AN NMDA RECEPTOR-MEDIATED MODEL OF ASSOCIATIVE OLFACTORY LEARNING

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by

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ABSTRACT

These experiments support a critical role of the NMDAR in early odor preference learning. First, learning is associated with a significant increase in phosphorylation of the NMDAR at odor-specific glomerular synapses following training and blocking the NMDAR at these glomerular synapses during training prevents learning. These results establish a causal role of the NMDAR in the *induction* of early odor learning. Second, early odor preference learning induces a transient decrease in synaptic NMDAR GluN1 subunit expression at 3 h post-training and a slower change of synaptic GluN2B subunit expression at 24 h. Transient decreases in synaptic NMDAR expression are thought to be important for *maintaining* the existing memory while reducing the plasticity for competing new memory. Finally, using *ex vivo* electrophysiology, it was shown that learning significantly increases the AMPA/NMDA ratio of mitral cell EPSC components. Together, these experiments support an NMDAR-mediated mitral cell-LTP model of early odor preference learning.

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2-DG	2-deoxyglucose
AC	Adenylyl cyclise
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AMPAR	AMPA receptor
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CREB	Cyclic adenosine monophosphate response element-binding protein
CS	Conditioned stimulus
D-APV	D-amino-5-phosphonopentanoate
EPSC	Excitatory post-synaptic current
EPSP	Excitatory post-synaptic potential
ET	External tufted
GABA	Gamma-aminobutyric acid
GC	Granule cell
HSV	Herpes simplex virus
ISO	Isoproterenol
JG	Juxtaglomerular cell
LC	Locus coeruleus
LOT	Lateral olfactory tract
LTD	Long-term depression
LTP	Long-term potentiation
MC	Mitral cell
mCREB	Mutant CREB
NBQX	2,3-dihydroxy-6-ntiro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NE	Norepinephrine
NMDA	N-methyl-d-aspartate
NMDAR	NMDA receptor
OB	Olfactory bulb
OD	Optical density
ON	Olfactory nerve
OR	Odorant receptor
ORN	Olfactory receptor neuron
pCREB	Phosphorylated CREB
PD	Postnatal day
PG	Periglomerular
pGluN1	Phosphorylated GluN1
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PPR	Paired-pulse ratio

PSD-95	Post-synaptic density-95
ROI	Region of interest
RT-PCR	Reverse transcription polymerase chain reaction
SA	Short axon
SEM	Standard error of measurement
TBS	Theta burst stimulation
TTX	Tetrodotoxin
UCS	Unconditioned stimulus

CHAPTER 1 – INTRODUCTION

1.1 Overview

Neonates of all species are highly vulnerable and dependent on maternal care. In many species, rapid formation of an attachment to the dam or caregiver is essential in ensuring survival. Most animals display a sensitive period of enhanced learning during early life that heightens their ability to form this critical attachment. In the neonate rat, learning associations between maternal care stimuli and maternal odors are critical in inducing approach responses to the dam and ensuring pup survival. Early odor preference learning is a mammalian model of imprinting in the neonate rat that allows investigation of the neurobiology of this attachment (Sullivan, 2001).

Neonate rats will learn to prefer a novel odor following a brief pairing with a stimulus that mimics maternal care, such as stroking (Sullivan and Leon, 1986). At this early age, the eyes and ears remain closed and the neonatal rat pup must rely completely on olfactory and somatosensory stimuli to navigate their environment and ensure survival (Wilson and Sullivan, 1994). As a result, the pairing of tactile and olfactory stimuli in this neonatal olfactory learning model is highly associative, and is an ideal model to study the synaptic mechanisms of associative plasticity.

Specifically, the protocol for this simple associative learning model involves removing pups from the nest on postnatal day (PD) 6 and placing them on clean,

unscented bedding for a 10 min habituation period. Next, pups are transferred to scented bedding where they receive repeated pairings of the novel odor with stroking (administered using a small paint brush) over a 10 min period before being returned to the nest. Odor preference learning is assessed 24 h later using either a Y-maze or a two-odor choice task (see Figure 1). Animals that received stroking paired with odor will spend more time in the arm of the maze scented with the odor, or more time over odor-scented bedding in a two-choice task (Wilson and Sullivan, 1994).

Neonatal rat pups show a sensitive period for acquiring an odor preference before PD 10-12. After this time, pairing of an odor with stroking does not induce odor preference learning (Woo and Leon, 1987). The end of the sensitive period for odor preference learning coincides with maturation of the pups and an increase in their locomotive abilities as they begin to be able to explore outside the nest (Woods and Bolles, 1965; Sullivan et al., 2000).

Perhaps the most important advantage of the neonatal odor preference model is that learning-induced changes in synaptic plasticity are thought to occur predominantly within the olfactory bulb (OB) circuitry (Sullivan et al., 2000). At this early age, many of the higher cortical structures that are involved in olfactory learning and memory in the adult (Staubli et al., 1986) have not yet fully matured (Math and Davrainville, 1980; Wilson, 1984). Therefore, this model provides us with a specific locus of learning-induced synaptic changes to focus our investigations.

However, despite years of research, the underlying circuitry changes and their synaptic location(s) within the OB remain poorly understood. In order to further understand the mechanisms of early odor preference learning there are two fundamental questions that need to be addressed. First, whether a long-term potentiation (LTP)-like phenomenon (considered to be the neural correlate of learning and memory) can account for odor preference learning. Second, which synapse(s) within the OB circuitry are involved in learning. While there has been significant research investigating this learning model and several controversial proposals have been made, the answers to these fundamental questions remain unsolved.

The experiments described in my thesis sought to uncover learning-induced synaptic changes and their location following early odor preference learning. Many associative learning models have been shown to rely critically on the activity of the N-methyl-D-aspartate ionotropic glutamate receptor (NMDAR). The NMDAR is an ideal candidate in associative learning because it can act as a coincidence detector of simultaneous pre- and post-synaptic activity (Erreger et al., 2004), which is achieved by the synchrony of unconditioned stimulus (UCS) and conditioned stimulus (CS) presentation during learning behaviour. As early odor preference learning is a highly associative model, requiring coincident occurrence of the UCS (stroking) and CS (odor), we hypothesized the NMDAR may very well be a critical mediator in this model.

The goal of my project was to investigate a role for the NMDAR in early odor preference learning. Specifically, I sought to determine: 1) Whether the NMDAR was activated at OB synapses during early odor preference learning; 2) Whether activation of

the NMDAR was necessary for odor preference learning to occur; 3) Could early odor preference learning induce significant changes in the expression or subunit composition of the NMDAR; 4) Whether AMPA/NMDA LTP-like changes were evident at the olfactory nerve-mitral cell (ON-MC) synapse following learning.

1.2 OB Circuitry

Odorants are volatile compounds in the air that enter the nose and bind to receptors within the nasal epithelium. Odorants bind to a specific receptor based on its structure (Reed, 1992). As a result, an odorant can bind to several different receptors with varying affinities (Krautwurst et al., 1998). There are approximately 1000 different odorant receptors (ORs) expressed in the rat (Buck and Axel, 1991; Ressler et al., 1993). ORs are expressed on the dendrites of olfactory receptor neurons (ORNs) in the nasal epithelium. Interestingly, a given ORN will express only one type of OR (Young and Trask, 2002). Within the nasal epithelium, ORNs expressing the same OR are randomly dispersed throughout the epithelium (see Figure 2), thus increasing the likelihood of odorant binding when inhaled (Ressler et al., 1993). From the nose, ORNs send their axons through the cribriform plate to the main OB to form the first synapse in olfactory processing. This bundle of ORN axons forms the olfactory nerve (ON). ORNs expressing the same OR converge and terminate together within a few specific glomeruli (Mombaerts et al., 1996). Glomeruli are spherical structures on the surface of the OB that contain the dendritic arbors from several cell types in the OB. Specifically, in situ hybridization studies have shown that all ORNs expressing the same OR terminate in just

one to two glomeruli on both the lateral and medial aspects of the OB (Ressler et al., 1994; Vassar et al., 1994). Gene targeting experiments have shown that ORNs expressing the same OR terminate in glomeruli within the same location from bulb to bulb and from animal to animal, with minimal local variability (Mombaerts et al., 1996). Such experiments have also revealed that a given OR is represented at similar positions in the lateral and medial portions of each OB. These experiments demonstrate the high degree of convergence and specificity within olfactory processing and show that odors are represented at specific topographic locations in the OB so that a given odor will activate a specific pattern of glomerular activity (Jourdan et al., 1980).

1.2.1 Layers of the OB

The OB is a highly organized and well defined structure that consists of several distinct layers. From the most superficial to deep, these layers are the ON layer, glomerular layer, external plexiform layer, mitral cell (MC) layer, internal plexiform layer, granule cell (GC) layer, and the subependymal layer.

1.2.1.1 Glomerular layer

The glomerular layer lies along the surface of the OB, deep to the ON layer and is comprised of spherical neuropil-filled structures called glomeruli. Importantly, ON terminals synapse within glomeruli, making them the first site of synaptic integration within the OB. Each glomerulus has a diameter ranging between 80-160 μm and there are

approximately 2000-3000 glomeruli in each bulb of the rat (Meisami and Safari, 1981). Surrounding a single glomerulus are thousands of small neurons and glial cells.

Within the glomerular layer, glial cells serve several functions. During embryonic development, glia-neuron interactions are critical in synapse formation and in the structured formation of individual glomeruli (Bailey et al., 1999). As development progresses, glial cells remain important in regulating and stabilizing existing as well as newly forming synapses within the glomerular layer. Furthermore, glial cells serve an important role in isolating individual glomeruli and preventing the spread of cellular activity from glomerulus to glomerulus, effectively making each glomerulus an isolated functional unit (Chao et al., 1997).

There are three main types of neurons within the glomerular layer including periglomerular (PG) cells, external tufted (ET) cells and short axon (SA) cells (Pinching and Powell, 1971a). These are collectively referred to as juxtaglomerular (JG) cells of which there are approximately 1500-2000 associated with each glomerulus (O'Connor and Jacob, 2008). Together, these JG cells form a complex glomerular inhibitory network.

PG cells are small spherical cells surrounding the glomeruli. These are the most abundant cells in the glomerular layer and are inhibitory. PG cells can be either GABAergic, dopaminergic, or both (Halasz et al., 1977; Mugnaini et al., 1984a; Mugnaini et al., 1984b; Shipley and Ennis, 1996, review). GABAergic PG cells make up over 20% of all cells within the glomerular layer while dopaminergic PG cells make up one of the largest populations of dopaminergic neurons in the brain (O'Connor and Jacob,

2008). PG cells receive excitatory input from ON terminals, MCs and ET cells (Pinching and Powell, 1971c, b; Hayar et al., 2004a). In return, PG cells form inhibitory synapses onto ON terminals, MCs, and ET cells (Pinching and Powell, 1971b). Both GABAergic and dopaminergic PG cells mediate *intra*glomerular inhibition.

ET cells are excitatory in nature and are the largest cells of the glomerular layer. They are located deeper to the glomerular layer and typically have a single dendrite which extends into a glomerulus to form an extensive dendritic arborisation (Pinching and Powell, 1971b; Hayar et al., 2004a; Hayar et al., 2004b). Electrophysiological studies have shown that ET cells receive monosynaptic ON input and in turn relay this input onto PG and SA cells via excitatory glutamatergic transmission (Hayar et al., 2004a; Liu and Shipley, 2008). In addition, there is evidence that ET cells can also excite MCs directly (Najac et al.; Gire and Schoppa, 2009).

Similarly, SA cells are also fairly large in size but are identified by their multiple dendrites that extend across several glomeruli (Hayar et al., 2004a). They do not receive direct synaptic input from ON terminals but do receive input from other SA and ET cells (Hayar et al., 2004a). Within the glomerular layer, the dendrites of SA cells can extend as far as 20-30 glomeruli away and form synapses with PG cells (Aungst et al., 2003). In contrast to PG cells which mediate *intra*glomerular inhibition, SA cells mediate *inter*glomerular inhibition.

Within glomeruli, odor information is transmitted from ON terminals to the apical dendrites of MCs via glutamate (Berkowicz et al., 1994; Ennis et al., 1998). MCs can

excite other MCs in the same glomerulus in two ways: through the release of glutamate from apical dendrites which can act on the apical dendrites of other MCs within that glomerulus, and/or through electronic coupling via gap junctions with neighbouring apical dendrites (Schoppa and Westbrook, 2001).

In summary, within the glomerular layer alone, excitatory olfactory transmission from ON terminals to MCs is modulated by several influences. This complex glomerular network can significantly modify olfactory processing at the very first site of synaptic integration.

1.2.1.2 External plexiform layer

The external plexiform layer lies deeper to the glomerular layer and has a very low cell density, including external, middle and internal (deep) tufted cells (Macrides and Schneider, 1982). It consists predominantly of dense neuropil that includes dendrites from both the MC layer and the GC layer. It is within the external plexiform layer that inhibitory GCs and excitatory MCs form reciprocal dendrodendritic synapses that play an important modulatory role in olfactory processing (Price and Powell, 1970b, a, c; Mori et al., 1983).

1.2.1.3 MC layer

The MC layer lies deeper to the external plexiform layer and mainly contains the somata of glutamatergic MCs, of which there are over 50,000 per bulb in the rat (Bonthius et al., 1992). MCs are the primary output of the OB. Their axons converge to form the lateral olfactory tract (LOT) which projects to the primary olfactory cortex. Each MC sends a single apical dendrite through the external plexiform layer to innervate a single glomerulus. Within a glomerulus, this apical dendrite branches to form a diffuse dendritic tuft. While each MC sends its apical dendrite into only one glomerulus, a single glomerulus receives innervation from at least 25-50 MCs (Allison, 1953). In addition to the single apical dendrite, each MC extends secondary dendrites laterally into the external plexiform layer to form reciprocal dendrodendritic synapses with GCs. These secondary dendrites can extend as far as 2 mm in the external plexiform layer, which is approximately 25% of the circumference of the OB (Mori et al., 1983; Shipley and Ennis, 1996, review).

1.2.1.4 Internal plexiform layer

The internal plexiform layer is a very thin layer that lies deeper to the MC layer and contains very few cells. The internal plexiform layer consists mainly of dendrites from GCs and centrifugal inputs including serotonergic (McLean and Shipley, 1987b), noradrenergic (McLean et al., 1989) and cholinergic (Nickell and Shipley, 1988) inputs.

1.2.1.5 GC layer

The GC layer is deeper to the internal plexiform layer and contains the largest number of cells in the OB. In fact, there are over 2 million GCs in each bulb of a young rat which increases to over 5 million per bulb in adult rats (Bonthius et al., 1992). GCs are small, axonless cells that are often arranged in 3-5 row-like aggregates within the GC layer. Studies suggest that these aggregates are electronically coupled by gap-junctions allowing for highly synchronized firing of neighbouring cells (Reyher et al., 1991). GCs are inhibitory GABAergic cells (Ribak et al., 1977) that form dendrodendritic synapses with MCs in the external plexiform layer, as described above. Within the GC layer, GCs receive synapses from centrifugal afferents predominantly from primary olfactory cortex, but also from the nucleus of the horizontal limb of the diagonal band (Price and Powell, 1970a, c).

1.2.1.6 Subependymal layer

The subependymal layer is also known as the subventricular zone as it is the area of the OB lining the ventricles. This is a region virtually devoid of cells in the adult rat. However, it is from progenitor cells in this layer that new interneurons arise postnatally, the vast majority of which are GABAergic GCs (~ 95%) with a small proportion being GABAergic PG cells (~ 3-5%) (Hinds, 1968b, a; Altman, 1969; Lledo et al., 2006).

1.2.2 Neuromodulatory inputs to the OB

1.2.2.1 Noradrenergic inputs to the OB

The source of noradrenergic input to the OB arises from the arousal centre of the brain, the locus coeruleus (LC). In fact, approximately 40% of all LC neurons project to the OB in the rat (Shipley et al., 1985), highlighting the importance of olfaction in rodents. The majority of these LC neurons project to the internal plexiform layer and GC layer, with moderate noradrenergic innervation to the external plexiform layer and MC layer, and limited innervation to the glomerular layer (McLean et al., 1989; McLean and Shipley, 1991).

