inhibitors) buffer. P2 samples were sonicated and then heated to 90^{0}C for 3 min to solubilize the pellet.

5.3.8 Synaptic and Cytosolic +Nuclear (CY+NU) Protein Extraction Protocol

Purification of synaptic membrane followed previously published procedures[446]. Tissue samples were homogenized using a Teflon glass tissue homogenizer (Thomas Scientific) in ice-cold sucrose buffer (300 μL, same composition as aforementioned). Teflon glass tissue homogenizer was wiped with 70% ethanol after each sample preparation. The homogenized samples were centrifuged at 1000 xg for 10 min. The pellet (P1) was collected as the nuclear fraction. The supernatant (S1) was further spun at 10,000 xg for 25 min to obtain a second supernatant (S2, cytosolic fraction) which was then mixed with the nuclear fraction (P1) to get the cyto+nuclear fraction (S2+P1). The pellet (P2) was re-suspended in 120 μL sucrose buffer using a pestle mixing/grinding rod (Thomas Scientific) directly in the microfuge tube. Then, 8 volumes of a non-ionic detergent Triton X-100 buffer (final 0.5% v/v; (in mM) 10 Tris (pH 7.4), 1 EDTA, 1 EGTA, 1× protease and phosphatase inhibitors) were added for detergent extraction. This suspension was incubated at 4°C for 35 min with gentle rotation. Then, the suspension was centrifuged at 28 000 xg for 30 min. This pellet (P3, postsynaptic densities and synaptic junctions that are insoluble in Triton X-100) was re-suspended in 100 μL of TE buffer and sonicated. Then samples were boiled for 5 min and stored at −80°C until further processing.

Protein concentrations for all those samples (Synaptic + extra-synaptic protein, synaptic and cyto+nuclear protein) were determined by using a bicinchoninic acid (BCA) protein assay kit (Pierce).

5.3.9 Western-Blotting

Samples were mixed with 5x sample buffer (50mM Tris-HCl, pH6.8, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.125% Bromophenol Blue) and de-ionized water (to adjust total volume to 25 µl). Samples were heated for 5 min at 100^0C prior to loading onto either 10% or 15% polyacrylamide gel. Samples were then transferred to nitrocellulose membrane (Millipore) at 100 volts for 60 min at 4^0 C. Following transfer, nitrocellulose membranes were washed in Tris-buffered saline with 0.1% Tween (TBST) for 3x5 min. Blots were blocked in 5% non-fat dry milk + TBST for 1 h at room temperature. After washing, blots were incubated in primary antibody overnight at 4^0C with continuous shaking. For determining NMDA receptor (GluN1), total calcineurin (PP2B), and catalytic calcineurin (cat PP2B) expression, immunoblots were cut horizontally, and the upper portions were probed with a rabbit antibody for GluN1 (1:3000, Cell Signalling; Cat. No. 5704S), total PP2B (1:5000, cell signaling; Cat. No. 2614S), and cat PP2B (1:5000, EMD Millipore; Cat. No. 07-068-I). The lower portions were probed with β-Actin (purified rabbit anti-β-actin, 1:5000, Cell Signaling; Cat. No.4967S) as a loading control.

For determining histone 3 phosphorylation at serine 10 (pH3), immunoblots were probed with a rabbit antibody for PH3 (1:2500, Cell Signaling; Cat. No. 9701S), with total histone3 (tH3, 1/5000, Cell Signaling; Cat. No. 4499S) as a loading control.

Blots were then washed in TBST (3x10 min) and incubated in secondary antibody (goat anti-rabbit conjugated with horseradish peroxide; Thermoscientific, Cat. No. 31460) with 5% non-fat dry milk +TBST, for 1.5 h at room temperature and then washed in TBST (3x10 min). Specific protein bands were visualized using chemiluminescence (Supersignal West Pico; Thermoscientific, USA). Blots were exposed for different times to film (Kodak Clinic Select Green; Eastman Kodak company, USA) and the optical density (OD) of each band were measured using Image J software.

Given that there have been reports questioning the suitability of β-actin as a loading control in certain model system[526], we show that in our synaptic fraction preparation, both GluN1 and β-actin show adequate dynamic ranges and the amount of loading we used (30 µg protein) is optimal and within the linear range of concentration detection curves (Supplementary Figure 5.1).

5.3.10 Statistical analysis

Paired t-test were used in Fig. 1, 2, 4, and 5. One-way ANOVA was used in Fig. 3. Two-way ANOVA was used in Fig. 6 and 7. Data were presented as mean ± S.E.M.

5.4 Results

In the following sections I will describe my results from the Chapter 5.

5.4.1 Calcineurin blockade in the OB prevented GluN1 down-regulation

We first replicated our previous data that early odor preference learning down-regulates OB GluN1 subunits[476]. Using unilateral naris occlusion, we showed that decreased GluN1 expression in the OB with open naris 3 h following odor training (normalized relative optical density (ROD): 0.91 ± 0.12) compared to the OB with occluded naris (ROD: 1.26 ± 0.12, n = 9, t = 2.82, p = 0.02; Fig. 5.1). We have shown that early odor learning can be lateralized by unilateral naris occlusion[451]. Our result also shows that GluN1 down-regulation induced by learning occurs at synaptic membrane.

We infused FK-506 (a calcineurin antagonist) into one OB and vehicle into the other OB 20 min before the odor conditioning and measured NMDAR GluN1 subunit levels 3 h following the training (Fig. 5.2A). We observed an increased level of synaptic GluN1 expression in the FK-506 infused OB (ROD: 1.16 ± 0.22, n = 8) compared to the vehicle infused OB (ROD: 0.57 ± 0.07, n = 8; t = 2.56, p = 0.04; Fig. 5.2B). FK-506 infusion did not change basal level of GluN1 in naïve pups (O/S(-); ROD saline: 1.33 ± 0.30, n = 5; ROD FK-506: 1.30 ± 0.08; t = 0.12, p = 0.91; Fig. 5.2C). Unilateral infusion

of a PP1 inhibitor Okadaic acid, on the other hand, did not change the level of OB GluN1 (Okadaic acid: 0.90 ± 0.13; vehicle: 0.92 ± 0.09; n = 10; t = 0.14, p = 0.89; Fig. 5.2D). These results suggest that calcineurin mediates the early down-regulation of GluN1 in the OB following early odor preference training. Interestingly, calcineurin blockade also prevents GluN1 down-regulation in the aPC at 3 h[446].

5.4.2 Calcineurin blockade in the OB prevented unlearning

Calcineurin inhibition during one-trial training maintained the odor preference memory up to 96 hs from the normal 24 hs[381]. Calcineurin blockade prolongs NMDAR opening [527] and enhances NMDAR current[528], which could account for its effect in promoting learning. Interestingly, at the same time, calcineurin inhibition prevents NMDAR internalization [514] and down-regulation [446] (see also Fig. 5.2B). We next tested whether FK-506 OB infusion had any effect on unlearning at 3 h (Fig. 5.3A). One-way ANOVA revealed a significant group effect ($F_{4,21} = 57.4$; $p < 0.001$; Fig. 5.3B). Single odor+stroking (O/S) training led to successful preference learning (63.0 ± 1.11% over peppermint) compared to the odor only (O/O) control group (29.20 ± 1.69%; t = 8.08; p < 0.001). Two trials of O/S training separated by 3 h abolished the odor preference memory (34.56 ± 3.38%; t = 7.45; p < 0.001 compared to the one trial O/S training). However, OB infusion of FK-506 prevented the unlearning induced by two trials of training in vehicle infused pups. Pups in the FK-506 group spent significantly more time over peppermint during testing (68.1 ± 2.09%) compared to the vehicle infused pups (t = 9.80, p < 0.001).