1.2.2.2 Serotonergic inputs to the OB

Serotonergic afferents to the OB arise from the medial and dorsal raphe nuclei (McLean and Shipley, 1987b). All layers of the OB receive serotonergic innervation, with the glomerular layer being the most densely innervated and the external plexiform layer being the least densely innervated (McLean and Shipley, 1987b).

1.2.2.3 Dopaminergic inputs to the OB

There are no known central dopaminergic afferents to the OB (but see McLean and Shipley, 1987b). The source of dopamine within the OB is a subpopulation of

dopaminergic PG cells within the glomerular layer (Halasz et al., 1977). There is evidence that these neurons can co-release both GABA and dopamine(Maher and Westbrook, 2008).

1.2.2.4 Cholinergic afferents to the OB

The dominant source of central cholinergic input to the OB arises from the nucleus of the horizontal limb of the diagonal band (Shipley and Adamek, 1984). Electrophysiological studies investigating the effects of electrical stimulation of the nucleus of the horizontal limb of the diagonal band suggest its primary function in the OB is in modulating the transmission of olfactory information between hemispheres via the anterior commissure (Nickell and Shipley, 1993).

1.2.3 Cortical Projections of the OB

Axons of MCs forming the LOT project to several cortical areas that collectively form the primary olfactory cortex. These structures include: the anterior olfactory nucleus, the anterior hippocampal continuation, olfactory tubercle, taenia tecta, piriform cortex, periamygdaloid cortex, transitional cortex and entorhinal cortex (Shipley and Ennis, 1996, review). In return, many of these structures send afferents back to the OB.

1.2.4 Olfactory Processing

A remarkable feature of olfactory processing compared to other sensory modalities is that olfactory information can reach primary olfactory cortex without being relayed through the thalamus. In fact, olfactory information synapses just once between the nasal epithelium and primary olfactory cortex. This is the ON synapse on primary dendrites of MCs in the OB, whose axons go on to form the LOT and synapse on pyramidal cells in the piriform cortex.

Another important feature of olfactory processing is the high degree of specificity and convergence of information. Each ORN in the nasal epithelium expresses only one type of OR and all ORNs expressing a given OR converge on just a couple glomeruli within a specific topographical location in the OB. In rodents, the convergence ratio of this ORN to glomerular projection is >5000:1 (Shepherd & Greer, 1998). This high degree of convergence has been proposed to serve an amplification role, effectively increasing the signal-to-noise ratio of olfactory transmission (Wilson and Mainen, 2006).

A remarkable characteristic of OB circuitry is the high degree of inhibitory modulation. Within the OB, inhibitory interneurons outnumber excitatory output neurons by 50-100:1 (Shepherd & Greer, 1990). This is the highest relative inhibitory:excitatory ratio in the brain, suggesting an important role of inhibition in olfactory processing. Within the OB there are two separate inhibitory networks that significantly modulate MC activity, and thus the output of the OB. At the first site of synaptic integration in olfactory processing within the glomerular layer, PG cells can provide both feedforward and feedback inhibition of MC activity. Either through direct ON-PG (~30%) or indirect ON-

ET-PG (~70%) activation, PG cells can inhibit MC activity via GABAergic transmission at dendrodendritic synapses (Najac et al.; Murphy et al., 2005; Shao et al., 2009). In this way, PG cells can reduce the ability of MCs to respond to odor input via feed forward inhibition. Alternatively, once MCs become activated in response to ON input, PG cells can exert feedback inhibition onto MCs via GABAergic transmission at dendrodendritic synapses, decreasing MC activity in response to odor input (Murphy et al., 2005). Furthermore, ON axons express $GABA_B$ receptors. During feedback inhibition following ON activation, GABA released from PG cells can act on presynaptic $GABA_B$ receptors and result in presynaptic inhibition of the ON (Aroniadou-Anderjaska et al., 2000). In addition, dopaminergic PG cells can co-release dopamine onto ON terminals (Maher and Westbrook, 2008), acting on D2 receptors to inhibit the pre-synaptic release of glutamate induced by odor stimuli (Hsia et al., 1999; Berkowicz and Trombley, 2000; Ennis et al., 2001). In this way, PG cells can effectively shut down transmission at the ON-MC synapse (see Figure 2).

Within the external plexiform layer, GABAergic GCs mediate feedback and lateral inhibition of MC activity. Once activated, MCs release glutamate onto GCs at reciprocal dendrodendritic synapses in the external plexiform layer. On GC dendrites, glutamate binds to both α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) and NMDA receptors resulting in calcium influx and the release of GABA back onto input MCs as well as neighbouring MCs to provide both feedback and lateral inhibition of MC activity in response to odor input (Isaacson and Strowbridge, 1998; Schoppa et al., 1998; Chen et al., 2000b; Halabisky et al., 2000). Each MC sends several

secondary dendrites, which individually can branch many times, outward in all directions radially through the external plexiform layer (Orona et al., 1984). Meanwhile, the arbour of each GC dendrite can range from 100-200 μm, allowing a single GC to make several synaptic contacts with secondary dendrites of many MCs from many glomeruli (Orona et al., 1983). In this way, GCs can significantly modulate the overall pattern of MC activity in response to a given odor across a very large region of the OB (see Figure 2).

An odorant is comprised of several different chemical molecules that bind to and activate an array of ORs, dependent on their chemical properties such as functional groups, hydrocarbon structure and overall molecular properties (see Johnson and Leon, 2007 for review). As a result, a single odor stimulus activates a distributed pattern of ORs, in turn leading to a distributed pattern of glomerular activation. As individual MCs receive sensory input from a single glomerulus, MC activity maintains this distinct and distributed spatial pattern of activity in response to a given odor. This chemotopographical organization implies that structurally similar odorants will induce similar but distinct patterns of activity within the OB (Johnson and Leon, 2007).

Axons of MCs forming the LOT carry olfactory information on to higher centres of olfactory processing, predominantly terminating on dendrites of pyramidal neurons in the piriform cortex (Haberly, 1983). *In vivo c*alcium imaging and patch clamping experiments have shown that a given odor will activate a small ensemble of pyramidal neurons that are dispersed across the piriform cortex. Importantly, different odors will activate different ensembles of neurons, yet these ensembles show overlapping activity. Individual neurons within an ensemble can respond to several different odors and show

varying levels of response to each odor (Poo and Isaacson, 2009; Stettler and Axel, 2009). Such studies suggest a sparse and distributed encoding of odor representations within the piriform cortex (Isaacson, 2010). Recent work by Davison & Ehlers (2011) used *in vivo* photostimulation to drive patterned activation of glomeruli within the OB. This work has shown that individual pyramidal neurons within piriform cortex respond to specific topographic patterns of OB activity but not to single glomerular activation. The piriform cortex also receives strong associational inputs from other cortical structures such as the amygdala, which can play an important modulatory role in olfactory processing and associative olfactory learning in adults (Haberly, 2001).

Taken together, olfactory processing is quite complex with a multitude of excitatory and inhibitory connections significantly influencing the activity pattern and output of the OB and higher olfactory structures. A general consensus that has emerged after decades of research is that olfactory information is encoded based on both topographical and temporal patterns of activity from the level of the OB to primary olfactory cortex.

1.3 Neonatal odor preference learning model

During early life, pup behaviour is predominantly governed by associations with odor cues (Hofer et al., 1976; Teicher and Blass, 1977). This provides us with an excellent model for the study of associative learning. In fact, a wide range of stimuli can be classically conditioned in the neonate rat pup when paired with a novel odor. For

instance, stroking (Sullivan and Leon, 1986; McLean et al., 1993), warmth (Pedersen et al., 1982), milk (Johanson and Teicher, 1980; Sullivan and Hall, 1988), tail pinch (Sullivan et al., 1986), mild foot shock (Camp and Rudy, 1988), and intracranial brain stimulation of the medial forebrain bundle (Wilson and Sullivan, 1990) have all been used as the UCS in classical conditioning of neonate rats. In response to pairing of any of the above UCS with a novel odor CS, pups will acquire a preference for the odor and will show an approach response to the odor after training during the first 10 days of life.

It may seem strange that rat pups learn a preference for an odor paired with aversive stimuli, such as foot shock or tail pinch, however the acquisition of an approach response to such stimuli is actually essential for their survival. Often the dam may step on her pups, bite them, or handle them roughly. However, no matter how rough the dam is, she is still the only means of food and protection for young pups. The heightened ability of neonatal rat pups to learn an approach response to odors paired with both aversive and appetitive stimuli is important for their survival during this early critical developmental period. It is important to note that this appetitive learning to aversive stimuli is not due to a decreased pain threshold in neonatal rats. In fact, during association of an odor with tail pinch or mild foot shock, pups show outward signs of pain detection. Indeed, the detection threshold for foot shock does not change with age (Haroutunian and Campbell, 1979; Camp and Rudy, 1988). In contrast, neonatal pups can learn aversive associations when odor is paired with harmful stimuli that could threaten their survival such as LiCl injection, which induces severe malaise, or very high intensity foot shock (Haroutunian and Campbell, 1979; Camp and Rudy, 1988). This suggests that during early life, when

pups are completely dependent on the dam for survival, they are predisposed to acquire associations with both rewarding and mildly aversive stimuli.

1.3.1 Neural changes induced by early odor preference learning are observed within the glomerular layer of the OB

1.3.1.1 Enhanced 2-deoxyglucose (2-DG) uptake in odor-specific glomeruli

As previously mentioned, ORNs expressing the same OR all project to only one or two glomeruli in the OB at specific topographical locations and this pattern is consistent across animals (Mombaerts et al., 1996). As a result, a given odor produces a specific spatial pattern of glomerular activity in the OB (Jourdan et al., 1980).

In a report published by Sullivan & Leon (1986), rat pups received either stroking paired with peppermint odor, stroking alone, or peppermint odor alone, each day from PD 1-18. On PD 19, they investigated 2-DG uptake in the OBs of pups from each condition. 2-DG uptake is often used as a measure of metabolic activity, with enhanced 2-DG uptake indicating enhanced cellular metabolism and activity. Sullivan et al. (1986) report that pups who received the paired stroking+odor showed an approach response to the odor in addition to significantly heightened focal 2-DG uptake in peppermint odor-specific glomeruli in the mid-lateral OB. In contrast, pups who received only stroking or

odor exposure did not show odor preference learning or enhanced 2-DG uptake. Importantly, further studies confirmed that 2-DG uptake in this odor-encoding region was not modified by either CS only, UCS only, random pairings, or backward pairings (Sullivan et al., 1989a). Following extinction of the conditioned response (via repeated presentations of the CS odor alone), the enhanced uptake of 2-DG in odor-specific glomeruli was eliminated, as was the conditioned behavioural approach response to the odor (Sullivan and Wilson, 1991). These results suggest that early odor preference learning significantly modulates glomerular activity.

1.3.1.2 Enhanced c-*fos* expression in odor-specific glomeruli

Following early odor preference learning, Johnson and colleagues (1995) reported significantly higher expression of the immediate early gene c-*fos* observed within the glomerular layer of learning animals compared to controls. These focal areas of heightened c-*fos* expression in the mid-lateral bulb aligned with those highlighted in 2-DG studies showing heightened uptake following learning. As c-*fos* expression is considered to be an indicator of cellular activity, these results suggest a significant increase in glomerular activity induced by odor preference learning (Johnson et al., 1995). However, which cell types within the glomerular layer show heightened c-*fos* expression following learning is not known and therefore the implications of these results are unclear. This heightened activity may reflect increased inhibitory neuron activity within learning-associated glomeruli, possibly resulting in either increased PG cell-

mediated intraglomerular inhibition or increased SA cell-mediated interglomerular inhibition, as described previously.

1.3.1.3 Increased size of odor-encoding glomeruli and number of associated JG cells following early odor preference learning

Woo et al. (1987) investigated whether glomerular foci associated with enhanced 2-DG uptake underwent significant morphological modifications following early odor preference learning. Specifically, they used silver and Nissl staining to examine structural changes within odor specific glomeruli that aligned with glomeruli showing enhanced 2-DG uptake in response to the conditioned odor. Animals that had received odor-stroke pairings from PD 1-18 showed significantly larger glomerular structures that protruded into the external plexiform layer in focal areas associated with enhanced 2-DG uptake. Specifically, the glomerular layer of learning animals was approximately 30% wider, while the cross-sectional area of individual glomeruli was over 20% larger in learning animals compared to controls (Woo et al., 1987). In a further study, Woo & Leon (1991) counted the number of JG cells and measured their size within the odor-specific region of the midlateral OB. They found that while there were no differences in cell size between learning and control animals, learning animals showed approximately 20% more JG cells compared to control animals in the odor-specific region (Woo and Leon, 1991).

1.3.1.4 Single unit MC recordings

Following odor preference learning, single unit recordings from the MC layer show an altered response pattern to the conditioned odor (Wilson et al., 1987; Wilson and Leon, 1988). In this study, pups received odor-stroke pairings from PD 1-18. On PD 19 pups were anaesthetized and single-unit responses to the conditioned odor were recorded from the MC layer. In response to the conditioned odor, a higher proportion of sampled MCs showed suppressive responses than excitatory responses in learning animals compared to controls (Wilson and Leon, 1988). However, whether this reflects suppressed activity of odor-encoding MCs or enhanced lateral inhibition to surrounding MCs (via activation of odor-encoding MCs) is not clear. Either way, these results suggest that odor preference learning can significantly modulate the output of the OB.

1.3.2 Neurotransmitters involved in olfactory learning

1.3.2.1 Norepinephrine (NE)

1.3.2.1.1 LC & the sensitive period for learning

Approximately 40% of neurons in the noradrenergic LC project to the rodent OB (Shipley et al., 1985). This suggests a significant role for NE in olfactory processing and learning. In the neonatal odor preference learning model, it was hypothesized that

stroking would activate the LC arousal centre, inducing NE release into the OB. Using microdialysis, the Leon group confirmed that OB NE levels increase dramatically during odor preference learning (Rangel and Leon, 1995). Furthermore, pharmacological activation of the LC during novel odor exposure to induce NE release was shown to be sufficient in inducing odor preference learning (Sullivan et al., 2000).

Odor preference learning in the neonate rat occurs within a critical time period. Pairing of a novel odor with stroking can only induce the acquisition of an odor preference before PD 10-12. It is thought that the closing of this sensitive period for learning coincides with a maturation of the LC (Sullivan, 2001). Indeed, the properties of the neonatal and the adult LC are very different. Early during development the LC is highly sensitive and more responsive to sensory stimuli than in the adult. This is likely related to the fact that the LC shows electronic coupling early in life (Christie et al., 1987). The duration of induced LC firing is much longer in the neonate compared to the adult (Nakamura and Sakaguchi, 1990) and firing of the neonatal LC does not habituate with repeated presentations of sensory stimuli while the adult LC does (Kimura and Nakamura, 1985; Vankov et al., 1995). Furthermore, the neonatal LC is highly sensitive to both noxious as well as non-noxious stimuli during early life but becomes significantly less sensitive to non-noxious stimuli and more sensitive to noxious stimuli over the course of development (Nakamura et al., 1987).

By PD 10-12, the infant LC begins to mature and display characteristics typical of the adult LC. This maturation of LC functioning is mediated by the functional development of inhibitory α2-autoreceptors within the LC (Kimura and Nakamura, 1987).

In the neonate, recurrent collaterals feedback onto the LC potentiating LC firing. In contrast, through the activation of inhibitory α2-autoreceptors, these recurrent collaterals provide feedback inhibition of LC firing in the mature LC (Kimura and Nakamura, 1987; Nakamura and Sakaguchi, 1990). Therefore, maturation of the LC is associated with reduced NE release into the OB in response to sensory stimuli. After the end of the sensitive period and maturation of the LC, odor preference learning can be induced either by direct infusion of a β-adrenergic receptor agonist into the OB or by infusion of an α2-autoreceptor antagonist together with acetylcholine into the LC (Moriceau and Sullivan, 2004a).

1.3.2.1.2 β-adrenoceptor activation in the OB is necessary and sufficient for odor preference learning

Within the OB, noradrenergic activation of the β-subtype of adrenoceptors in particular is critical in inducing odor preference learning. Specifically, intrabulbar infusion or systemic injection of a β-adrenoceptor antagonist can completely prevent odor preference learning-induced by odor and stroke pairing (Sullivan et al., 1989b; Sullivan et al., 1992; Sullivan et al., 2000). In fact, either intrabulbar infusion or systemic injection of a β-adrenoceptor agonist paired with odor is sufficient to induce odor preference learning in neonate rats (Sullivan et al., 2000). Therefore, a subcutaneous β-adrenoceptor agonist injection, for example isoproterenol, can be used to replace stroking in inducing odor preference learning in the neonate rat. Interestingly, there is an inverted U dose response

curve for the efficacy of OB β-adrenoceptor activation in mediating odor preference learning. Meaning there exists optimal, suboptimal, and supra-optimal dosages of a β-adrenoceptor agonist such as isoproterenol in this learning model. Work by Sullivan and colleagues (1991) has shown that a moderate level of stroking or a moderate dose of isoproterenol injection (2 mg/kg) can induce odor preference learning when paired with odor, but neither a 1 mg/kg nor a 4 mg/kg isoproterenol injection can do so. As well, they found that combining both stroking and isoproterenol paired with odor does not induce learning unless suboptimal levels of both stimuli are used, in other words a reduced level of stroking together with a low 1 mg/kg dose of isoproterenol can produce odor preference learning (Sullivan et al., 1991).

1.3.2.2 Serotonin

Serotonergic innervation of the OB originates in the dorsal and median raphe nuclei (McLean and Shipley, 1987b) and is present in the OB within the first 4-5 days of life (McLean and Shipley, 1987a). Within the OB, the densest serotonergic innervation occurs in the glomerular layer, with lesser innervation to the external plexiform layer, GC layer and internal plexiform layer (McLean and Shipley, 1987b). Using an intrabulbar infusion of a neurotoxin, the McLean group (1993) has shown that selective depletion of OB serotonin can completely prevent odor preference learning in pups receiving odor and stroke pairings. However, this effect can be overcome with a stronger UCS. For instance, pairing odor with systemic injection of 4 mg/kg isoproterenol together with stroking can produce odor preference learning in serotonin depleted animals. Alternatively, pairing

odor with 6 mg/kg isoproterenol can induce odor preference learning in serotonin depleted animals (Langdon et al., 1997). This suggests that OB serotonin is not necessary for odor preference learning, but may play a significant role in facilitating learning.

1.3.2.3 GABA

GABAergic PG cells and GCs can significantly influence the activity of MCs in response to odor input through the inhibitory networks previously described. Okutani and colleagues (1999) found that intrabulbar infusion of a $GABA_A$ receptor agonist on PD 11 prevented the acquisition of aversive odor learning in a foot shock paradigm. When a $GABA_A$ receptor antagonist infusion was paired with odor exposure alone, they observed a preference for the odor in pups who had received a low dose of the antagonist and an aversion to the odor in pups that had received a high dose of the antagonist. Interestingly, aversion induced by the high dose of $GABA_A$ antagonist was non-specific, in that these pups showed an aversive response not only to the conditioned odor but to other novel odors (Okutani et al., 1999). From these experiments, they concluded that disinhibition of MCs may play an important role in olfactory learning and that the degree of disinhibition could significantly influence the valence of the conditioned odor (ie., whether an aversion or preference would be formed). In addition, the Okutani group has shown that infusion of a $GABA_B$ receptor agonist during odor and foot shock pairing completely prevents learning. Yet infusion of a $GABA_B$ receptor antagonist paired with odor promotes aversive learning to the conditioned odor as well as other novel odors (Okutani et al., 2003). In summary, the Okutani group has shown that GABAergic transmission in the

OB plays an important role in aversive olfactory learning, however the effects are not straight-forward.

Recent work in our lab has shown that local intrabulbar infusion of a $GABA_A$ receptor antagonist into an odor-specific region of the lateral glomerular layer paired with odor can induce odor preference learning in neonate rats (Lethbridge et al., submitted). This suggests that blockade of the PG-mediated inhibitory glomerular network may play a role in early odor preference learning.

1.3.2.4 Dopamine

The OB does not receive extrinsic dopaminergic input, therefore the sole source of dopaminergic transmission within the OB arises from a population of PG cells in the glomerular layer (Halasz et al., 1977). Using microdialysis, the Leon group showed that OB levels of dopamine increase as high as 400% of baseline measures during odor and stroke pairing in neonate rats (Coopersmith et al., 1991). Weldon et al. (1991) report that systemic injections of a D1 receptor antagonist immediately following odor and stroke pairing completely prevented odor preference learning. In contrast, pre-training injection of the antagonist did not affect the acquisition of an odor preference (Weldon et al., 1991). Therefore, the learning-induced increase in OB dopamine is thought to play a role in the consolidation, not acquisition, of odor preference memory. In the OB, dopaminergic transmission can reduce glutamate release from ON terminals (Hsia et al., 1999; Berkowicz and Trombley, 2000; Ennis et al., 2001). It is possible that following

training, dopaminergic modulation of ON-MC transmission may promote consolidation and synaptic strengthening.

1.3.2.5 Opioids

Either systemic injection or intracranial administration of morphine can induce an odor preference in neonate rats when paired with a novel odor (Kehoe and Blass, 1986b, a). Furthermore, the Sullivan group has shown that systemic injection of a general opioid antagonist before odor preference training can completely block learning. Interestingly, injection of the opioid antagonist after training can also prevent odor preference learning and in fact induces an aversion to the conditioned odor (Roth and Sullivan, 2001). Further studies confirmed that the opioid system is involved in the acquisition, consolidation and expression of odor preference memory in the neonate rat (Roth and Sullivan, 2003).

1.3.2.6 Glutamate

Glutamate plays an essential role in early odor preference learning as it is the transmitter at the ON-MC synapse (Berkowicz et al., 1994). In response to odor, ON terminals release glutamate onto MC dendrites within the glomerular layer, this is the first step in odor processing. Glutamate release from ON terminals is considered to represent the CS within the OB while the UCS is mediated by NE-induced β-adrenoceptor activation. The site of CS-UCS convergence in the OB has been proposed to be the MC (Yuan et al., 2003a).

1.3.3 Learning-induced changes are confined to the OB

OB connections to the anterior olfactory nucleus, piriform cortex, and connections from the entorhinal cortex to the hippocampus do not become functional until approximately PD 14 (Math and Davrainville, 1980). The fact that these pathways are critical for olfactory learning in adult animals (Staubli et al., 1986; Slotnick and Schoonover, 1992), yet are undeveloped at an early age when neonatal rats show robust olfactory associative learning, suggests that these higher structures are not involved in early odor preference learning. In contrast, olfactory projections to the amygdala are present and functional at birth (Schwob and Price, 1984). While bilateral lesions of the amygdala on PD 4 have been shown to prevent odor preference learning, this effect can be overcome with increased training (Sullivan and Wilson, 1993). This suggests that while amygdala connections are present and functional at birth, they play an important *modulatory* role in early odor preference learning, facilitating its acquisition or expression, but are not necessary for learning to occur. In addition, while the connections with higher structures underlying the appetitive drive that induces neonatal rats to approach a learned odor must be functional during the critical period, their identity remains unknown.

Proposed synaptic changes in olfactory processing induced by early odor preference learning are thought to occur primarily within the OB itself based on several facts: 1) higher centres involved in olfactory learning in adults are not yet functional in the immature brain (Staubli et al., 1986; Slotnick and Schoonover, 1992); 2) noradrenergic activity in the OB is sufficient to produce learning (Sullivan et al., 2000);

and 3) all reported changes in neural activity to date have been observed within the OB itself (Sullivan and Leon, 1986; Wilson et al., 1987; Woo et al., 1987; Woo and Leon, 1991; Johnson et al., 1995). This suggests that the neonatal OB is highly plastic and able to undergo significant synaptic plasticity and modulation in response to olfactory learning.

1.3.4 Advantages of the neonatal odor preference learning model

Neonatal odor preference learning provides an excellent model to study the mechanisms of synaptic plasticity in associative learning. First, the learning-induced changes in synaptic plasticity are thought to occur predominantly within the OB, an easily accessible structure that is highly structured and well-defined. Furthermore, a simple pairing of novel odor with either stroking or β-adrenoceptor agonist produces robust learning that is easily quantified. The robust and transient nature of this learning is excellent for the study of the molecular mechanisms underlying the induction, expression, and loss of this memory.

1.4 Underlying circuitry and cellular mechanisms of early odor preference learning

While noradrenergic activation of OB β-adrenoceptors has been shown to be necessary and sufficient for neonatal odor preference learning to occur (Sullivan et al., 2000; Harley et al., 2006), how this activation mediates learning is not yet fully

understood. While considerable research has been focused on determining the underlying mechanisms of this learning model, where specifically β-adrenoceptor activation occurs in the OB and which synapses are modified in response to odor conditioning remain debated. Currently there are two models of how β-adrenoceptor activation mediates synaptic plasticity in the OB, each model proposing different circuitry changes in the OB with learning.

1.4.1 GC-MC disinhibition model

LC noradrenergic afferents into the OB terminate densely in the GC layer (McLean et al., 1989). For this reason, it has been speculated that NE release during early odor preference learning exerts its primary effect on GC activity. Recall that GCs form reciprocal dendrodendritic synapses with MC lateral dendrites. When activated, MCs release glutamate onto GCs, which in turn exert GABAergic feedback inhibition onto the MC as well as lateral inhibition onto neighbouring MCs (Isaacson and Strowbridge, 1998). Wilson & Sullivan (1994) proposed that the UCS-triggered NE release activates β-adrenoceptors on GCs during CS-UCS pairing to release MCs from granule-to-mitral cell dendrodendritic inhibition. They propose that this NE-induced disinhibition of MCs, together with odor-induced excitation, prevents habituation of odor-responsive MCs during training and results in potentiation at the GC-MC synapse. As a result, during a later presentation of the conditioned odor alone, for example during testing, learning is expressed as *suppressed* activity of odor-encoding MCs due to *heightened* feedback inhibition mediated by GCs. This model proposes learning-induced synaptic changes that

strengthen the reciprocal GC-MC synapse. Support for this model comes from single unit recording studies of MCs showing a higher proportion of suppressive than excitatory responses to the conditioned odor following training (Sullivan et al., 1989b). However, conflicting evidence from *in vitro* electrophysiological studies suggest that β-adrenoceptor activation exerts little influence on GC activity, while α-adrenoceptor activation is implicated in mediating the majority of NE-induced changes in GC activity (Trombley, 1992; Trombley and Shepherd, 1992; Hayar et al., 2001).

1.4.2 Enhanced MC excitation model

While disinhibition of MCs from the inhibitory GC network may play a role in olfactory processing and learning, there exists considerable evidence to suggest a learning-induced potentiation at the *glomerular* level of olfactory processing. In particular, glomeruli in odor-specific foci show heightened 2-DG uptake (Sullivan and Leon, 1986), larger glomerular size (Woo et al., 1987), increased cell number (Woo and Leon, 1991), and enhanced c-*fos* expression (Johnson et al., 1995) after conditioning. As well, CS-UCS pairing has been shown to significantly increase the glomerular intrinsic optical signals and ON-evoked field excitatory post-synaptic potentials (EPSPs) (Yuan et al., 2000; Yuan et al., 2002). Together, these data suggest significant potentiation of synaptic strength at the level of glutamatergic transmission from ON terminals to MCs within the glomerular layer.

A model proposed by Yuan et al. (2003a) suggests that neonatal odor preference learning induces activation of an intracellular signalling cascade at the level of MCs that leads to heightened excitation of odor-encoding MCs and synaptic strengthening of the ON-MC synapse such that MC excitation in response to the learned odor is significantly *enhanced*. In support of this hypothesis, the McLean group has demonstrated the expression of β-adrenoceptors on the MC membrane, suggesting that the UCS (β-adrenoceptor activation) occurs directly on MCs (Yuan et al., 2003a). In addition, neonatal odor preference learning has been shown to significantly increase MC levels of the second messenger cyclic adenosine monophosphate (cAMP) and the transcription factor phosphorylated cAMP response element-binding protein (pCREB) (McLean et al., 1999; Yuan et al., 2000; Yuan et al., 2003b). Thus this associative learning model appears to be critically mediated by a post-synaptic cAMP-cAMP dependent protein kinase A (PKA)-CREB signalling cascade similar to that shown to be involved in associative learning presynatically in *Aplysia*, *Drosophila* and honeybee (Davis et al., 1995; Hildebrandt and Muller, 1995; Connolly et al., 1996; Kandel, 2001). The activation of presynaptic cAMP cascades promotes calcium-mediated neurotransmitter release and supports a presynaptic expression mechanism. Postsynaptically in MCs, , it is proposed that β-adrenoceptor activation, via LC-NE release, converges on MCs with odor-induced glutamate release to activate an evolutionally conserved intracellular signalling cascade, resulting in immediate early gene transcription and long-term memory formation.

1.4.2.1 An intracellular signalling cascade in MCs underlies early odor preference learning

The first evidence to suggest that a cAMP-PKA-CREB signalling cascade similar to that described in *Aplysia* was involved in neonatal rat odor preference learning was reported by McLean et al. (1999). In this study, they showed that stroking paired with odor significantly increased OB levels of the transcription factor phosphorylated cAMP response element binding protein (pCREB) while odor only or stroking only did not. Using immunocytochemistry they localized this enhancement of pCREB in learning animals to the dorsal-lateral MC layer, a foci of peppermint odor-encoding (Johnson and Leon, 1996). They later confirmed that an isoproterenol (2 mg/kg) presentation of the UCS to induce learning similarly induced an increase in pCREB expression, while saline or 6mg/kg isoproterenol paired with odor did not (Yuan et al., 2000). These studies demonstrated that odor preference learning, whether induced via stroking or direct activation of β-adrenoceptors, is mediated by enhanced phosphorylation of CREB within the output neuron of the OB, the MC.

Yuan and colleagues (2003a) went on to confirm that the β1-adrenoceptor is indeed expressed within the MC membrane, providing substantial support for a direct effect of NE on MC activity during learning. Using a cAMP assay they demonstrated that following either stroking, 2 mg/kg or 4 mg/kg isoproterenol paired with odor, cAMP levels within the OB are significantly elevated immediately after the end of training (Yuan et al., 2003a). Interestingly, either stroking alone, stroking paired with odor, isoproterenol (2 mg/kg, 4 mg/kg) alone or isoproterenol paired with odor were all similar

in inducing elevations in cAMP expression. This suggests that the UCS alone initiates an increase in cAMP levels that is not further altered by pairing with the CS, at least when examined immediately after training (Yuan et al., 2003a). Using immunocytochemistry, they localized significant cAMP expression predominantly to MCs in the learning versus non-learning conditions, which is in line with previous work showing a learning-induced increase in MC pCREB expression. From this study, a role for cAMP and CREB signalling within MCs during early odor preference learning was evident.

To evaluate a causal role of CREB signalling in this learning paradigm, Yuan and colleagues (2003b) employed the use of Herpes simplex virus wild-type CREB (HSV-CREB) and expression of mutant CREB (HSV-mCREB) within the OB and determined the effects on learning. Infusion of HSV-mCREB was found to significantly impair learning in pups that received stroking paired with odor or the learning dose (2mg/kg) of isoproterenol paired with odor. However, HSV-mCREB infused pups that received a supra-optimal dose of isoproterenol (4mg/kg) paired with odor showed odor preference learning. This suggests that a higher level of β-adrenoceptor activation could activate CREB enough to produce sufficient pCREB for learning. In contrast, overexpression of CREB via HSV-CREB infusion prevented learning in pups that received stroking, 2mg/kg or 4 mg/kg isoproterenol paired with odor. Interestingly, pups that received HSV-CREB infusion and 1mg/kg isoproterenol showed odor preference learning. As CREB phosphorylation is the first step in activation of CREB and down-stream gene expression, the investigators performed pCREB assays to quantify changes in pCREB levels induced in these conditions. In animals that received HSV-CREB and 1 mg/kg isoproterenol or

HSV-mCREB and 4mg/kg isoproterenol, the level of pCREB expression in the OB was higher than that of control (naive) animals (Yuan et al., 2003b). These results suggest that there is an optimal range in which CREB expression can facilitate learning and long-lasting synaptic changes and this is dependent on its phosphorylation.