5.4.3 Inhibiting OB Histone Deacetylation by TSA Prevented GluN1 Downregulation and Unlearning

Similar to the calcineurin effect, histone deacetylation blockade by TSA extends odor preference memory up to 9 days[216]. Here we studied whether TSA infusion affected GluN1 expression at 3 h, and unlearning induced by re-training at 3 h (Fig. 5.4A). TSA bilateral OB infusions prevented unlearning. The TSA infused group given two trials of training separated by 3 hs spent significantly more time over peppermint during testing (TSA: 62.30 ± 7.98%, vehicle: 26.24 ± 3.80%; $n = 7$; $t = 4.08$, $p < 0.01$; Fig. 5.4B). In parallel, the TSA infused OB group (ROD: 1.62 ± 0.23) showed significantly higher levels of GluN1 than the vehicle infused OB (0.68 ± 0.02; $n = 8$; $t = 4.08$, $p < 0.01$; Fig. 5.4C). Thus both FK-506 and TSA, which normally produce prolonged odor preference memory, prevented unlearning induced by a 2nd training trial.

5.4.4 No Simple Interactions of Calcineurin and Histone Deacetylation Occurred in Down-regulating GluN1

Do calcineurin and histone deacetylation act independently or synergistically in regulating GluN1 levels at 3 h following associative learning? To study whether there is an interaction between the effects of calcineurin and histone deacetylation, we first tested if there was any additional effect on GluN1 expression when TSA was added to FK-506

(Fig. 5.5A). FK-506 and TSA co-infused OBs showed no difference in GluN1 level (ROD: 0.64 ± 0.13) compared to the FK-506 only OBs (ROD: 0.64 ± 0.05; n = 8; t = 0.02, p = 0.98; Fig. 5.5B). This result implies one of two possibilities: 1) FK-506 and TSA work synergistically through the same pathway to influence GluN1 expressions; or (2) they do not interact synergistically. However, GluN1 down-regulation is saturated by either calcineurin or histone deacetylation signaling, thus no additional effect was observed by combining the two.

HDAC inhibition alters hippocampal calmodulin kinase II (CaMKII) activity[529,530], which is the upstream substrate of calcineurin[531,532]. We next explored whether TSA had any effect on the total level of calcineurin in the OB following odor preference learning (Fig. 5.6A). We measured total calcineurin levels in the nuclear/cytoplasmic fractions (Fig. 5.6B) and synaptic fractions (Fig. 5.6C), as well as the synaptically activated calcineurin levels (Fig. 5.6D). A 2 x 3 Group x Time repeated ANOVA was carried out in each case. For nuclear/cytoplasmic total calcineurin, there was no significant effect of group ($F_{1,28}$ = 0.03; p = 0.87), but there was a significant effect of time ($F_{2,28}$ = 5.22; p = 0.01) with no group x time interaction ($F_{2,28}$ = 0.18; p = 0.84) (Fig. 5.6B). For total synaptic calcineurin, there was no significant effect of group ($F_{1,28}$ = 0.02; p = 0.90), time ($F_{2,28}$ = 1.81; p = 0.18), or group x time interaction ($F_{2,28}$ = 0.18; p = 0.84) (Fig. 5.6C). The same results occurred for activated synaptic calcineurin found by measuring catalytic subunits. There was no significant effect of group ($F_{1,28}$ = 0.43; p = 0.52), time ($F_{2,28}$ = 1.33; p = 0.28), or group x time interaction ($F_{2,28}$ = 0.09; p = 0.91) (Fig. 5.6D). Overall, there is no effect of TSA on calcineurin levels at various times

following early odor preference learning. Thus, TSA infusion does not alter calcineurin or activated calcineurin.

We then investigated the possibility that calcineurin could potentially alter histone acetylation through an effect on histone phosphorylation. In the hippocampus, histone H3 phosphorylation and acetylation co-occur with contextual learning via extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways[533,534]. Dephosphorylation of ERK pathway by calcineurin [535] therefore likely alters histone signaling. We tested the effect of FK-506 on H3 phosphorylation following early odor preference learning (Fig. 5.7A). A 2 x 3 Group x Time repeated ANOVA was carried out. There was no significant effect of group ($F_{1,30} = 0.18$; $p = 0.67$), but there was a significant effect of time ($F_{2,30} = 8.48$; $p < 0.01$), with no group x time interaction ($F_{2,30} = 0.62$; $p = 0.54$) (Fig. 5.7B).

5.5 Discussion

In this study, we discovered that manipulations that were previously established to produce stronger memory (memory lasting longer than 24 h that is normally provided by one-trial training), such as those induced in the presence of the calcineurin inhibitor FK-506 [381] or the histone deacetylation inhibitor TSA[216], do not exhibit a metaplastic behavioral unlearning phase at 3 h post-training. Such pharmacological manipulations also prevent NMDA GluN1 down-regulation at 3 h. There was no direct interaction of the

calcineurin and histone deacetylation substrates during or following odor preference learning, even though nuclear/cytoplasmic calcineurin level decreased at 3 h, while phosphorylated H3 increased at the same time.

5.5.1 NMDAR Plasticity Following Olfactory Learning and its Metaplastic Effect

NMDAR plasticity has been well characterized in the olfactory system. NMDAR subunit composition in the piriform cortex is altered following both development [481] and associative learning in adult animals[213,536], indexed by increased ratio of NMDAR GluN2A/2B in both scenario. The amount of NMDARs, indexed by the essential unit GluN1, and NMDAR current, are altered following neonatal odor learning in both the OB [475,476] and aPC[505]. However, the impact of NMDAR plasticity on future learning capacity has been understudied.

Previously we have shown that one-trial training, which normally induces a 24 h preference memory, undergoes a metaplastic unlearning-promoting phase in the aPC during memory consolidation at 3 h. This metaplastic phase was induced by aPC NMDAR down-regulation[505]. While odor preference learning is mediated by NMDARs, NMDAR plasticity and metaplastic unlearning are independent of NMDARs since NMDAR blockade prevents odor learning, but does not affect unlearning[505]. This implies that the lack of unlearning metaplasticity we observe here with odor training is an independent process from the original learning itself. Here we show that GluN1 down-

regulation occurs in the OB as well and that preventing OB GluN1 down-regulation also abolishes unlearning. Both OB and aPC are implicated in the encoding of the early odor preference memory[329,373,476]. Our data reveal the same dual circuitries (in the OB and in the aPC) for metaplastic unlearning.