Further work in the McLean lab went on to characterize learning-induced changes in cAMP within the OB during and immediately after odor preference training. In 2007, Cui et al. reported a learning-induced oscillation in cAMP levels that was not evident in control animals. Specifically, 2 mg/kg isoproterenol pairing with odor produced an oscillatory pattern of cAMP (determined by a cAMP assay) consisting of a peak followed by a trough in cAMP expression at 5 min intervals beginning at the start of odor exposure through to 10 min after the end of training. In contrast, animals that had received saline paired with odor failed to show an oscillatory pattern of cAMP expression, while animals that had received 6 mg/kg isoproterenol paired with odor showed a linear increase in cAMP levels that continued to rise from the beginning of training through to 10 min after the end of training. Pairing of the CS-UCS was shown to be necessary to induce this oscillation in cAMP levels, as presentation of either the CS or UCS alone did not significantly affect cAMP expression. The peak in cAMP expression at the end of training followed by a decrease 5 min later was confirmed in natural learning with odor-stroke pairings. Specifically, Cui et al. (2007) proposed that the temporal pattern of peak cAMP expression at the end of training was critical in inducing intracellular plasticity changes. Using immunocytochemistry, learning-induced changes in cAMP expression were localized to the MCL, further supporting a MC-cAMP mediated intracellular signalling

cascade in this learning model (Cui et al., 2007). More recent work in the McLean lab has gone on to confirm the involvement of downstream regulatory enzymes, providing further support for a role of cAMP-PKA-CREB signalling in early odor preference learning (McLean et al., 2005; Christie-Fougere et al., 2009; McLean et al., 2009).

Work in *Aplysia* has shown that activation of G-protein coupled receptors results in the activation of the adenylyl cyclase (AC) enzyme which is required for the production of cAMP from adenosine triphosphate (ATP) (Kandel, 2001). In turn, cAMP can recruit PKA which goes on to phosphorylate several downstream targets including synaptic receptors and transcription factors. Work by Yovell and colleagues (1992) has shown that the temporal sequence of pairing of the UCS and CS significantly affects the rate of cAMP production, while the peak of cAMP to either alone did not differ. These results are in line with those of Cui et al. (2007) suggesting that pairing of the CS and UCS can dramatically alter the rate of cAMP production resulting in a critically timed peak in cAMP that is essential for the recruitment of downstream signalling substrates involved in learning.

In summary, these studies have significantly contributed to our understanding of neonatal odor preference learning and have provided further evidence for the conservation of a cAMP-PKA-CREB cascade originally described in the *Aplysia* to be involved in mammalian learning and memory. Specifically, in neonatal odor preference learning, it is proposed that odor-induced activation of glutamate receptors (CS) and resultant calcium influx together with NE-mediated activation of β-adrenoreceptors (UCS) would result in activation of AC to increase cAMP production and recruit PKA

leading to phosphorylation of several downstream targets including synaptic receptors and transcription factors such as CREB (Cui et al., 2007).

1.4.2.2 Early odor preference learning is associated with increased AMPAR expression at glomerular synapses

AMPAR insertion into the synapse is a widely accepted mechanism of learning and plasticity that has been demonstrated across a variety of paradigms (Malinow and Malenka, 2002). PKA-mediated phosphorylation of the AMPAR has been proposed to target the receptor to extrasynaptic sites from where it can be laterally trafficked into active synapses during LTP (Oh et al., 2006). Hence a two-stage hypothesis of AMPAR insertion into the synaptic membrane during LTP has been proposed that critically involves PKA activity. This recently led Cui et al. (2011) to hypothesize that a similar trafficking of the AMPAR to the synapse may be involved in the robust cAMP-PKA-CREB-dependent model of neonatal odor preference learning. They specifically tested whether early odor preference learning involved PKA-mediated phosphorylation of the AMPAR and subsequent insertion at OB synapses. Using Western blotting they showed the AMPAR to undergo significant phosphorylation at the PKA site (ser-845) from the end of training up to 1 h afterwards in animals receiving isoproterenol (2 mg/kg) paired with odor, but not naive controls (Cui et al., 2011). A peak in AMPAR phosphorylation was observed at 10 min post-training, which is consistent with the previously reported

peak in PKA-mediated phosphorylation of CREB (McLean et al., 1999; Yuan et al., 2000).

To investigate synaptic changes in AMPAR expression following learning, Cui et al. (2011) performed Western blotting of OB synaptoneurosomes, a tissue extraction enhanced for synaptic proteins (Hollingsworth et al., 1985; Quinlan et al., 1999a). At 24 h after conditioning, learning (2 mg/kg isoproterenol + odor) animals showed significantly higher expression of the synaptic GluA1 subunit of the AMPAR compared to naive or saline + odor controls. Immunohistochemistry revealed higher expression of the GluA1 subunit of the AMPAR within the glomerular layer of learning animals at both 3 and 24 h following training, but not at 48 h. The fact that AMPAR insertion had occurred at 3 and 24 h following training suggests that enhanced AMPAR function may mediate the odor preference memory observed at these time points. Moreover, the fact that this enhanced expression was observed in the glomerular layer suggests it may occur within MC apical dendrites. Cui et al. (2011) directly tested whether AMPAR insertion was necessary for learning using an intrabulbar infusion of an interference peptide (Yu et al., 2008). They showed that prevention of synaptic AMPAR insertion completely prevented learning in these animals, suggesting that AMPAR insertion is indeed necessary for early odor preference learning.

Over the past decade, there has been significant evidence suggesting early odor preference learning to be a cAMP-PKA-CREB signalling model, with significant learning-induced changes in the expression of involved signalling molecules observed within MCs. Furthermore, the fact that PKA-mediated phosphorylation and trafficking of

AMPARs occurs at glomerular synapses further supports a MC localization of learning-induced changes. These studies, together with 2-DG and optical imaging studies demonstrating dramatic changes at the glomerular layer with learning, and are consistent with a learning-induced potentiation of the ON-MC synapse in neonatal associative odor preference learning.

1.4.2.3 An *in vitro* model of early odor preference learning

Recently, Yuan (2009) has proposed an *in vitro* model mimicking the *in vivo* parameters of early odor preference learning. Specifically, in the OB slice, sniffing of an odor is mimicked by theta burst stimulation (TBS) of the ON. As theta (4-12 Hz) is the frequency associated with sniffing in the live animal (Kepecs et al., 2006), TBS of the ON is considered to be a realistic representation of the *in vivo* response to odor presentation. *In vitro,* UCS presentation is represented by application of the β-adrenoceptor agonist isoproterenol. Together, TBS paired with isoproterenol is thought to mimic learning in the live animal and is expected to induce learning-associated LTP-like changes in OB circuitry (Yuan, 2009).

Using calcium imaging, Yuan (2009) showed that pairing of TBS of the ON with bath application of isoproterenol significantly potentiated MC calcium responses to ON stimulation when tested 30 min post-induction. Importantly, neither TBS nor isoproterenol application alone were sufficient to significantly alter MC calcium responses. These results correlate very well with the *in vivo* learning model as both β-

adrenoceptor activation and odor exposure are required to induce odor preference learning. Importantly, these results provide direct support for an enhanced MC excitation model of early odor preference learning.

In another set of experiments Yuan (2009) investigated TBS and isoproterenol effects on JG cell activity. TBS of the ON was shown to significantly increase the excitatory post-synaptic current (EPSC) and decrease the paired-pulse ratio (PPR) recorded in whole-cell mode from JG cells. An increase in JG cell EPSCs would be expected to result in increased inhibition onto MCs, thereby reducing their activity. However calcium imaging revealed significant enhancement of MC calcium responses following TBS pairing with isoproterenol. When the effects of isoproterenol on JG cell activity were examined it was observed that isoproterenol significantly reduced both JG cell EPSCs and calcium signals in response to ON stimulation (Yuan, 2009). Based on these results, Yuan (2009) hypothesized that isoproterenol can influence MC excitability through its activation of β-adrenoceptors on inhibitory glomerular interneurons, releasing MCs from the glomerular inhibitory network. Indeed, work by Hayar and colleagues (2001) has shown that while β-adrenoceptor activation induces an inward current in MCs, this current is dependent on synaptic transmission as it is completely abolished by synaptic transmission blockers, suggesting β-adrenoceptor activation to have no *direct* effect on MC excitability. Alternately, it has been shown that a subpopulation of PG cells is dopaminergic (Halasz et al., 1977). These cells co-release dopamine and GABA (Maher and Westbrook, 2008). Isoproterenol suppression of PG cells could reduce GABA and/or dopamine release during ON stimulation, thereby reducing $GABA_B$ or D2

receptor-mediated presynaptic inhibition (Hsia et al., 1999; Aroniadou-Anderjaska et al., 2000) and enhancing presynaptic glutamate release from ON terminals. While NE has been proposed to disinhibit MCs via its action on inhibitory GCs (Jahr and Nicoll, 1982; Trombley, 1992; Trombley and Shepherd, 1992), this effect has been attributed primarily to the α-adrenoceptor, not the β-adrenoceptor (Trombley, 1992; Trombley and Shepherd, 1992). As β-adrenoceptor activation has been shown to be necessary and sufficient (Sullivan et al., 2000) for early odor preference learning, and does not mediate a direct effect on MC excitability nor an indirect effect via the GC-MC synapse, it has been proposed that β-adrenoceptor activation may occur on inhibitory JG cells in the glomerular layer (Yuan, 2009). As β-adrenoceptors are indeed expressed in JG cells (Woo and Leon, 1995; Yuan et al., 2003a) and isoproterenol application significantly reduces their ON-evoked EPSC and calcium signals (Yuan, 2009), it seems likely that β-adrenoceptor mediated disinhibition of MCs via actions on JGs may be involved in changes in synaptic plasticity with neonatal odor preference learning. Specifically, during odor preference learning β-adrenoceptor activation on JG cells may reduce their activity, thus reducing GABAergic input to MCs, releasing them from the inhibitory network and allowing significant excitation to occur when β-adrenoceptor activation occurs coincidentally with ON odor input.

Recent work in our lab investigating this hypothesis in the *in vitro* learning model has shown that pairing of isoproterenol with TBS of the ON significantly increases MC spiking in response to ON stimulation at 30 min following induction, as determined by loose-patch recordings of MCs (Lethbridge et al., submitted). This suggests that β-

adrenoceptor activation and sniffing-related stimulation of the ON significantly increase MC firing, and thus OB output. In this same study, activation of the glomerular inhibitory network via local glomerular puff application of a $GABA_A$ receptor agonist completely blocked the TBS+isoproterenol-induced increase in MC evoked spiking. Meanwhile, local glomerular disinhibition via local puff application of a $GABA_A$ receptor antagonist was sufficient to potentiate MC spiking when paired with TBS of the ON. Together, these experiments suggest that in an *in vitro* model of learning, β-adrenoceptor activation can disinhibit MCs from the inhibitory glomerular network, allowing significant potentiation of MC activity in response to odor-sniffing-related stimulation of the ON.

ON-evoked potentials in MCs have been shown to consist of two main components, a fast AMPAR-mediated component followed by a slower and long-lasting NMDAR-mediated component. The NMDAR is widely accepted as a critical mediator of synaptic plasticity. Specifically, the long-lasting depolarization and calcium influx mediated by NMDAR activation is proposed to be essential in inducing plasticity. In fact many models of LTP, primarily in the hippocampus (Bliss and Collingridge, 1993; Malenka and Nicoll, 1993) and amygdala (Maren, 1999; Blair et al., 2001), have been shown to be dependent on the activation of NMDARs. Recent experiments in our lab investigated whether the NMDAR could play an important role in mediating the isoproterenol and TBS pairing induced potentiation of MC activity observed in our *in vitro* model of neonatal olfactory learning (Lethbridge et al., submitted). We found that when an NMDAR antagonist, D-amino-5-phosphonopentanoate (D-APV), was added to the bath during isoproterenol+TBS induction, it completely prevented the increase in MC

spiking previously observed at 30 min (Lethbridge et al., submitted). In line with this, when D-APV was added to the bath during glomerular disinhibition (via local puff application of $GABA_A$ antagonist to the glomerular layer, as described above), the previously observed potentiation of MC spiking was not produced. Together, these results suggest an important role for the NMDAR in β-adrenoceptor mediated plasticity within the OB circuitry and further suggest a role for the NMDAR in early odor preference learning.

1.5 The NMDAR

1.5.1 Structure and function

The NMDAR is an ionotropic glutamate receptor that consists of a heteromeric complex of four subunits (Cull-Candy et al., 2001). There are three families of NMDAR subunits: GluN1, GluN2 (A, B, C and D) as well as GluN3 (A and B). The functional NMDAR is thought to be a tetramer comprised of two obligatory GluN1 subunits in combination usually with two GluN2 subunits, typically existing as a dimer of GluN1 and GluN2A or GluN2B dimers (McBain and Mayer, 1994). GluN3 subunits cannot form functional synapses alone, but can combine with a GluN2 subunit to form a functional receptor (Cull-Candy et al., 2001).

Each subunit of the receptor contains an intracellular C-terminus domain of variable length, three transmembrane domains, a pore-forming domain, and a long

extracellular N-terminus domain. The intracellular C-terminus domain of the subunit interacts with scaffolding proteins and is subject to intracellular modulation, such as phosphorylation. The co-agonist glycine binds to the extracellular N-terminus domain of the GluN1 subunit while the extracellular N-terminus domain of the GluN2 subunit contains the binding site for glutamate as well as binding sites for allosteric modulators (Mayer, 2005). Therefore, functional NMDARs require both a GluN1 and GluN2 subunit (Erreger et al., 2004).

The NMDAR is permeable to sodium, potassium, and most importantly calcium. It is widely accepted that intracellular calcium is essential in inducing LTP and plasticity (Lynch et al., 1983). The NMDAR is present within the post-synaptic density and is anchored to the synapse via a large macromolecular complex including various proteins, kinases and phosphatases that are involved in an array of intracellular signalling cascades (Husi et al., 2000). In this way, the NMDAR is easily poised to induce fast and significant effects on intracellular signalling and activity.

The subunit composition of the NMDAR confers significant variability in the cellular expression pattern, as well as the physiological and pharmacological properties of the receptor (Cull-Candy and Leszkiewicz, 2004). As GluN2A and GluN2B subunits predominate in functional NMDARs in the forebrain and play well-characterized roles in development and plasticity, a detailed description of their properties is important here.

GluN2A containing NMDARs exhibit a high open probability, in addition to fast deactivation, decay, and rise times (Erreger et al., 2005). In contrast, GluN2B containing

NMDARs display a low open probability and slow deactivation, decay, and rise times (Erreger et al., 2005). In other words, in response to glutamate binding and membrane depolarization, GluN2A containing receptors tend to open and close faster than GluN2B containing receptors. These properties translate into a larger EPSC, greater calcium influx and higher charge transfer associated with GluN2B containing NMDARs compared to GluN2A containing receptors (Erreger et al., 2005; Sobczyk et al., 2005). As a result, synaptic GluN2B containing NMDARs are poised to exert a more significant influence on post-synaptic cellular activation and synaptic plasticity.

1.5.2 Activation requirements

The NMDAR is unique in that it has two distinct activation requirements that must be met to activate the receptor and allow ion flux through the channel. At the resting membrane potential the ion pore of the channel is blocked by a magnesium ion. To remove the magnesium block and open the channel, the NMDAR requires simultaneous glutamate binding to the receptor in addition to fairly strong membrane depolarization (Mayer et al., 1984; Nowak et al., 1984). Once activated, the NMDAR mediates a long-lasting depolarization and allows significant influx of calcium into the post-synaptic cell.

1.5.3 Role of the NMDAR in plasticity

Several characteristics of the NMDAR described above allude to its importance in learning and plasticity processes including its: 1) slow kinetics and resultant long-lasting

depolarization; 2) high permeability to calcium, a critical mediator of LTP; 3) post-synaptic localization and structural association with key modulators of intracellular signalling pathways; and 4) dual activation requirements of coincident pre- and post-synaptic activity. Many forms of LTP and long-term depression (LTD) in the brain have been shown to be dependent on NMDAR activation. In fact, NMDAR-dependent LTP is widely accepted to be the dominant model of plasticity in the brain (Yashiro and Philpot, 2008). Whether a given stimulus will induce LTP or LTD has been shown to critically depend on the amount of calcium influx that occurs. It is thought that moderate activation of NMDARs may allow only moderate influx of calcium and result in LTD, whereas robust depolarization and activation of NMDARs allows significant calcium influx and triggers cellular events leading to LTP (Bear and Malenka, 1994; Malenka and Bear, 2004).

In support of this idea, blockade of NMDAR activity has been shown to completely prevent the induction of LTP in CA1 and DG of the hippocampus, the most studied model of synaptic plasticity (Collingridge et al., 1983; Bliss and Collingridge, 1993; MacDonald et al., 2006). Correspondingly, intraventricular infusion of an NMDAR antagonist selectively prevents spatial learning in the Morris Water Maze (Morris et al., 1986; Morris, 1989). This NMDAR dependence of hippocampal plasticity and learning has been translated to several models of learning. Interestingly, NMDAR blockade does not affect memory retrieval in tasks of spatial learning (Riedel et al., 1999). This is in line with a wealth of evidence highlighting an essential role of the NMDAR in the *induction* of learning and LTP. Instead, normal information processing as well as memory retrieval

and maintenance is thought to be mediated by the activity of AMPARs, another ionotropic glutamate receptor (Malenka and Bear, 2004).

1.5.3.1 Developmental pattern of NMDAR expression and synaptic contribution

The importance of NMDAR expression in learning and plasticity is evident in its pattern of expression during critical developmental periods across several cortical areas. Overall, NMDAR expression at birth is very high and NMDAR-only "silent" synapses are abundant (Durand et al., 1996; Isaac et al., 1997; Rumpel et al., 1998; Franks and Isaacson, 2005). However, over the course of sensory experience and development, there appears to be a gradual loss of these NMDAR-only synapses and a reduction in the synaptic contribution of NMDARs (Tsumoto et al., 1987; Carmignoto and Vicini, 1992; Durand et al., 1996; Wu et al., 1996). The maintenance of synaptic transmission with a reduced NMDAR presence is mediated by an insertion of AMPARs. A widely accepted model of LTP and plasticity is that with learning or LTP induction, there is an NMDAR-dependent insertion of AMPARs into previously NMDAR-only synapses. Specifically, LTP is thought to reflect NMDAR-dependent exocytosis of AMPARs into the synaptic membrane while LTD is thought to reflect NMDAR-dependent endocytosis of synaptic AMPARs out of the synaptic membrane (Heynen et al., 2000; Malinow and Malenka, 2002). At an early age, when many cortical structures remain quite immature, NMDAR expression is high. At this time, synaptic plasticity is critical for the formation of

functional synapses and for learning critical associations early after birth. Therefore, the NMDAR is proposed to be critical for synaptogenesis, LTP and LTD (Collingridge et al., 2004; Lau and Zukin, 2007).

An important functional consequence of this developmentally- or plasticity-induced reduction in synaptic NMDAR contribution is a significant increase in the induction threshold for LTP. Specifically, this shift from NMDAR-only synapses to AMPAR-dominated synapses induces a shift in the modification threshold for LTD/LTP, meaning that after such changes the induction of LTD may be more likely in response to a given stimulus than LTP (Kirkwood et al., 1996).

1.5.3.2 NMDAR functioning in visual cortex during development and plasticity

The visual cortex has been extensively studied as a model of experience-induced synaptic plasticity. After birth, bilateral input from both eyes is required to form normal binocular connections within the visual cortex. Importantly, this must occur within a critical postnatal developmental period which can be delayed by rearing in complete darkness. This has provided researchers an excellent model for the study of experience-induced plasticity as sensory experience and deprivation can be easily modulated in a neonatal visual model. Kirkwood and colleagues (1995) demonstrated that LTP plays a critical role in experience-dependent maturation of the visual cortex by investigating the susceptibility to LTP induction in visual cortices of animals at different developmental

ages. They observed that LTP was easily induced in visual cortices of younger animals compared to older animals, and that this critical period of LTP susceptibility could be delayed by dark rearing (Kirkwood et al., 1995). This supports the idea that LTP is an important mechanism in experience-dependent synaptic modifications during development. Furthermore, they went on to show that experience in the visual cortex directly shifts the modification threshold for LTD versus LTP induction. Specifically, they showed that the same induction protocol that could elicit LTP in dark-reared cortices produced little potentiation in control cortices from animals of the same age. In contrast, LTD was reliably induced in control cortices by low frequency stimulation but the same low frequency stimulation produced much lower magnitude LTD in cortical slices from dark-reared animals (Kirkwood et al., 1996). Together, these results suggest that visual experience during a postnatal critical period significantly shifts the modification threshold for LTP-LTD induction such that LTP is more difficult to induce and LTD is easier to induce following experience.

As the induction of LTD versus LTP depends on the amount of calcium influx into the post-synaptic cell through NMDARs (Bear and Malenka, 1994), these results suggest a significant developmental change in NMDAR expression and/or functioning. Indeed, Rumpel et al. (1998) reported the existence of functionally silent NMDAR-only synapses in the immature visual cortex that were converted to functional AMPAR-expressing synapses by pairing of pre-synaptic and post-synaptic activity. Importantly, they showed that the number of silent NMDAR-only synapses within visual cortex decreases dramatically with age from PD 3 to PD 11, decreasing by almost 80% over this

short time window (Rumpel et al., 1998). They also showed that pairing of pre- and post-synaptic activity significantly increased the number and amplitude of AMPAR-mediated post-synaptic currents in pyramidal cells and this was dependent on activation of NMDARs (Rumpel et al., 1998). Together, these results show an essential role of the NMDAR in neonatal synaptogenesis and maturation in the visual cortex. Importantly, this conversion of NMDAR-only silent synapses into functional AMPAR-containing synapses over the course of a critical developmental period has been replicated in many areas of the brain, including the hippocampus (Durand et al., 1996), somatosensory cortex (Isaac et al., 1997), and olfactory cortex (Franks and Isaacson, 2005).

1.5.3.3 Developmentally- and plasticity-induced modulation of NMDAR subunit composition

The subunit composition of NMDARs appears to be highly plastic and to undergo significant developmentally- and plasticity-induced changes. Specifically, as the vast majority of NMDARs in the forebrain are composed of either GluN2A or GluN2B subunits, focus has been on their role in NMDAR-mediated plasticity (Yashiro and Philpot, 2008). These subunits show a dramatic change in expression over the course of development in many areas of the brain, including the hippocampus (Liu et al., 2004a), visual cortex (Chen et al., 2000a), somatosensory cortex (Mierau et al., 2004), thalamus (Liu et al., 2004b), brainstem (Hestrin, 1992), and olfactory cortex (Quinlan et al., 2004). As previously described, the GluN2B subunit confers more plastic properties on the

NMDAR compared to the GluN2A subunit, primarily due to differences in kinetics and intracellular interactions (Yashiro and Philpot, 2008). At birth, GluN2B expression is abundant in most areas of the neonatal brain while GluN2A expression is very low or non-existent. However, as development progresses there is a rapid increase in, and inclusion of, GluN2A-containing NMDARs at central synapses (Wenzel et al., 1997).

Investigation of the subunit composition of the NMDAR within the visual cortex over a critical developmental period has revealed significant experience-dependent changes in GluN2A and GluN2B expression. Quinlan and colleagues (1999) reported that animals that were light-deprived over the first 6 weeks of life showed significantly lower expression of the GluN2A subunit in synaptic preparations of the visual cortex compared to control animals raised under a normal 12h light:dark cycle. As age progressed from 1 to 6 weeks of life, this difference in GluN2A expression between groups increased. Meanwhile, expression of the obligatory GluN1 subunit and the GluN2B subunit did not differ significantly over the 6 week period between dark-reared and control animals. When the developmental ratio of GluN2A/GluN2B synaptic expression was compared to the developmental change in NMDAR-mediated currents in visual cortical neurons, there was an obvious symmetry, suggesting that changes in the subunit of the NMDAR could account, at least partially, for the decrease in NMDAR-mediated currents observed over the course of development (Carmignoto and Vicini, 1992; Quinlan et al., 1999a). Carmignoto and Vicini (1992) reported a progressive shortening of NMDAR-mediated EPSCs in developing rat visual cortex that could be significantly attenuated by dark-rearing. In line with these results, Quinlan et al. (1999) report a progressive increase in

GluN2A expression in developing rat visual cortex that could be attenuated by dark-rearing. A similar switch in the dominant NMDAR subunit at synapses over development has been reported for cortical (Sheng et al., 1994), thalamic (Liu et al., 2004b) and piriform synapses (Quinlan et al., 2004). Importantly, this switch in GluN2B to GluN2A predominance is thought to reflect primarily a dramatic increase in GluN2A translation and expression at the synapse, more so than a significant change in the translation and expression of GluN2B (Carmignoto and Vicini, 1992; Quinlan et al., 1999a; Hoffmann et al., 2000; Yashiro and Philpot, 2008).

The fact that the GluN2B subunit confers significantly greater plastic properties to the NMDAR and that it is highly expressed at birth, followed by an experience and developmental swap to predominant GluN2A expression, suggests the presence of the GluN2B subunit plays an important role in NMDAR-mediated plasticity. As the GluN2A subunit confers much faster kinetics and reduced calcium influx to the NMDAR, it has been proposed that a GluN2B to GluN2A switch is important for maintaining plasticity at the synapse in a similar way as NMDAR-mediated insertion of AMPARs (Quinlan et al., 1999a; Quinlan et al., 2004; Yashiro and Philpot, 2008). In fact, *in vivo* infusion of an anti-sense GluN2B oligonucleotide directly into the hippocampus completely prevented the acquisition of a spatial learning task in rats (Clayton et al., 2002). Similarly, when a selective GluN2B antagonist was infused into the lateral amygdala of rats before training, it completely blocked the acquisition of both auditory and contextual fear conditioning (Rodrigues et al., 2001).

Direct evidence for a LTP-induced synaptic switch from GluN2B to GluN2A subunits was recently reported by Bellone & Nicoll (2007). In this report, they confirmed a developmental predominance of the GluN2B subunit within the hippocampus that gradually decreased in parallel with an increase in the GluN2A subunit. EPSCs recorded from neonatal hippocampal slices showed a higher sensitivity to a selective GluN2B antagonist and slower kinetics compared to those recorded from older slices. Following LTP induction in neonatal slices, the NMDAR-mediated EPSC showed significantly faster decay compared to control. This change in kinetics was found to last at least one hour and was found to be less sensitive to GluN2B blockade compared to controls, suggesting a loss of GluN2B subunits following LTP induction (Bellone and Nicoll, 2007). To establish whether the increase in EPSC kinetics post-LTP induction was mediated by a decrease in GluN2B alone or in conjunction with an increase in GluN2A subunit expression, the experimenters conducted the entire LTP experiment in the presence of a selective GluN2B antagonist. This revealed that when GluN2B subunits are blocked, the size of the NMDAR-mediated EPSC was significantly larger post-LTP induction compared to baseline, suggesting that LTP induces an increase in synaptic GluN2A expression. Interestingly, when a low frequency stimulation was applied post-LTP to depotentiate the synapse, the LTP-induced acceleration of NMDAR EPSCs was reversed as was the reduced sensitivity of the EPSC to a GluN2B antagonist, suggesting that changes in synaptic NMDAR subunit expression are reversible. Importantly, the authors were unable to replicate these results in adult animals, suggesting that this heightened plasticity and trafficking of NMDAR subunits may be restricted to the developing animal (Bellone and Nicoll, 2007). They suggest that, at least during an early

age, NMDARs are highly plastic and changes in their subunit composition can significantly contribute to synaptic plasticity. Specifically, replacement of GluN2B subunits with GluN2A subunits may function to decrease plasticity by shortening the time window in which summation of synaptic events can induce LTP.

1.5.3.4 Role of NMDARs in olfactory plasticity

We propose a role for the NMDAR in the neonatal odor preference learning model, but is there evidence for an important role of NMDAR-mediated plasticity in olfaction? Specifically, does the NMDAR undergo similar developmentally- and plasticity-induced changes in the olfactory system as has been reported for other areas such as the hippocampus and visual cortex? Quinlan and colleagues (2004) first reported a significant learning-induced modification of synaptic NMDAR subunit composition following the acquisition of an olfactory discrimination task. Specifically, they investigated the subunit composition of NMDARs within the intracortical projections of the piriform cortex. First, they evaluated the NMDAR-dependence of olfactory discrimination learning by determining the effects of chronic peripheral administration of an NMDAR antagonist on acquisition. They found that chronic blockade of the NMDAR significantly attenuated acquisition of olfactory discrimination learning (Quinlan et al., 2004). Next, in slices taken from animals post-learning, they showed that the ability to induce LTP in the piriform cortex was significantly reduced in trained animals compared to control. Specifically, high frequency stimulation of intracortical afferents reliably induced potentiation of the EPSP amplitude in slices from pseudo-trained and naive

animals, but not trained animals. In addition, field EPSPs recorded in the piriform cortex of slices from naive animals were highly sensitive to reduction by the selective GluN2B antagonist ifenprodil, while those recorded from trained animals were not (Quinlan et al., 2004). These results suggest that following olfactory discrimination learning there is a significant decrease in synaptic GluN2B expression at intracortical connections in the piriform cortex.

To further evaluate changes in NMDAR subunit expression following learning, Quinlan and colleagues (2004) conducted immunoblotting of synaptic piriform cortex samples following training. They observed a significantly higher ratio of GluN2A/GluN2B synaptic expression in trained animals compared to naive or pseudo-trained controls. Importantly, this difference in subunit composition was learning-specific, as it was only evident in the piriform cortex and was not observed in synaptic samples prepared from the frontal cortex or hippocampus. Moreover, when levels of NMDAR subunits were examined in animals at 5 days following learning, when the memory was no longer expressed, there was no longer a significant difference in the synaptic ratio of GluN2A/GluN2B subunit expression in the pirifrom cortex between trained and control animals. These results imply that synaptic NMDAR subunit composition in the piriform cortex can be acutely and reversibly altered by olfactory learning (Quinlan et al., 2004).

In addition to plasticity-induced changes in NMDAR subunit composition within the olfactory system, recent evidence suggests a developmentally-induced general reduction in overall NMDAR expression at olfactory synapses. Specifically, Franks and

Isaacson (2005) investigated the effects of early experience on the glutamate receptor composition of synapses between the LOT and the piriform cortex. They report a significant increase in the relative contribution of AMPA/NMDA receptors at LOT synapses in the piriform cortex from PD 8 to PD 22. This is consistent with maturation of other cortical afferent synapses described previously. They next investigated whether this increase in AMPA/NMDA ratio was mediated by an increase in AMPARs, a decrease in NMDARs, or both. First, they found no evidence for a developmental increase in AMPAR expression at LOT synapses as the amplitude of AMPAR quantal events was not significantly different between ages PD 8 and PD 22. However, when they examined the contribution of NMDAR-only silent synapses at PD 8 and PD 22 they observed a significantly higher proportion of silent synapses at the younger age. The observed maturation of silent NMDAR-only synapses from PD 8 to PD 22 suggests that the increase in the AMPA/NMDA ratio could be mediated by an elimination of NMDAR-only synapses.