5.5.2 Mechanisms for Calcineurin and Histone Deacetylation in NMDAR Down-regulation

Acetylation of histone H3 can loosen DNA-histone interactions to enhance the transcription of target genes[537]. Suppressing HDAC activity has been correlated with enhanced histone acetylation at the NMDAR subunit promoters in primary neocortical cell culture[538], hippocampal CA1 neurons[539], frontal cortex[540], and striatum[525]. On the other hand, calcineurin may promote NMDAR down-regulation via dephosphorylation of GluN2B at Tyr1472 and triggering clathrin-mediated endocytosis of NMDARS[514].

Do calcineurin and histone deacetylation act independently or synergistically in down-regulating NMDAR GluN1? We first tested what effect enhanced histone acetylation had (by TSA administration) on calcineurin expression or activity during odor preference learning. Possible interaction could occur through CaMKII activation by HDAC inhibitor [529,530] and CaMKII activation of calcineurin[532]. We showed no change in the levels of nuclear and synaptic calcineurin, and synaptically activated calcineurin production in the TSA group compared to the control at multiple times following learning. We then investigated whether calcineurin promoted histone deacetylation

through its potential influence on H3 phosphorylation. H3 and H4 are major forms of histone that are critically involved in learning. In an olfactory aversive learning model, both H3 and H4 acetylation are increased in the OB following conditioning[454]. Contextual fear conditioning increases H3 phosphorylation at Ser10 [534] and acetylation at Lys14 [533] 1 h post-training. The proximity between Ser10 and Lys14 sites implies a possible interaction between H3 phosphorylation and acetylation[541]. Indeed, studies have suggested that H3 phosphorylation may be a prerequisite for H3 acetylation in certain scenarios[542,543]. Both H3 phosphorylation and acetylation are regulated by ERK/MAPK pathways (Chwang et al., 2006). ERK/MAPK is a target of calcineurin mediated dephosphorylation[535]. Calcineurin could reduce H3 phosphorylation via decreasing ERK/MAPK signaling. However, our results showed no difference in H3 levels in FK-506 treated OBs compared to the vehicle infused ones. Interestingly, H3 phosphorylation significantly increased 3 h following odor preference conditioning, suggesting histone activation was enhanced by natural associative learning, consistent with other models[534].

5.5.3 Functional implications for NMDAR metaplasticity in learning

Metaplasticity permits dynamic regulation of synaptic changes (threshold and direction) [496] during complex learning and is likely critical for long-term memory formation. Metaplasticity can be manifested in different forms in behavior. Stress, and associated high levels of corticosterone, induces acute or chronic synaptic changes that can alter learning capacity. Behavioral stress impairs NMDAR-dependent LTP and hippocampal-dependent learning[501]. In both visual [480] and olfactory [481] cortices, changes

in LTP or LTD capability are observed following periods of sensory deprivation or enrichment. Metaplastic manipulations can be used for therapeutic purposes. A recent study shows that metaplastic activation of ryanodine receptors restores long term LTP and its associated synaptic properties in an APP/PS1 mouse model of Alzheimer's disease[544].

Our studies in early odor preference learning provide direct evidence in support of NMDAR plasticity as a mechanism for metaplasticity in natural learning[446,505]. We have provided some of the first evidence that learning itself affects future learning capacity at the very same synapses. Previously we have shown that metaplasticty is critically dependent on timing (i.e. intervals between training trials). Here we demonstrate that the metaplastic effect is influenced by the strength of the initial learning, such that only weak learning down-regulates NMDARs but strong learning prevents NMDAR down-regulation and its associated metaplastic effect. In an amygdala-dependent fear conditioning paradigm, a weak training trial, which does not produce fear memory, can prime future learning. Another weak trial delivered hours later results in long-lasting and robust fear memory[502]. Together, these studies suggest that the strength of the priming event may determine the nature of the synaptic changes and the behavioral output.

5.6 Conclusion

We show that the metaplastic effects of initial learning are dependent on learning strength. A weak learning that only produces a 24 h duration memory results in NMDAR

down-regulation and primes unlearning by the same training at 3 h. NMDAR up-regulation at 24 h following the initial learning, on the other hand, is associated with effective re-training at the 24 h interval enhancing and prolonging learning. Here a calcineurin inhibitor, as well as a histone deacetylation inhibitor given during training and producing strong learning, prevents the 3 h NMDAR down-regulation event, abolishing synaptic weakening and preventing unlearning. Understanding behavioral metaplasticity, and its associated properties, has significant therapeutic implications for enhancing training and improving adaptive learning and for diminishing maladaptive behaviors following traumatic experience.

5.7 Figures of Chapter 5

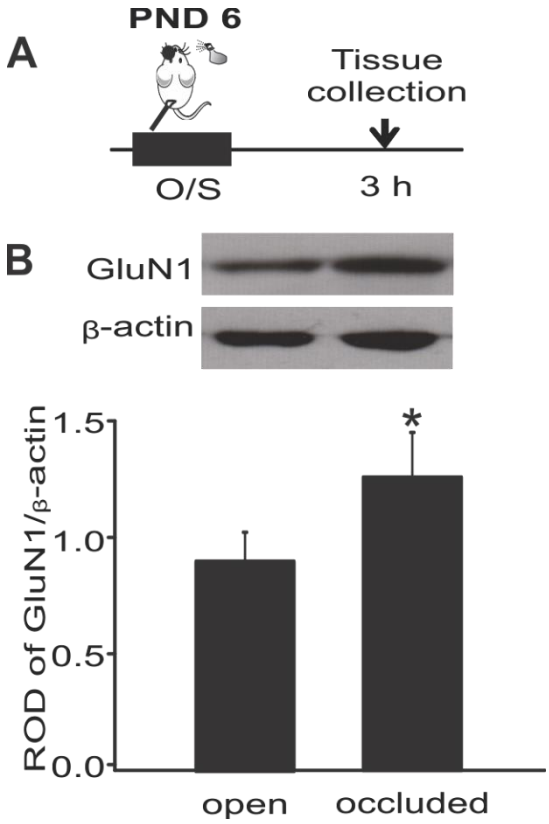

Figure 5.1. Early odor preference learning down-regulated GluN1 expression in the OB

A. Schematic of training and tissue collection. O/S(+): Odor+Stroke training. B. GluN1 expression is reduced in the OB of open naris 3 h following odor preference learning.