The authors proposed that the observed changes in glutamate receptor contribution at LOT synapses was due to experience-dependent plasticity and maturation of these synapses. To test this idea, they performed unilateral nasal occlusion on PD 1 and examined whether this could alter the increase in relative AMPA/NMDA mediated transmission of LOT inputs over the following 5 weeks of life. They observed a significant difference in the relative contribution of AMPA/NMDA receptors at LOT synapses between 2-4 weeks of life, with the ratio observed in spared hemispheres being

over 50% greater than that observed in occluded hemispheres (Franks and Isaacson, 2005).

They next compared synaptic input-output relationships for LOT-evoked AMPAR-mediated field EPSPs in both spared and occluded hemispheres. Specifically, they plotted the EPSP amplitude against the pre-synaptic volley (which is indicative of afferent fibre recruitment). This allowed comparison of the AMPAR-mediated response to a given synaptic input between spared and occluded hemispheres. They observed a greater but more variable AMPAR-mediated response to a given pre-synaptic input in spared, compared to occluded, hemispheres. From these experiments, they concluded that the developmental increase in AMPA/NMDA ratio could not be accounted for solely by an increase in post-synaptic AMPAR expression. From here, the investigators went on to examine post-synaptic changes in NMDAR expression. To do this, they similarly examined an input-output relationship for NMDAR-mediated responses to LOT input by selectively isolating NMDAR field EPSPs in low magnesium aCSF in the presence of an AMPAR antagonist. They observed a significantly lower NMDAR-mediated response to given synaptic input in spared compared to occluded hemispheres, suggesting a loss of NMDARs at post-synaptic sites. In addition, when they examined the existence of NMDAR-only silent synapses in minimal stimulation experiments, they observed a significantly lower success rate in cells sampled from spared hemispheres compared to occluded hemispheres, suggesting a higher existence of NMDAR-only synapses in occluded hemispheres. Together, these results suggest that early sensory experience

significantly reduces the post-synaptic expression of NMDARs at LOT synapses in the piriform cortex (Franks and Isaacson, 2005).

In a final experiment, Franks & Isaacson (2005) investigated the effect of early sensory experience on the induction of LTP at LOT synapses. TBS of the LOT in slices from spared hemispheres induced significant potentiation of field EPSPs. In contrast, field EPSPs of the occluded hemispheres from the same rats showed significantly greater potentiation in response to TBS. The authors tested whether this difference in the magnitude of TBS-induced LTP was due to a difference in the ability to *express* LTP or a difference in the threshold to *induce* LTP. When LTP was induced with strong stimulation (16X TBS) field EPSPs recorded from spared and occluded hemispheres displayed similar levels of potentiation, suggesting that sensory experience significantly increases the *threshold* for LTP induction but does not significantly affect the ability to express LTP (Franks & Isaacson, 2005).

This report suggests that sensory experience during early life induces a strong activity-dependent decrease in synaptic NMDAR expression and a moderate increase in AMPAR expression at LOT synapses in the piriform cortex. Together with the learning-induced increase in GluN2A/GluN2B synaptic expression at intracortical piriform synapses reported by Quinlan et al. (1999), these studies suggest significant experience- and plasticity-induced modifications to the NMDAR in the olfactory system. These investigators propose that following strong olfactory stimulation, NMDAR activation triggers the selective insertion of AMPARs to form functional synapses from previously silent synapses. Concurrent selective downregulation of synaptic NMDARs and

replacement of GluN2B subunits with GluN2A subunits may reduce plasticity at affected synapses and increase the threshold for further synaptic strengthening. Together, these changes could increase the threshold for LTP induction, preventing further synaptic strengthening and promoting the maintenance of encoded memory (Quinlan et al., 2004; Franks and Isaacson, 2005).

1.5.4 Is there a role for NMDAR modifications in early odor preference learning?

Early odor preference learning is a model of the critical imprinting of maternal odors that occurs during neonatal mammalian development (Sullivan, 2001). This is a period during which the NMDAR has been shown to be highly plastic and to undergo significant modifications in response to sensory experience in the olfactory cortex, as well as other sensory cortices (Kirkwood et al., 1996; Quinlan et al., 1999a; Quinlan et al., 2004; Franks and Isaacson, 2005). Therefore it is straightforward to hypothesize that odor preference learning during early life may involve significant contribution from, and modifications of, the NMDAR.

Furthermore, early odor preference learning is a highly associative learning model. At this age, the eyes and ears of the rat pup are sealed and they receive only tactile and chemosensory stimuli (Moriceau and Sullivan, 2004b; Raineki et al., 2011). Therefore, the pairing of a novel odor with a tactile stimulus such as stroking is very powerful. As the NMDAR requires pre-synaptic glutamate release and binding to the

receptor, in addition to strong post-synaptic membrane depolarization, it can act as a coincidence detector of simultaneous pre- and post-synaptic activity (Erreger et al., 2004; Malenka and Bear, 2004; Mayer, 2005). This coincidence detecting function has determined a critical role for the NMDAR in several models of associative plasticity (Morris, 1989; Kandel, 2001; Rodrigues et al., 2001).

Early odor preference learning has been shown to be a robust cAMP-PKA-CREB signalling model (McLean et al., 1999; Yuan et al., 2000; Yuan et al., 2003b; McLean and Harley, 2004) and while calcium influx is important in this signalling cascade and is indisputably critical in mediating long-lasting cellular potentiation (Lynch et al., 1983; Bear and Malenka, 1994; Malenka and Bear, 2004), the avenue(s) of calcium influx in this model have not yet been determined. While the cAMP-PKA-CREB-mediated enhanced MC excitation model of early odor preference learning is well supported by behavioural, anatomical and electrophysiological evidence, and while the NMDAR has been hypothesized to be involved in mediating this excitation, direct investigation or confirmation of its involvement has yet to be conducted. Furthermore, a recent study published by Cui et al. (2011) has shown a significant increase in synaptic AMPAR expression at glomerular synapses following early odor preference learning. A wealth of evidence exists showing the dependence of AMPAR insertion on NMDAR activation (Malenka and Nicoll, 1993; Malinow and Malenka, 2002; Malenka and Bear, 2004), further suggesting a role for the NMDAR in the early odor preference learning model. Within the OB, the NMDAR is highly expressed in the glomerular layer, predominantly

on MC dendrites as well as PG and glial cell processes. The NMDAR is also expressed on GC dendrites in the GC layer (Giustetto et al., 1997).

To date, few studies have investigated the role of the NMDAR in early odor preference learning. In one study, animals received daily systemic injections of an NMDAR antagonist before odor preference training each day from PD 1-18. NMDAR blockade was reported to block the acquisition of an odor preference when tested on PD 19 (Lincoln et al., 1988). However, major flaws in this study include a lack of consideration of the effects of chronic daily injection of an NMDAR antagonist on central nervous system development during a critical postnatal period, as well as a lack of mechanistic considerations of NMDAR function during learning. More recently, supplemental data published by Cui et al. (2011) have shown elevated levels of NMDAR phosphorylation in the OB shortly after the end of training, as determined by whole bulb Western blotting analysis. This recent study more strongly suggests a role of the NMDAR in early odor preference learning.

Direct support for a role of the NMDAR in this learning model comes from recent electrophysiological experiments in our lab showing that a TBS+isoproterenol-induced increase in MC spiking is dependent on NMDAR activation (Lethbridge, et al., submitted). This directly supports a role for the NMDAR in an *in vitro* model of early odor preference learning.

1.5.5 Objectives and hypothesis

The experiments described in this thesis sought to investigate whether the NMDAR is involved in the neonatal odor preference learning model. Overall, we hypothesized a critical role of the NMDAR in mediating early odor preference learning. Specifically, we hypothesized that NMDAR activation on MC apical dendrites at the ON-MC synapse during learning may result in critical and long-lasting LTP-like changes in MC processing of ON input that could act as a fundamental mechanism of early odor preference learning.

Objective 1: We first sought to determine whether the NMDAR plays a role in the *induction* of early odor preference learning. Specifically, we asked if the NMDAR was activated during learning, and if so, was activation of the NMDAR necessary for learning to occur? Based on the coincidence detection role of the NMDAR and the highly associative nature of this learning model, we hypothesized that the NMDAR would indeed be activated during early odor preference learning. Furthermore, as the NMDAR mediates a long-lasting depolarization and significant influx of calcium, we hypothesized that these two occurrences within the OB were necessary to induce odor preference learning in the neonate rat and therefore NMDAR activation would be necessary for learning to occur.

Objective 2: Next, we sought to determine whether the *expression* of odor preference memory in the neonate rat was mediated by changes in the synaptic expression and/or subunit composition of the NMDAR within the OB. Based on a wealth of evidence in other cortical areas describing dramatic modifications in synaptic expression and subunit

composition of the NMDAR, we hypothesized that early odor preference learning would induce a significant reduction in overall synaptic expression of the NMDAR and/or significantly increase the GluN2A/GluN2B subunit composition of the receptor.

Objective 3: Finally, we sought to determine the synaptic locus of NMDA and AMPA receptor changes following early odor preference memory. As an abundance of previous research has highlighted a critical role of an intracellular cAMP-PKA-CREB signalling pathway in MCs, we hypothesized that learning induced changes in NMDA and AMPA receptor properties or expression would be detectable using whole cell electrophysiology at the ON-MC synapse.

CHAPTER 2 – MATERIALS & METHODS

2.1 Animals

Sprague Dawley rat pups (Charles River) of both sexes were used in this study. Day of birth was considered to be PD 0 and litters were culled to 12 pups on PD 1. Dams were maintained under a reverse 12 h light/dark cycle at 22°C in polycarbonate cages with *ad libitum* access to food and water. All experimental procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland and follow the guidelines set by the Canadian Council on Animal Care. Behavioural conditioning and testing occurred in a temperature controlled room maintained at approximately 28°C.

2.2 pGluN1 immunohistochemistry

NMDAR activation following early odor preference learning was examined by immunohistochemistry of the subunit GluN1 phosphorylation at Ser897, a PKA-mediated phosphorylation site.

Behavioural conditioning followed the standard protocol previously established for early odor preference learning (Sullivan et al., 1989b). On PD 6 animals underwent odor preference training where they were individually removed from the nest briefly to receive a subcutaneous injection (50 µl) of either saline or the β-adrenoceptor agonist

isoproterenol (2 mg/kg, made in saline), and then returned to the nest. At 30 min following injection, each pup was individually placed on unscented clean bedding for a 10 min habituation period before being transferred to peppermint scented bedding (0.3 ml peppermint extract per 500 ml clean bedding) for a 10 min odor exposure period. A third group received only isoproterenol (2 mg/kg) injection with no exposure to peppermint odor, remaining on unscented bedding for 20 min. At 5 min following the end of the training period, animals were deeply anaesthetized with chloral hydrate (1.5 g/kg, Sigma-Aldrich) and perfused transcardially with ice-cold saline solution followed by ice-cold fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were removed from the skull with OBs intact and post-fixed for 1 h in the same fixative solution, after which they were immersed in 20% sucrose solution overnight at 4°C.

The next day, brains were quick-frozen on dry ice and 30 μm coronal sections of the entire OB were cut in a cryostat at -20°C. Sections were directly mounted onto slides and sections from animals in each treatment group within the same experiment were mounted together on the same slide. This was in order to ensure uniform staining development across experimental groups. A pGluN1 rabbit antibody (1:500, Abcam) was used to probe for phosphorylation of the NMDAR at the Ser897 PKA-mediated phosphorylation site. The antibody was dissolved in phosphate buffered saline with 2% Triton-X-100, 0.002% sodium azide, and 5% normal goat serum and applied to sections overnight at 4°C in a humidified chamber. The next day, sections were incubated in a biotinylated anti-rabbit secondary antibody (Vectastain Elite) followed by diaminobenzidine tetrahydrochloride reaction. Staining progress was visually monitored

using an upright microscope (Olympus) and typically required between 2-3 min. Afterwards, sections were dehydrated and coverslipped with permount (Fisher Scientific).

2.2.1 Image analysis for pGluN1 immunohistochemistry

Staining for pGluN1 was analyzed using a Bioquant image analysis system (R&M Biometrics). Images of sections were captured with a CCD camera connected to a Leitz microscope. The light intensity of the microscope was kept at the same level for all sections from all animals analyzed. For each section analyzed, the optical density (OD) of the ON layer was used as a measure of background OD. After taking a captured image of a section, regions of interest (ROI) were selected using a hand tracing tool. The relative OD of each ROI was obtained using the following formula: (OD of ROI – OD of background)/OD of background. Regions analyzed included the lateral and medial portions of the glomerular layer, as well as the lateral and medial portions of the GC layer lying directly subjacent to those areas of the glomerular analyzed (see Figure 3A). This was an attempt at specifically targeting training odor-induced changes, as previous studies have reported peppermint encoding "hotspots" to be located on the lateral surface of the OB (Coopersmith and Leon, 1986; Sullivan and Leon, 1986; Johnson et al., 1995; Johnson and Leon, 1996).

It is important to note that when cryostat cutting was performed, every section was taken from the very beginning of the OBs until the accessory OB was reached for each animal. Image analysis was conducted on every 3rd- 4th section beginning from the most

rostral extent of the OB until the accessory OB was reached caudally, resulting in the analysis of approximately 8-10 slices per animal. The average OD of pGluN1 staining for the lateral and medial portions of the glomerular layer and GC layer from the whole rostrocaudal range of the OBs was calculated. Separate one-way ANOVAs and post-hoc Tukey tests were carried out to determine whether differences existed between experimental groups in the average pGluN1 staining across the rostrocaudal range of the lateral glomerular layer, medial glomerular layer, lateral GC layer and medial GC layer. Values reported are mean ± standard error of measurement (SEM) of the relative OD for each ROI measured.

2.3 Intrabulbar infusion of a NMDAR antagonist

2.3.1 Cannulae assembly and implantation surgery

Two guide cannulae (Vita Needle Company Inc.; 23 gauge tubing cut to 6 mm) were anchored in dental acrylic (Lang Dental) such that they were separated laterally by approximately 4 mm and extended beyond the acrylic approximately 0.5-1 mm. Insect pins were placed inside the guide cannulae to prevent blocking.

On PD 5 rat pups were anaesthetized via hypothermia and placed in a stereotaxic apparatus with bregma and lambda in the same horizontal plane. The skull was exposed and two small holes were drilled over the dorsal-lateral surface of each OB. The cannulae were lowered into the OB and the assembly was fixed to the skull with dental acrylic (see

Figure 4). The skin was sutured together and pups were allowed to recover from anaesthesia on warm bedding before being returned to the dam and littermates.

Infusion cannulae were made from 30 gauge stainless steel tubing (Small Parts Inc.), cut to a length of approximately 13 mm and inserted into PE20 polypropylene tubing (Intramedic). Each infusion cannula was inserted into a piece of tubing so that 7 mm of cannula extended beyond the end of the tubing, this allowed the infusion cannulae to extend beyond the guide cannulae by approximately 1 mm into OB tissue. For bilateral OB infusion, the other end of PE20 tubing was secured over the needle of a 10 µl microsyringe (Hamilton Company). The two syringes attached to the infusion cannulae were placed in a multi-syringe pump (Chemyx). At time of infusion, the insect pins were removed from the guide cannulae and the infusion cannulae were gently inserted into the OB through the guide cannulae assembly fixed to the animal's skull (see Figure 4).

2.3.2 Intrabulbar infusion experiments

In these experiments animals received the β-adrenoceptor agonist isoproterenol (50 µM), administered directly into the OBs via intrabulbar infusion, as the UCS (Sullivan et al., 2000). All drugs for infusion were made in artificial cerebrospinal fluid (aCSF) containing the following (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. During training on PD 6, animals received bilateral intrabulbar infusion of either aCSF, isoproterenol (50 µM), or isoproterenol together with the NMDAR antagonist D-APV (500 µM). Infusion occurred at a rate of 0.05µl/min for

20 min over the course of both the habituation and odor exposure periods, the total volume infused was 1 µl/bulb. The next day, pups were tested for odor preference memory as described below.

2.3.3 Two-odor choice testing

On PD 7 each pup was tested for odor preference memory. A stainless steel box (30 X 20 X 18 cm) was placed on top of two training boxes separated by a 2 cm neutral zone. One box contained peppermint scented bedding while the other box contained clean, unscented bedding. Each pup was removed from the nest and underwent five separate 1 min trials during which they were placed in the neutral zone of the test box and allowed to move freely (see Figure 1). After each trial the pup was removed from the test box and placed on normal unscented bedding during the inter-trial interval (1 min). During testing, when the pup's nose moved from the neutral zone to either the peppermint side or the unscented side, the experimenter began recording time. The total amount of time spent over peppermint scented bedding and unscented bedding was calculated, time spent in the neutral zone was not recorded. Values reported are mean ± SEM of the percentages of time animals spent over the peppermint scented bedding divided by the total time spent over peppermint and unscented bedding combined. A one-way ANOVA was carried out and post-hoc Tukey tests were used to evaluate differences between experimental groups in this index of odor preference learning.

2.3.4 Cannulae placement verification

After testing, those animals who had received intrabulbar infusions during training received a final intrabulbar infusion of methylene blue dye (4%, Fisher Scientific) at the same rate and volume as during training. Following infusion, pups were sacrificed and the OBs were examined to ensure correct placement of cannulae in the glomerular layer of the dorsal-lateral OB (see Figure 5A1-3). In a subset of cases the cannulae were blocked on PD 7, in these animals the OBs were visually examined to locate the cannulae injection sites. Pups with incorrect cannulae placements were excluded from the analysis.

2.4 Synaptic expression of AMPA and NMDA receptors following odor preference learning

2.4.1 Western blot on OB synaptoneurosomes

2.4.1.1 Behavioural procedure and sample collection

On PD 6 animals received a subcutaneous injection of either saline or isoproterenol (2 mg/kg) and underwent odor preference conditioning, as previously described, while a separate group received isoproterenol alone without odor exposure. Following training, pups were returned to the nest. At 3 h or 24 h following training, animals were sacrificed and OBs were rapidly removed and flash frozen on dry ice. All

samples were stored at -80°C until use. Littermates were tested at 24 h for odor preference learning as described above.

2.4.1.2 Synaptoneurosome isolation

In order to assess changes in AMPA and NMDA receptor expression at the synapse, synaptoneurosome extractions were isolated from collected OBs. This is a method of protein extraction that has previously been shown to be enriched with synaptic proteins (Hollingsworth et al., 1985; Quinlan et al., 1999a). Briefly, whole OBs were homogenized using Teflon-glass tissue homogenizers (Thomas Scientific) in ice-cold HEPES buffer containing (in mM): 50 HEPES, 124 NaCl, 26 NaHCO3, 1.3 MgCl2, 2.5 CaCl2, 3.2 KCl. 1.06 KH2PO4, 10 glucose, 1 EDTA, 1 PMSF, complete protease inhibitor cocktail (Roche), complete phosphatase inhibitor cocktail (Roche), saturated with 95% O2/5% CO2, pH 7.4. Following a 10 min incubation period on ice, homogenates were passed through a series of filters held in syringe filter holders (Millipore); first through two 100 μm nylon filters (Small Parts Inc.), then through a 5 μm filter (Millipore). Next, the filtrate was centrifuged at 1000 x g for 20 min at 4°C. After centrifugation, the synaptoneurosome pellet was resuspended in ice-cold HEPES buffer and protein concentrations were determined using a BCA protein assay kit (Pierce). Samples, standards and reagents were added to a 96 well plate and incubated at 37°C for 30 min. Next, the plate was read at 540 nm on a BIO-RAD Model 3550 Microplate Reader. Using a standard curve generated from values of standards run on the same plate, the concentration of protein in each sample was calculated. The volume of lysate required

to obtain 40 μg of protein for each sample was determined according to the calculated protein concentrations of each sample.

2.4.1.3 Western blot

Sample solutions were prepared using 4 μl of 5X sample buffer (0.3 M TRIS-HCl, 10% SDS, 50% glycerol, 0.25% bromophenol blue, 0.5 M dithiothreitol), lysate (volume determined to contain 40 μg protein), and dH_2O to bring the total volume to 20 μl. Sample solutions were then boiled for 5 min before being loaded into lanes of a 7.5% SDS-PAGE gel. Samples were separated by SDS-PAGE and then transferred to a nitrocellulose membrane (Amersham). After transfer was complete, membranes were cut horizontally at the 72 kDa level. For experiments investigating expression of GluN1 and GluA1, the top portion was probed with a rabbit antibody recognizing GluN1 (1:1000, Cell Signalling) while the bottom portion of the membrane was probed with a rabbit antibody recognizing β-actin (1:2000, Cedarlane). Membranes were immersed and agitated in primary antibody overnight at 4°C. The next day, antibodies were detected using a horseradish peroxidase-conjugated secondary antibody (anti-rabbit, Pierce), visualized with Super West Pico Chemiluminescent Substrate (Pierce), and developed on X-ray film (AGFA). Following visualization, membranes were washed and stripped using Restore buffer (ThermoScientific). The top portion of the membrane was then probed for GluA1 and developed again as described above.

For experiments investigating expression of GluN2A and GluN2B subunits of the NMDAR, the experimental protocol was identical to that of the GluN1/GluA1 experiments except the top portion was first probed for GluN2A (1:1000, rabbit antibody, Millipore), then stripped and probed for GluN2B (1:1000, rabbit antibody, Millipore). For all Western blotting experiments, samples collected from the same litter and within the same experiment were processed together, protein determination was completed in the same plate, and the samples were all run in the same gel and transferred to the same nitrocellulose membrane during Western blotting.

For experiments investigating the developmental profile of GluA1, GluN1, GluN2B and GluN2A naive animals aged either PD 6 or PD 21 were used. OB samples were processed and synaptoneurosome extractions were performed as described above. These samples were run on the same gel as experimental samples and were processed and analyzed in the same way.

Using an image scanner (CanoScan LiDE 200), the films for each blot were scanned and the OD of each band was measured using ImageJ software. The OD of the band of interest for each sample was normalized to the OD of the β-actin band for that sample run on the same gel. Next, for each post-training experiment this value was normalized to that of control (saline+odor) animals to determine differences in expression in learning animals compared to non-learning littermates. Therefore, for these experiments values reported are mean ± SEM of the relative expression of the subunit of interest normalized to expression of that subunit in saline+odor non-learning control animals.

For developmental experiments, a student's *t*-test was used to evaluate differences in the mean OD of the GluN1 and GluA1 subunit between PD 6 and PD 21 animals. A one-way ANOVA and subsequent post-hoc Tukey tests were used to evaluate whether subunit expression or the ratio of subunits differed between experimental groups at 3 h and 24 h post-training.

2.5 Electrophysiological investigation of relative AMPA:NMDA contribution to ON-evoked MC EPSCs following learning

2.5.1 Electrophysiology experiments

On PD 6-9 animals underwent odor preference training. Specifically, they were briefly removed from the nest, given a subcutaneous injection of 2 mg/kg isoproterenol (50 µl, made in saline), and returned to the nest. Thirty minutes later, pups were again removed from the nest and placed on clean unscented bedding for a 10 min habituation period. At the end of this habituation period, unilateral nasal occlusion was performed by applying a medical grade odourless silicone grease plug to one nostril. Animals were then placed on peppermint scented bedding for a 10 min odor exposure period. At the end of the odor exposure period, the grease plug was removed from the occluded nostril and pups were returned to the nest.

2.5.2 Slice preparation and electrophysiology

At either 1-3 h or 24 h following odor preference training, rats were anaesthetized via halothane inhalation and decapitated. The brains were quickly dissected and placed into ice-cold aCSF containing the following (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 and equilibrated with 95% O_2/5% CO_2. Horizontal OB slices were cut at 400 μm using a vibrating slicer (Leica VT 1000P). Slices were hemisected and those from occluded and non-occluded OBs were separated and incubated at 34°C for 30 min in the same high glucose aCSF. Slices were then left at room temperature until use. During recording, slices were superfused with magnesium-free aCSF containing the following (in mM): 122 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose and equilibrated with 95% O_2/5% CO_2. Magnesium-free aCSF was used during recording to minimize blockade of the NMDAR and optimize the probability of NMDAR activation. Slices were viewed with an upright microscope (Olympus BX51) using differential interference contrast (DIC) optics. Whole cell patch recordings were obtained using glass pipettes (2-6 MΩ) filled with internal recording solution containing the following (in mM): 123 K-gluconate, 2 $MgCl_2$, 8 KCl, 0.2 EGTA, 10 HEPES, 4 Na_2-ATP, 0.3 Na-GTP, pH 7.35. Recording pipettes were positioned at the cell body of MCs within the mid-lateral OB whose primary dendrites could be followed to the glomerular layer. A stimulation pipette was placed at the ON layer adjacent to the glomeruli that were innervated by the primary dendrites of the recorded MCs (see Figure 10 for recording positioning). The ON layer was stimulated by a single test stimulus (20–100 μA) using a concentric bipolar stimulating pipette (FHC).

The intensity of the stimulation was adjusted to evoke a MC response when the cell was held in voltage clamp (VC) at both -70 mV and +40 mV. 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 (Molecular Devices) and Igor Pro 6.10A (WaveMetrics). All experiments were conducted at 30–32°C. The membrane resistance and access resistance for each cell was monitored throughout each experiment. All cells had an access resistance between 10-25 MΩ and any cells whose access resistance changed >30% during recording were discarded.

2.5.3 Drug application

The NMDAR antagonist D-APV (50 μM, Tocris) and the AMPAR antagonist 2,3-dihydroxy-6-nitro-7-sulfanoyl-benzo[f]quinoxaline-2,3-dione (NBQX, 20 μM, Tocris) were used in bath application to verify AMPA and NMDA mediated components of MC-evoked responses. For mEPSC recording experiments, the synaptic transmission blocker tetrodotoxin (TTX, 1 μM,Sigma) and the $GABA_A$ receptor antagonist gabazine (2-5 μM, Tocris) were bath applied.

2.5.4 The AMPA/NMDA ratio of ON-evoked MC EPSCs

The AMPAR and NMDAR mediated components of ON-evoked MC EPSCs were dissociated and measured during recording (see Figure 10). The AMPAR component of a MC EPSC was recorded when the cell was held at -70 mV and consisted of a large

negative-going peak immediately following ON stimulation. The NMDAR component of a MC medicated EPSC was recorded when the cell was held at +40 mV in the presence of the AMPAR antagonist NBQX (20 μM). This NMDAR-mediated EPSC consisted of a slower, longer lasting positive-going peak measured as the average value between 50-100 ms following ON stimulation. Following acquisition, the peak amplitudes of the AMPAR and NMDAR components of MC EPSCs were measured to obtain an AMPA/NMDA ratio for each cell. Values reported are mean ± SEM of the AMPA/NMDA ratio for occluded (control) and non-occluded (learning) slices and student's t-tests were used to evaluate differences between experimental conditions at each time point examined.

2.5.5 Paired pulse ratio

To examine whether early odor preference learning modifies pre-synaptic release, the PPR of two ON-evoked EPSCs with an inter-stimulation interval of 50 ms was measured while the cell was held in VC mode at -70 mV. A PPR of ON-evoked MC EPSC peaks for each cell was calculated (ratio of EPSC2/EPSC1).Values presented are mean ± SEM for occluded (control) and non-occluded (learning) slices and student's t-tests were used to evaluate differences between experimental conditions at each time point examined.

2.5.6 Miniature EPSCs

To further examine the synaptic site(s) of MC-LTP expression, TTX-insensitive mEPSCs were examined in both learning and control slices. mEPSCs were recorded at a holding potential of -70 mV. TTX (1 μM) was included in the bath to block action potentials, as was gabazine (5 μM) to block inhibitory synaptic transmission.

2.5.7 Biocytin staining

For post-hoc visualization of recorded MCs, biocytin (1 mg/ml, Sigma) was added to the internal solution of the recording pipette in a subset of slices. When recording experiments were complete, the slice was removed from the recording chamber and placed in cold fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) for a minimum of 24 h at 4°C. Next, each slice was transferred to a separate well in a 24 well plate and was developed on slow rotation using a free-floating protocol. First, slices were washed for 10 min in PBS. Next, slices were placed in 1% H_2O_2 at room temperature for 4 h before being transferred to blocking solution (10% normal goat serum, 2% bovine serum albumin, 0.4% Triton) for 24 h at 4°C. The next day slices underwent 3 x 10 min washes in PBS before being developed using a standard ABC kit (Vectastain Elite) followed by diaminobenzidine tetrahydrochloride reaction. Staining development was visually monitored using a microscope (Olympus). Sections were then dehydrated, mounted onto slides and coverslipped with permount (Fisher Scientific).

CHAPTER 3 – RESULTS

3.1 Early odor preference learning induces rapid phosphorylation of glomerular NMDARs

To investigate whether the NMDAR is activated following early odor preference learning and the location of activation, PKA-mediated phosphorylation of the receptor was examined immediately following training using immunohistochemistry. Significantly stronger staining was observed in the mid-lateral glomerular region of the OB in the learning group (2 mg/kg isoproterenol+odor), consistent with previous reports showing 2-DG activation in the same area following peppermint odor conditioning in pups (Coopersmith and Leon, 1986; Sullivan and Leon, 1986; Johnson and Leon, 1996). Specifically, the relative OD of pGluN1 staining in the lateral and medial portions of both the glomerular and GC layers of the OB were calculated for animals in each group (see Figure 3A). Animals that received subcutaneous isoproterenol paired with odor showed significantly higher levels of phosphorylation of the obligatory GluN1 subunit within the mid-lateral portion of the glomerular layer (0.080 ± 0.010) compared to saline+odor (0.044 ± 0.007) or isoproterenol only littermates (0.045 ± 0.003; $F_{(2,10)} = 6.79$, $p < 0.05$, Figure 3B,C). However, there was no significant difference between groups in pGluN1 staining of the medial glomerular layer (saline+odor: 0.043 ± 0.006; ISO+odor: 0.058 ± 0.010; ISO only: 0.043 ± 0.002; $F_{(2,10)} = 1.54$, $p = 0.26$). These results suggest that glomerular NMDARs in an odor-specific region of the OB are activated during early odor preference learning.

Glomerular staining of pGluN1 was observed in processes within the glomerulus that may possibly be of MCs or PG cells (Giustetto et al., 1997). Staining of small glial-like cells across glomerular and external plexiform layers was also observed (Figure 3, inset). Glomerular glial cells express GluN1 (Giustetto et al., 1997) and their activities reflect MC activation (De Saint Jan and Westbrook, 2005).

Staining of pGluN1 was also observed and analyzed in the lateral and medial GC layer lying directly adjacent to the area of glomerular layer analyzed (see Figure 3A). Within the GC layer, there were no significant differences in pGluN1 staining between groups in either the lateral (saline+odor: 0.028 ± 0.003; ISO+odor: 0.041 ± 0.009; ISO only: 0.027 ± 0.004; $F_{(2,10)} = 1.6$, $p = 0.25$, Figure 3B,C) or medial (saline+odor: 0.025 ± 0.002; ISO+odor: 0.036 ± 0.008; ISO only: 0.026 ± 0.004; $F_{(2,10)} = 1.2$, $p = 0.35$) regions analyzed.