*$p<0.05$

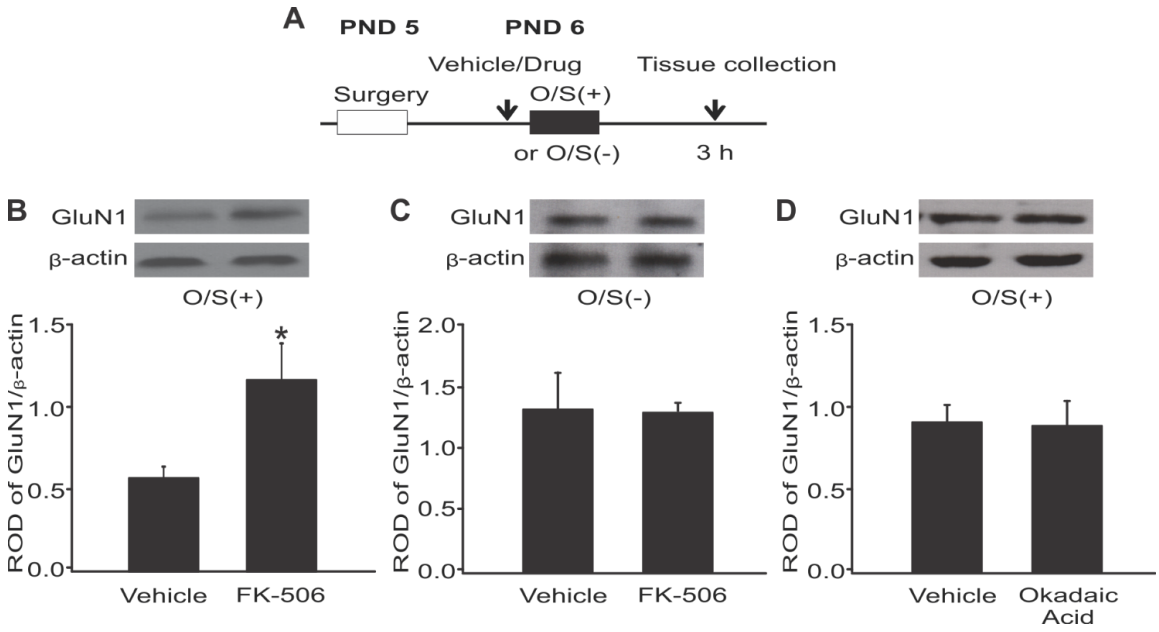

Figure 5.2. FK-506 increased GluN1 expression in the OB

A. Schematic of training and tissue collection. O/S(+): Odor+Stroke training; O/S(-): no training. B. FK-506 (a calcineurin inhibitor) treated OBs in the conditioning training group show more GluN1 expression. C. FK-506 does not alter basal level GluN1 in naïve, untrained pup OBs. D. Okadaic acid (a PP1 inhibitor) does not alter the GluN1 level in the OB in trained pups. *$p<0.05$

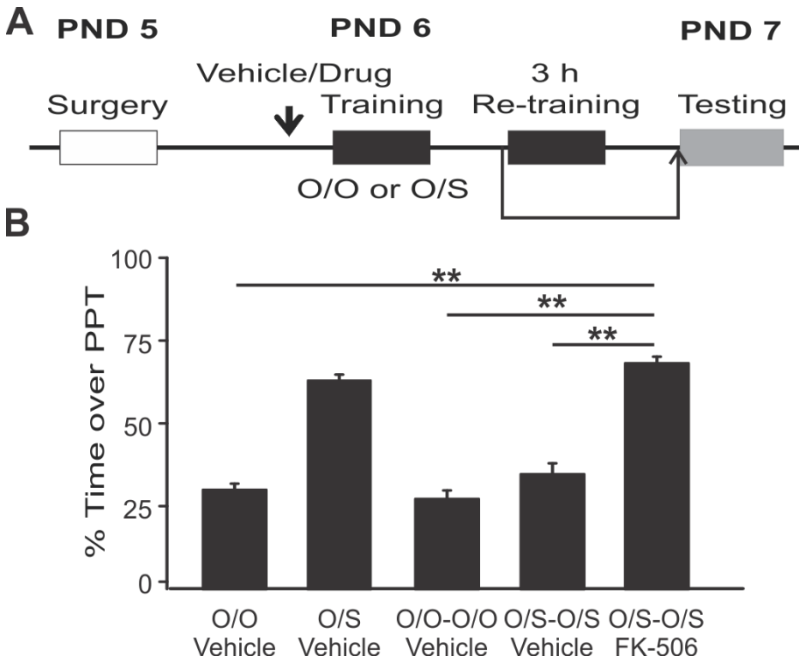

Figure 5.3. FK-506 prevented unlearning

A. Schematic of training and testing. B. FK-506 prevents unlearning induced by a 2nd training at 3 h. O/S: odor+stroking; O/O: odor only; **p<0.01

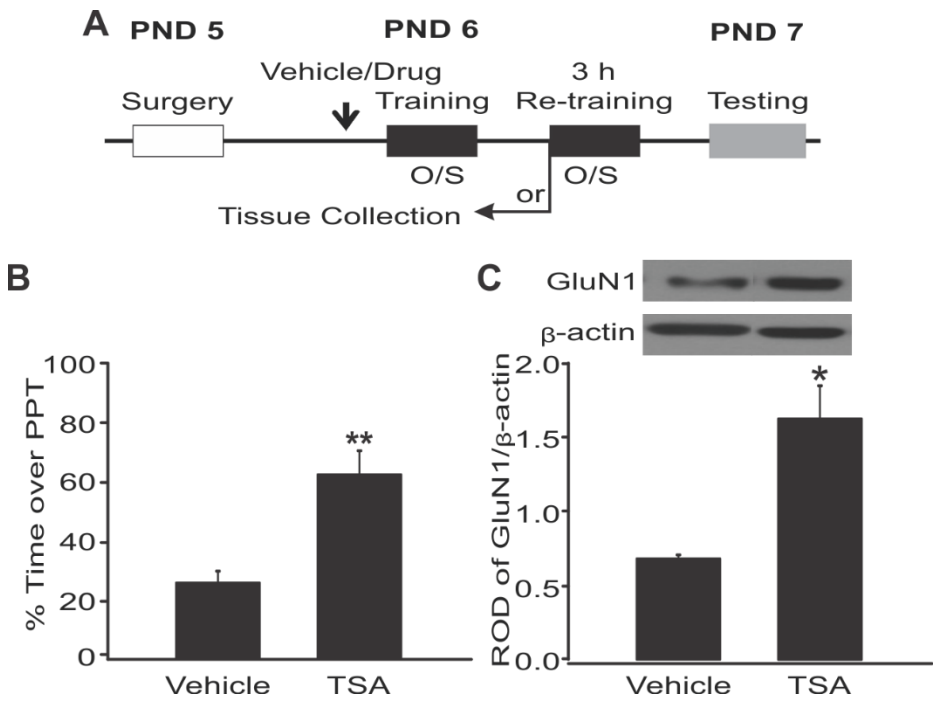

Figure 5.4. TSA prevented GluN1 down-regulation and unlearning

A. Schematic of training, testing and tissue collection. B. TSA prevents unlearning induced by a 2nd training at 3 h. C. TSA increases the GluN1 level in OB. *p<0.05; **p<0.01

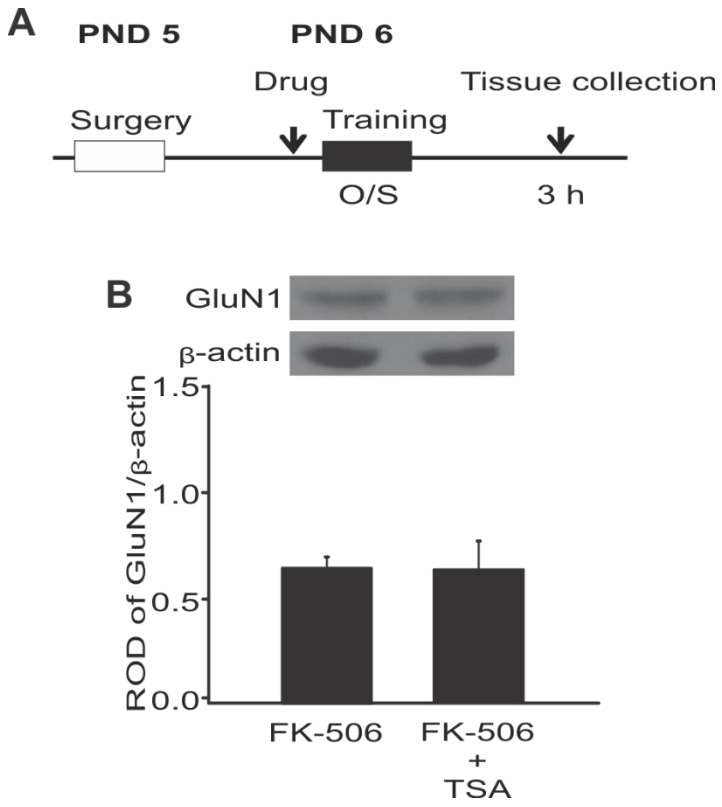

Figure 5.5. No additive effects of TSA and FK-506 on GluN1 levels in the OB
A. Schematic of training and tissue collection. B. FK-506+TSA OBs show similar levels of GluN1 as that of FK-506 only OBs.

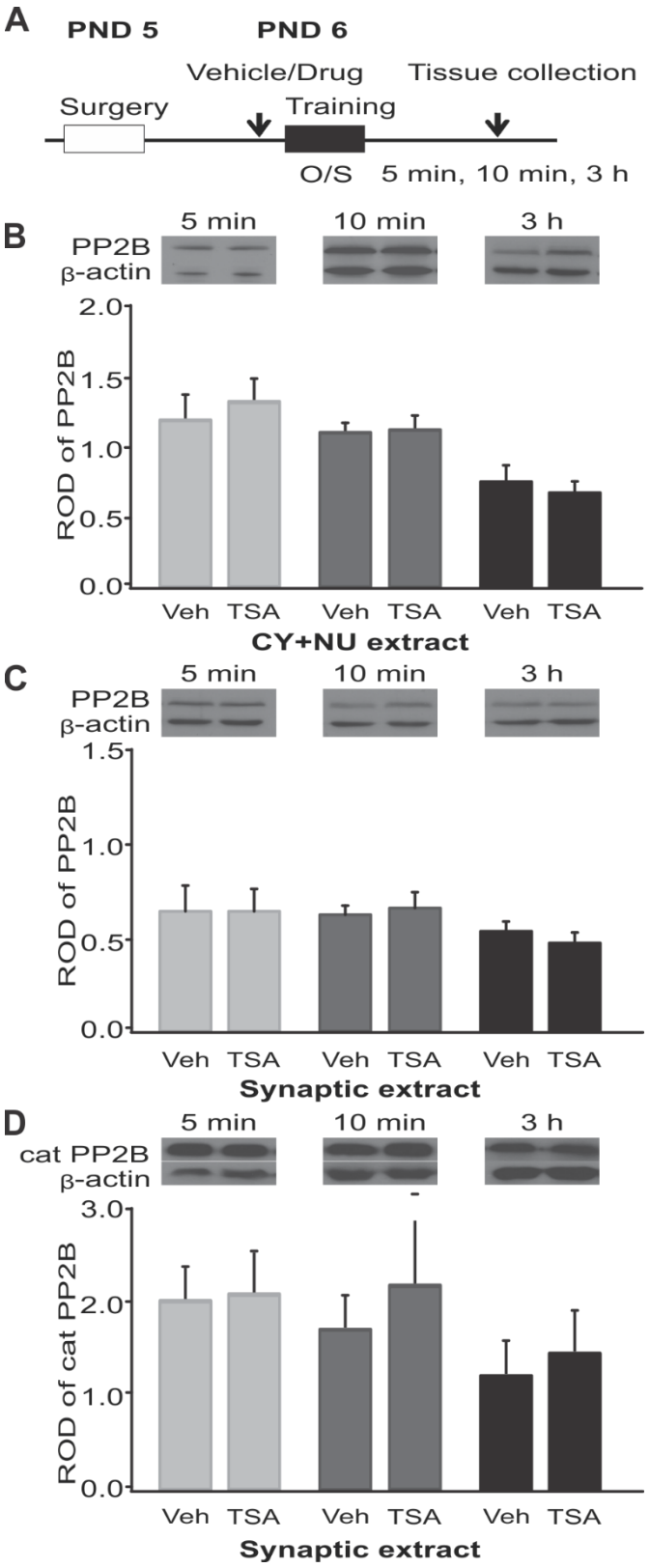
A
PND 5
PND 6
Vehicle/Drug
Tissue collection
Surgery
Training
O/S
5 min, 10 min, 3 h
B
5 min
10 min
3 h
PP2B
β-actin
ROD of PP2B
2.0
1.5
1.0
0.5
0.0
Veh TSA Veh TSA Veh TSA
CY+NU extract
C
5 min
10 min
3 h
PP2B
β-actin
ROD of PP2B
1.5
1.0
0.5
0.0
Veh TSA Veh TSA Veh TSA
Synaptic extract
D
5 min
10 min
3 h
cat PP2B
β-actin
ROD of cat PP2B
3.0
2.0
1.0
0.0
Veh TSA Veh TSA Veh TSA
Synaptic extract

Figure 5.6. TSA had no effect on calcineurin expression levels in the OB following early odor preference training

A. Schematic of training and tissue collection. B. Expression levels of calcineurin (PP2B) in the cytoplasmic and nuclear fraction (CY+NU) of the OB. C. Expression levels of PP2B in the synaptic extract of the OB. D. Expression levels of activated PP2B in the synaptic extract of the OB; Veh, vehicle.

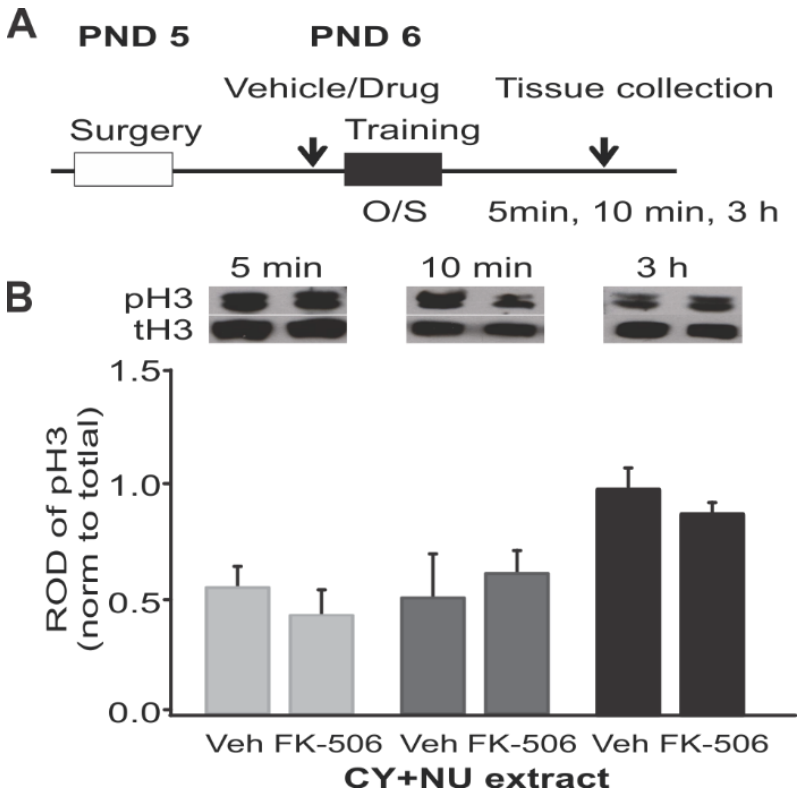

Figure 5.7. FK-506 had no effect on histone H3 phosphorylation

A. Schematic of training and tissue collection. B. Expression levels of phosphorylated H3 (pH3) in the cytoplasmic and nuclear fraction (CY+NU) of the OB.

6. Summary

In my thesis, I have investigated the role of NMDARs in early odor preference learning and how NMDARs mediate synaptic plasticity and metaplasticity. During my thesis, I have delineated molecular pathways underlying synaptic plasticity and metaplasticity in early odor preference learning, and these findings may have implications for general learning and learning optimization. In the following sections, I will summarize and discuss the findings from Chapter 2 to Chapter 5.

6.1 Differential roles of NMDAR and LTCC in Early Odor Preference Learning

Previously, researchers have characterized the functions of LTCCs and NMDARs separately by focusing on their independent contributions. The channel properties of NMDARs and LTCCs have been well-characterized [545] [546]. It had been shown that NMDARs plays a crucial role in LTP [171] and in the initiation of learning [185] whereas LTCCs helps in the maintenance of LTP [547]and LTM even though both allow Ca2+ into the cell [548]. Ca2+ entry through NMDARs and LTCCs can trigger different signaling cascades which are important for different aspects of learning [549,550]. There have also been detailed characterizations of the signaling pathways affected by Ca2+ entry through NMDARs and LTCCs, but evidence for the distinct yet complementary roles of these two channel proteins in learning are sparse.

Previously it had been shown that both long-term fear memory [455] and odor preference memory required CREB signaling [443] [393] [373]. In a recent study, our group also

reported that local AMPAR insertion into the synaptic membrane accompanies STM and this AMPAR insertion is mediated by CaMKII signaling [50]. An NMDAR-mediated CaMKII activation leading to AMPAR insertion mechanism for LTP effects has previously been described by R.C. Malenka in 2012 [485]. In my work (Chapter 2), I investigated the differential roles of NMDAR and LTCC in early odor preference learning.

My new research extends these findings and clearly shows that NMDARs and LTCCs are important for different phases of memory. The three temporal phases of early odor preference memory have been well characterized by Grimes et al. in 2011. Short-term memory (up to 3 hr) is independent of transcription and translation, intermediate memory (5 hr) requires transcription but not the translation, and long-term memory (24 hr) is dependent on both transcription and translation. I have shown that blocking NMDARs prevents both STM and LTM in early odor preference memory.

Blocking NMDARs decreased AMPAR expression relative to learning controls at synaptic sites at 3 h and at 24 h. In contrast, inhibition of LTCCs prevented LTM without affecting STM. However, the loss of NMDAR function, which prevents STM, can be compensated for by overdriving LTCCs and, again, increased synaptic AMPAR changes correlate with renewed STM memory expression.

These results suggest calcium changes can transiently enhance AMPAR expression at synaptic sites without requiring a change in protein synthesis. Further

investigation of the mechanism of increased synaptic expression will be of interest. Both insertion from the mobile cytoplasmic pool and lateral movement from extrasynaptic sites are candidate mediators [416], but the translocation must be transient without protein synthesis changes. These data also suggest that NMDARs play a crucial role in both STM and LTM whereas LTCCs normally exclusively support LTM. However, it remains to be clarified how these two calcium entry channels are differentially engaged with the downstream signalling pathways that lead to either STM or LTM.

Subcellular localization of NMDARs and LTCC s are likely critical for their roles in learning and memory. Researchers have shown that NMDARs are localized in the synaptic membrane [551] whereas LTCCs are abundant mainly in somatic areas [375 429]. These different subcellular localizations imply differential roles for NMDARs and LTCCs in intracellular signaling. Specifically, we have shown that the activation of somatic LTCCs is dependent on the initial activation of more distant NMDARs, which is consistent with early research on striatal neurons [552]. Somatic LTCC activation allows Ca2+ influx and leads to different protein kinase activation than that seen for NMDA receptors, for example, CaMKIV [553 554] and PKA [554]. The Ca^{2+} dependent kinase activation phosphorylates CREB for transcription of plasticity-related mRNAs.

Another intriguing finding from my research is that LTCC activation in the absence of NMDAR activation results in impaired discrimination of the conditioned odor from a similar odor mixture. AMPAR expression was increased in the group with LTCC

activation alone in the absence of NMDAR activation. We hypothesize that increased AMPARs in absence of NMDAR activation occurs in a more diffuse pattern due to missing synaptic tags. Diffuse insertion of AMPARs would explain the loss of input specificity, which further leads to impairment in pattern separation. Identifying spatial differences in AMPA-receptor insertion in these experiments remains a topic for further study. The present data suggest that NMDARs play a critical role in mediating stimulus specificity in early odor preference learning. There is compelling evindence that memory allocation to specific neurons and synapses is not a random process [555]. This requires specific mechanisms like, neuronal excitability, synaptic tagging and capture that determines where the specific memory will be allocated [555]. Similar odor mixture can activate different neurons and synapses in the neuronal circuitry [556]. Also, neurons get activated other than conditioned stimulus. This activation of neuron can reduce the distinctiveness of the encoding ensemble. During this activation NMDAR determines which synapse needs to be strenghtned and after that incorporation of AMPAR takes place in the specific synapses which could serve as a synaptic tag for maintainance of the memory or memory consolidation. On the other hand activation of LTCCs in absence of NMDARs leads to random incorporation of AMPAR and synaptic strengthening takes place in other synapses. This random incorporation leads to generalization of memory and complicay in pattern separation at the circuitry level.

6.2.2 NMDAR Plasticity following Early Odor Preference Learning

NMDARs play a crucial role as coincidence detectors for mediating synapse specific AMPAR plasticity in associative learning [145], including early odor preference learning [316 373]. Recent evidence has shown that NMDARs themselves undergo plastic changes, particularly, during development [477]. The data suggest that during development NMDAR subunit composition changes from mainly GluN2A to GluN2B subunits. In the piriform cortex, a reverse switch from GluN2B to GluN2A occurs in adult rats following olfactory rule learning [213]. In my research, I have shown a biphasic change in the obligatory NMDAR subunit GluN1 at the synaptic site following early odor preference training. We observed a downregulation of GluN1 at the synaptic site at 3 hr and an increase of GluN1 expression at 24 hr after training. We have also reported that LTD is inducible at 3 hr but LTP was impaired at the same time point. Unlike the adult rat rule learning model, we did not observe any reductions in GluN2B expression at the LOT-aPC pyramidal cell synapses following early odor preference training. This may relate to the already high levels of GluN2A expression at this developmental stage. These data suggest that GluN1 downregulation at LOT synapses is responsible for the reduced NMDAR synaptic transmission at 3 hr after training, which is associated with inducible LTD.

Interestingly, the NMDAR LTD at 3 hr post-training, co-exists with AMPAR LTP at the same LOT-aPC synapses [373]. A similar observation was reported in a study on nucleus accumbens [486]. In that report, simultaneous LTP of non-NMDA receptors and LTD of NMDAR in the nucleus accumbens was also described. With respect to other literature, it is very likely that AMPAR LTP and NMDAR LTD are both mediated by

Ca2+ concentration [477]. Kombian and Malenka showed that the same postsynaptic Ca2+ concentration might have an opposite effect on AMPAR and NMDAR mediated synaptic responses [486]. Interestingly, 24 hr following early odor preference training, there is an increase in GluN1 expression at the synaptic site in aPC which coincides with both enhanced LTP [373] and impaired LTD. We hypothesize that increased AMPARs and NMDARs 24 hr following training may have saturated the synapse with respect to AMPAR insertion, which leads to impaired LTP, while the high Ca^{2+} concentration occurring through the increased NMDARs might be responsible for the impairment in LTD.

6.2.3 Metaplasticity and Behavior

It has been shown that learning can result in increased neuronal excitability in different learning tasks and in different brain areas [214 557 212 558 559]. This intrinsic excitability change has been recognized as a metaplastic mechanism, which facilitates future learning through priming [560 561]. Priming itself does not induce behavioral changes but it changes neuronal intrinsic excitability which influences subsequent plasticity events and, thus, behavior. Also, it appears priming effects (changes in intrinsic excitability) do not last for long periods; eventually intrinsic excitability goes back to its baseline level even though learning persists [562 209 210].

Zelcer et al. (2006) first showed a priming effect on behavior. In this study, they reported that well-trained rats that had learned the rule for an odor discrimination paradigm,

performed more rapidly on subsequent discriminations between new odors [563]. They showed that an increase in intrinsic excitability followed rule learning and accompanied the more rapid performance. The intrinsic neuronal excitability lasts for approximately 24 hr after training. Surprisingly, in a hippocampus-dependent Morris water maze experiment, the odor rule learning rats exhibit superior performance compared to control rats that have never received prior odor discrimination training. They also reported that if the water maze training is delayed for 3 days after the odor discrimination training, there are no differences between the groups. At the same time, increased intrinsic excitability also goes back to baseline. This study suggests time windows for metaplastic changes, which is consistent with our own finding.

In another study, Clem et al. showed sensory experience can also regulate both future plasticity and learning [564]. After removing a single vibrissa, whisker experience resulted in synaptic strengthening in the spared barrel cortical neurons. This effect depended on glutamate signaling through NMDARs. *In vitro* studies showed that the spared barrel cortical neurons exhibited impaired or partially occluded LTP. The same study revealed that blocking NMDARs in the spared barrel cortical neurons leads to a form of synaptic strengthening which is dependent on mGluR. The single whisker removal not only altesr the synaptic state but also affect subsequent learning. Overall, the study indicates that behavioral experience alters synaptic state which further alters the mechanisms of later synaptic plasticity and learning.

In a Pavlovian classical conditioning study, Parsons and Davis (2012) [502] described the effect of metaplasticity on learning [502]. The behavioral paradigm was fear conditioning where a presentation of light was paired with shock for two trials, which generates a fear memory to the light. If the rat is given only one trial they do not form a memory as revealed by behavioral tests, but surprisingly this weaker stimulus primes the rats for subsequent learning. The same rat displays robust fear memory if a second identical trial is delivered within one hour following the first trial. This priming effect is dependent on PKA signaling. Blocking of PKA in the amygdala shortly before the first trial blocked fear memory formation after the second trial.

These studies and others have shown some forms of metaplasticity can lead to facilitation of memory and to associated synaptic strengthening. My research has shown a metaplastic effect which negatively regulates synaptic plasticity and memory. Attenuation of a cue-specific memory occurred via a metaplastic mechanism. In my study, I have shown that identical retraining at 3 hr after initial training leads to unlearning. This effect is time-dependent such that if the second training is given 24 hr later then the memory remains intact. I have shown that this attenuation of the odor preference memory by 3 hr retraining is dependent on the downregulation of NMDARs at aPC synapses. I have also shown that activating different synapses by using a new odor 3 hr after the first training does not affect the original cue-specific memory. Only activation of the same synapses via the same conditioned odor cue affects the odor preference memory and leads to unlearning. Blocking NMDARs before retraining blocks unlearning, which suggests again that attenuation of the memory after retraining is mediated by the NMDAR. I

subsequently characterized the molecular mechanisms of the NMDAR plasticity and metaplasticity in this model.

6.2.4 Molecular Mechanism of NMDAR Plasticity and Metaplasticity

In continuation of my previous project, I decided to further decipher the molecular mechanisms of NMDAR plasticity and metaplasticity (Chapter 4). Identifying molecular mechanisms will eventually be crucial for building models of learning and memory substrates and for manipulating learning and memory in clinical settings.

Previously, as reviewed, I had shown that 3 hr after the training there is a downregulation of the NMDAR-GluN1 subunit at the aPC-LOT synapse. Unlike learning itself, blocking NMDARs prior to the first training does not inhibit this downregulation of NMDARs. I then asked whether the physical NMDAR down-regulation is mediated by mGluR activation during training. There are two main types of mGluRs present in these postsynaptic cells specifically mGluR1 and 5 [565]. Both of them have the ability to activate TRP type channels which trigger calcium release inside cells [566] [567]. I have shown that inhibiting mGluR5 prior to first training inhibits the downregulation of NMDAR at 3 hr and blocks the NMDAR mediated unlearning after retraining. I further showed that the downregulation of NMDAR is mediated by the phosphatase calcineurin. Unlearning is prevented when calcineurin in the piriform cortex is blocked before the first training. But still, the question remains with respect to how the downregulated NMDARs cause

unlearning during the second training. Hypothetically, downregulation of NMDARs could lead to low Ca2+ concentrations, which would further lead to activation of the same protein, calcineurin. The activation of calcineurin produces dephosphorylation of the NMDAR and its subsequent downregulation in first place and its stronger activation by lower calcium levels (fewer NMDA receptors now) the second time would dephosphorylate AMPAR during retraining via activation of the phosphotase. Dephosphorylation of the AMPAR at aPC-LOT synapse could cause internalization of AMPAR and result in unlearning. The dephosphorylation of NMDARs and AMPARs needs to be checked to substantiate this hypothesis. It is also possible that due dephosphorylation, the AMPARs and NMDARs trafficked from synaptic sites to extra-synaptic sites. To understand the complete mechanism, more investigation is necessary. We do not know the entire molecular pathway yet but we show in our behavioral paradigm that timing and the repetition of training are very important for unlearning. In our study, we were able to capture the vulnerable period, or the state of the synapse where protein synthesis or mRNA synthesis is crucial to maintaining the long-term memory consolidated in our behavioral paradigm. We suggest we altered the consolidation state during retraining.

Other questions that need to be addressed is whether or not there are changes in the level of expression of the NMDAR-GluN1 after retraining. Is there another timed reduction of GluN1 subunit or does it remain the same. This needs assessment at different time point especially 3 hr and 24 hr following the retraining. As mentioned earlier Ca2+ plays a crucial role in signalling pathways and LTCC may also have a role in this

paradigm. LTCC can lead to calcineurin activation and could be manipulated after training to assess its contribution to the metaplastic effects we have discovered.

Another interest for future studies is the role of intracellular signaling molecules like PKA, CaMKII, and CaMKIV. The subcellular localization of these proteins suggests that PKA is mostly present in dendritic shafts when compare to somatic localization [568]. On the other hand, CaMKII is localized at the synaptic site and in the cytoplasm [569] but CaMKIV is localized mostly in the nucleus but can translocate to cytoplasm [570]. CaMKIV appears to plays a crucial role in transcription and it is associated with cAMP pathways [571]. Whether there is any translocation of CaMKIV due to retraining is of? What would be the effect of CaMKIV translocation? What are the other interacting molecules? These questions can be addressed rapidly in this model.

I have already discussed the effects of phosphorylation and dephosphorylation on AMPARs. In the early odor preference learning model, the phosphorylation and dephosphorylation of AMPARs not been well-described. We would like to investigate the phosphorylation and dephosphorylation of AMPARs at different time points and relate these phosphorylation changes to behaviour. A key question is AMPAR changes relate to membrane/cytosol trafficking or to synaptic to extra-synaptic trafficking.

In another project, I focused on delineating the relationship between memory strength and metaplastic changes. One trial of 10 min odor/stroking conditioning leads to a 24 hr memory, whereas other manipulations such as described below can generate

memories more than 24 hrs. Does this stronger training, which generates longer-lasting memories, have the same metaplastic effects on the synapse and on behaviour as normal single trial training? In my next section, I discuss my research related to this issue.

6.2.5 Memory Strength and Metaplasticity

In my last project, I have characterized the relationship between the strength of the initial learning and its metaplastic effect on subsequent training. Previously, my research showed that 3 hr following a single early odor preference training trial, there is a downregulation of the NMDAR-GluN1 subunit in the aPC. This downregulation is responsible for unlearning when the pups are retrained with the same parameters at 3 hr following initial training. As mentioned earlier this 10 min stroking with a paintbrush in presence of odor leads to a 24 hr stable memory. But this memory does not last beyond 24 hr. Previously, two protocols have been established that induce prolonged odor preference memories using the same single odor+stroking training protocol. Blocking calcineurin [381] or histone deacetylation to prior training [216] in the olfactory bulb (OB) extends a one-trial odor preference memory for days. Bhattacharya et al. (2017) established a model where they showed the same early odor preference memory can be extended to 9 days by blocking OB histone deacetylation. They also reported that the extended memory is correlated with extended increased AMPAR expression in the OB. The key question I asked here was whether a stronger induction protocol that produces prolonged memory has the same metaplastic effect on 3 hr re-training. We have established that both the OB and aPC are plastic sites that are critical for early odor preference learning. NMDAR

blockade in either site prevents odor preference memory formation [316 373]. The downregulation of NMDAR is also observed in the OB at 3 hr after early odor preference training [316]. We, therefore, investigated the modulation by the odor preference training of subsequent learning and subsequent NMDAR plasticity using OB blockade of histone deacetylation at the time of initial training to create stronger memory.

In this study, first, we observed that there was no NMDAR downregulation in the OB at 3hr following odor preference training when the pups were infused HDAC inhibitor prior to training. In parallel, we also observed that retraining of HDAC infused pups has no effect on the already formed memory.

A well-established fact is that histone acetylation loosens DNA-histone interactions to enhance the transcription of target genes [537]. It is also the case that in various studies histone acetylation leads to stronger memory [572 216 573 574]. Numerous studies have shown that inhibition of HDAC resulted in enhanced histone acetylation specifically at the NMDAR subunit promoters in hippocampal CA1neurons [539], primary neocortical cell cultures [538], frontal cortex [575], and striatum [525]. All these data suggest that histone acetylation results in more NMDAR synthesis and incorporation in the membrane. Beside NMDAR insertion there is another mechanism by which NMDARs either get trafficked from synaptic to extra-synaptic sites or internalized for degradation. The downregulation of NMDAR is mediated by calcineurin via clathrin-dependent endocytosis of NMDARs [514]. This previous report also suggests that H3 and H4 phosphorylation and acetylation are interconnected and responsible for gene regulation [543]

[542]. According to the Chwang et al., 2006 report, contextual fear conditioning leads to an increase in both H3 phosphorylation and acetylation [534]. The phosphorylation of H3 is mediated by the ERK/MAPK pathway [534] and ERK/MAPK mediated phosphorylation can be reversed by calcineurin [576]. However, my data suggest that there is no difference in total H3 expression levels in FK-506 treated OBs compared to vehicle-infused ones. We have noticed a significant increase in H3 phosphorylation at 3 hr following odor preference conditioning. These data suggest that natural associative learning has to activate histones, which is consistent with other models [534].

6.2.6 The Functionality of NMDAR Metaplasticity

Metaplasticity can be manifested in various forms of behavior. Research on stress showed that it induces acute or chronic synaptic changes that can alter learning capacity. Many studies have shown that NMDAR-mediated LTP is impaired in stressed rodents [577] [501]. Sensory deprivation has been shown to change LTP and/or LTD induction in both olfactory [578] and visual [579] [480] cortices. In PTSD and stress research, metaplastic manipulations may be used for therapeutic purposes. Metaplasticity can be used not only in stress research but also in Alzheimer's disease (AD). A recent study has shown that metaplastic activation of ryanodine receptors restores long-term LTP and its associated synaptic properties in an APP/PS1 mouse model of Alzheimer's disease [544].

In my research, I showed that the metaplastic effects have the ability to alter future learning and that this is dependent on the strength of the initial learning. My data

suggests that only weak training down-regulates NMDARs. On the other hand, strong learning prevents NMDAR down-regulation and its associated negative metaplastic effect.

Figure 6.0. Olfactory Circuitry

Here In the schematic representation olfactory nerve layer referred to as ONL (Green lines on top). ORN projects their axons to the Glomerular layer (GL-Red circle) of the olfactory bulb. Then they make synapses with Mitral and Tufted cell (MC/MT) dendrites in the GL. Granule cells (GC-Green cells near MC/MT cells) are present in the granule cell layer (GCL). The MC/MT projects their axons to the piriform cortex (PC) by lateral olfactory tract (LOT). The PC cells make synapses with LOT to receive the olfactory information for further processing.

7. References

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