3.2 Early odor preference learning is critically dependent on glomerular NMDAR activation

In order to directly test a causal role of the NMDAR in early odor preference learning, we designed experiments to investigate the effect of blocking the NMDAR during training. Animals received intrabulbar infusion of either aCSF, ISO (50 μM), or ISO together with the NMDAR antagonist D-APV (50 μM), directly into the lateral OB during training. Infusions were specifically targeted to the lateral glomerular layer in accordance with our pGluN1 results showing enhanced activation of the NMDAR at this

location (Figure 5A1-3). Lateral intrabulbar infusion of D-APV together with isoproterenol completely blocked the learning effect normally induced by isoproterenol infusion during odor training. Figure 5B shows that infusion of isoproterenol into the lateral OB during peppermint odor exposure successfully induced odor preference (63.5 ± 7.3%; $F_{(3,20)} = 6.14$, $p < 0.01$). In contrast, aCSF vehicle infusion (27.2 ± 6.8%) or isoproterenol infusion alone without odor exposure (32.9 ± 6.4%) did not induce an odor preference (Figure 5B). A student's *t*-test showed that combined infusion of isoproterenol with D-APV completely blocked the isoproterenol effect in inducing odor preference learning (39.4 ± 5.1%; $t_{(10)} = 2.703$, $p < 0.05$; Figure 5B). These results suggest that activation of glomerular NMDARs during training is necessary for early odor preference learning to occur.

3.3 Early odor preference learning induces a transient and reversible decrease in synaptic NMDAR expression

Using pGluN1 immunohistochemistry and intrabulbar infusion of an NMDAR antagonist, we have shown the NMDAR to play an essential role in the induction of early odor preference learning. Next, we investigated whether the NMDAR shows any long-lasting changes that may be involved in the maintenance or expression of odor preference memory. Developmental- and activity-dependent modifications of glutamate receptor expression and subunit composition dramatically alter receptor function and consequently

affect the properties of synaptic activity and plasticity (Rebola et al.; Malenka and Nicoll, 1993; Yashiro and Philpot, 2008).

We first investigated the developmental patterns of expression of these receptors at OB synapses, and then compared the developmental profiles with learning-induced changes of receptor expressions and compositions. Western blotting of OB synaptoneurosomes examining the obligatory GluN1 subunit of the NMDAR and the GluA1 subunit of the AMPAR were carried out. Synaptic expression of the NMDAR is significantly higher in neonate rats (PD 6: 1.53 ± 0.22) compared to juvenile pups only 2 weeks older in age (PD 21: 0.60 ± 0.23; $t_{(8)} = 2.97$, $p < 0.01$; Figure 6A), suggesting a dramatic developmental decrease in synaptic NMDAR expression in the OB. Concurrently, there seems to be a moderate but variable increase of AMPAR expression at OB synapses during development (PD 6: 0.95 ± 0.17; PD 21: 1.84 ± 0.8, $p = 0.15$) resulting in a significantly higher AMPA/NMDA synaptic ratio in juvenile pups (3.07 ± 0.41) compared to neonate pups (0.63 ± 0.08; $t_{(8)} = 5.8$, $p < 0.001$; Figure 6B). This switch of NMDAR to AMPAR expression is in line with several other studies reporting a similar increase in AMPA/NMDA ratio over the course of development in visual cortex, somatosensory cortex, hippocampus and thalamus (Wu et al., 1996; Isaac et al., 1997; Rumpel et al., 1998; Heynen et al., 2000; Mierau et al., 2004). We hypothesized that a similar change in NMDA and AMPA receptor expression may occur at OB synapses following early odor preference learning.

To investigate whether a similar change in the ratio of AMPA to NMDA receptor expression may be involved in early odor preference learning, we examined the

expression of the obligatory GluN1 subunit of the NMDAR and GluA1 subunit of the AMPAR at 3 h and 24 h following training. Animals received either saline paired with odor, ISO paired with odor, or ISO only. At 3 h following training, synaptic expression of the AMPAR GluA1 subunit (shown as relative OD) in both the ISO+odor (1.16 ± 0.13) and ISO only (0.97 ± 0.1) groups was not significantly different compared to the saline+odor group ($F_{(2,45)} = 1.26$, $p = 0.29$, Figure 7A). In contrast, when compared to the saline+odor group, synaptic expression of the GluN1 subunit of the NMDAR was significantly lower in the ISO+odor group (0.69 ± 0.08) but not in the ISO only group (0.82 ± 0.1) at 3 h following the end of training ($F_{(2,45)} = 4.22$, $p < 0.05$, Figure 7A). There was no significant difference in the AMPA/NMDA ratio between the ISO+odor (3.21 ± 1.46) or ISO only (1.49 ± 0.28) groups when compared to saline+odor controls at 3 h post-training ($F_{(2,45)} = 1.82$, $p = 0.17$, Figure 7B).

At 24 h following training, there was no significant difference in synaptic GluA1 expression in ISO+odor (1.03 ± 0.01) or ISO only (0.96 ± 0.06) groups compared to the saline+odor group ($F_{(2,42)} = 0.277$, $p = 0.759$; Figure 7C). Interestingly, the decrease in synaptic GluN1 expression observed in learning animals at 3 h following training was not present at 24 h (ISO+odor: 0.89 ± 0.12 compared to saline+odor; ISO only: 0.91 ± 0.18 compared to saline+odor; $F_{(2,42)} = 0.22$, $p = 0.81$; Figure 7C). There was also no significant difference in the AMPA/NMDA ratio at 24 h following training in ISO+odor (3.32 ± 2.2) or ISO only (1.92 ± 0.44) groups when compared to saline+odor controls ($F_{(2,42)} = 0.85$, $p = 0.44$; Figure 7D). These data suggest that early odor preference

learning induces a transient but reversible decrease in synaptic NMDAR expression that may be important for the induction of plasticity changes underlying the memory.

3.4 Early odor preference learning significantly alters NMDAR subunit expression

We next investigated whether the composition of NMDAR subunits is modified by early odor preference learning, as suggested by a GluN2A to GluN2B switch observed in other systems during development and/or following sensory activity (Flint et al., 1997; Chen et al., 2000a; Liu et al., 2004a; Liu et al., 2004b; Yashiro and Philpot, 2008). First, we investigated whether there was a significant change in the expression of these two subunits at OB synapses over the course of development. From PD 6 to PD 21 there seems to be a modest increase in synaptic GluN2B expression (PD 6: 0.81 ± 0.17; PD 21: 1.49 ± 0.24; $t_{(8)} = 2.29$, $p < 0.05$, Figure 8A), while synaptic GluN2A expression increases by over two-fold from PD 6 (0.78 ± 0.14) to PD 21 (2.21 ± 0.75; $t_{(8)} = 1.88$, $p < 0.05$, Figure 8A). Taken together, young neonatal pups tend to have a slightly lower ratio of GluN2A expressing NMDARs to GluN2B expressing NMDARs at synapses in the OB compared to juvenile pups approximately 2 weeks older, however this trend does not reach significance (PD 6: 1.21 ± 0.31; PD 21: 1.44 ± 0.31; $t_{(8)} = 0.53$, $p = 0.305$, Figure 8B).

Next, we investigated GluN2A and GluN2B changes following early odor preference learning. At 3 h following learning there were no significant differences in

synaptic GluN2A expression in ISO+odor animals (1.08 ± 0.09) or ISO only animals (0.98 ± 0.07) compared to saline+odor littermates ($F_{(2,51)} = 0.65$, $p = 0.53$; Figure 9A). Similarly, there were no significant differences in synaptic GluN2B expression in either ISO+odor (1.09 ± 0.14) or ISO only (0.94 ± 0.09) groups compared to saline+odor littermates ($F_{(2,52)} = 0.64$, $p = 0.53$; Figure 9A) at 3 h after training. Taken together, these results did not represent a significant difference in the GluN2A/GluN2B ratio between groups at 3 h following learning (ISO+odor: 1.38 ± 0.24 compared to saline+odor; ISO only: 1.22 ± 0.14 compared to saline+odor; $F_{(2,53)} = 0.03$, $p = 0.97$; Figure 9B). These results suggest that early odor preference learning does not induce significant changes in GluN2B and GluN2A expression at 3 h following training.

In contrast, at 24 h following training, synaptic expression of the GluN2B subunit was significantly lower in learning animals (0.79 ± 0.09) compared to saline+odor littermates as revealed by *t*-test ($t_{(18)} = 2.43$, $p < 0.01$; Figure 9C), even though one-way ANOVA did not show overall significance among three experimental groups ($F_{(2,54)} = 1.21$, $p = 0.30$). GluN2B expression in ISO only animals (0.94 ± 0.15; Figure 9C) was not significantly different from either saline+odor or ISO+odor animals, but expression seemed to be intermediate between the two groups. There was no evidence for a reciprocal increase in synaptic GluN2A expression in learning animals (0.90 ± 0.1) as they did not differ from ISO only (0.84 ± 0.07) or saline+odor littermates in synaptic GluN2A expression at 24 h ($F_{(2,54)} = 1.45$, $p = 0.24$; Figure 9C). When a student's *t*-test was conducted, learning animals showed a significantly higher ratio of synaptic GluN2A to GluN2B expressing NMDARs compared to saline+odor littermates at 24 h after

training (ISO+odor: 1.35 ± 0.21, compared to saline+odor, $t_{(36)} = 1.67$, $p = 0.05$). These data suggest the reduced expression of synaptic GluN2B in learning animals at 24 h following the end of training results in a higher GluN2A/GluN2B ratio in these animals. These changes would confer reduced plastic properties to the affected receptors which may help to stabilize learning-induced synaptic changes.

3.5 Early odor preference learning significantly alters the AMPA/NMDA ratio of ON-evoked MC EPSCs

To further investigate the cellular and synaptic locus of NMDAR and AMPAR changes, we carried out *ex vivo* whole cell electrophysiological recording experiments of MCs from OB slices collected at different time points post-training. Specifically, our experimental design targeted MCs within the mid-lateral OB, consistent with our pGluN1 immunohistochemistry and D-APV infusion studies. Figure 10A shows an example of a biocytin filled MC recorded from the lateral OB. From OB slices of unilaterally nasal occluded animals, we measured the AMPA/NMDA ratio of ON-evoked EPSCs from MCs of occluded (control) and non-occluded (learning) OBs to obtain a measure of learning-induced synaptic changes. We hypothesized that learning may involve a significant increase in the AMPA/NMDA ratio of ON-evoked MC EPSCs in odor-encoding MCs.

As is shown in Figure 10C, the AMPAR-mediated component of a MC EPSC was recorded at a holding potential of -70 mV and was measured as a fast negative-going peak

immediately following ON stimulation. This was confirmed to be AMPAR-mediated as it was completely abolished by addition of the AMPAR antagonist NBQX (20 μM) to the bath (Figure 10B). The NMDAR-mediated component of a MC EPSC was recorded at a holding potential of +40 mV in the presence of NBQX (20 μM). The NMDA component is a slower and longer-lasting positive-going EPSC measured as the average peak between 50-100 ms following ON stimulation (Figure 10C). This was confirmed to be NMDAR-mediated as it was completely abolished by the NMDAR antagonist D-APV (50 μM, Figure 10B).

At 1-3 h post-training, the AMPA/NMDA ratio of ON-evoked MC EPSCs from non-occluded OBs was significantly higher than that recorded from MCs of occluded OBs (non-occluded: 2.81 ± 0.72 ; occluded: 0.74 ± 0.20; $t = 2.54$, $p = 0.01$; Figure 11A). At 24 h post-training, the AMPA/NMDA ratio of MCs from non-occluded OBs appeared to be higher than that of MCs from occluded OBs (non-occluded: 2.56 ± 0.81; occluded: 1.17 ± 0.35), but this trend did not reach significance ($t = 1.43$, $p = 0.087$; Figure 11B). The AMPA/NMDA ratio of the occluded OB was also compared with naïve control slices of the same age and no difference was found (naïve: 0.9805 ± 0.242; 1-3 h occluded: 0.7363 ± 0.1992; t = 0.69, p = 0.2517; 24 h occluded: 1.168 ± 0.3494; t = 0.37, p = 0.36). Therefore, acute occlusion of the OB did not affect these receptor properties.

An increase in AMPA/NMDA ratio following early odor preference learning could be caused by an increase in AMPAR, a decrease in NMDAR, or both. Our Western blotting results provide supportive evidence that NMDAR removal from the synapse may occur in MCs. To test whether and how AMPARs change following learning we

performed miniature EPSC (mEPSC) recording from MCs. An increase in the number or sensitivity of post-synaptic AMPARs could be reflected by an increase in the amplitude of mEPSCs.

3.6 Absence of mEPSCs in MCs

To investigate possible AMPAR changes and/or pre-synaptic release changes (reflected by mEPSC frequency), we attempted to measure MC mEPSCs from occluded and non-occluded OBs. mEPSCs are independent of action potential firing and inhibitory input as both TTX (1 μM) and gabazine (5 μM) are added to bath solutions when recording mEPSCs. We found that MCs do not exhibit significant mEPSCs even though spontaneous activity is abundant. As is shown in Figure 12A, at a holding potential of -70 mV MCs show spontaneous EPSCs at a frequency of approximately 5 Hz. However, once TTX and gabazine are added to the bath solution (Figure 12B) the cell becomes completely quiet, showing no mEPSCs.

3.7 The PPR of ON-evoked MC EPSCs is transiently altered by odor preference learning

In an attempt to gain further insight into a potential pre-synaptic locus of learning-induced synaptic changes, the PPR of ON-evoked MC EPSCs was also examined at the same time points following training. At 1-3 h post-training there was a moderate but not significant decrease in the PPR of recorded MCs when comparing non-

occluded with occluded OBs (non-occluded: 0.65 ± 0.1; occluded: 0.88 ± 0.12; $t = 1.48$, $p = 0.077$; Figure 11C). When cells recorded at 1 h post-training were examined separately, a significant difference between occluded and non-occluded cells was evident (non-occluded: 0.63 ± 0.13; occluded: 1.05 ± 0.17; $t = 1.94$, $p = 0.042$). This suggests there may be a transient pre-synaptic change involved in the early stages of odor preference memory formation. In line with this idea, there was no difference between the PPRs of recorded MCs from non-occluded and occluded OBs at 24 h post-training (non-occluded: 1.04 ± 0.18; occluded: 0.82 ± 0.14; $t = 0.94$, $p = 0.182$; Figure 11D).

The properties of MCs sampled from occluded and non-occluded OBs were not significantly different as access resistance values and membrane resistance values were similar at both time points examined. Specifically, MCs in the occluded and non-occluded groups did not differ significantly in access resistance values at either 1-3 h (occluded: 12.8 ± 0.91 MΩ; non-occluded: 14.8 ± 1.98 MΩ; $t_{(17)} = 0.45$, $p = 0.33$, Figure 13A) or 24 h (occluded: 15.4 ± 2.76 MΩ; non-occluded: 14.8 ± 3.06 MΩ; $t_{(11)} = 0.14$, $p = 0.45$, Figure 13C). Similarly, there was no significant difference in membrane resistance values between groups at either 1-3 h (occluded: 193.3 ± 16.7 MΩ; non-occluded: 185.8 ± 22.0 MΩ; $t_{(17)} = 0.27$, $p = 0.40$, Figure 13B) or 24 h post-training (occluded: 231.4 ± 38.5 MΩ; non-occluded: 222.5 ± 18.9 MΩ; $t_{(11)} = 0.2$, $p = 0.42$, Figure 13D).

CHAPTER 4 – DISCUSSION

4.1 Summary of major findings

These experiments were designed to investigate the role of the NMDAR in early odor preference learning. First, we tested whether the NMDAR is critical for odor learning induction. We examined whether the NMDAR was activated following learning using immunohistochemical analysis of phosphorylation of the receptor at the PKA site. Learning animals showed significantly higher phosphorylation of the NMDAR within the lateral glomerular layer, a peppermint-encoding region of the OB. Then, we tested whether activation of the NMDAR was necessary for learning to occur. We found that blocking NMDAR activity within the lateral glomerular layer completely blocked learning, suggesting early odor preference learning is an NMDAR-dependent model. These data fit with current literature showing that the NMDAR mediates associative LTP formation and memory in others systems (Malenka and Nicoll, 1993; Kandel, 2001; Malenka and Bear, 2004).

Next, we tested if early odor preference learning alters NMDAR expression and subunit composition in the OB and searched for the cellular locus of such changes. Using Western blotting of OB synaptoneurosomes, we showed that early odor preference learning causes a transient and reversible reduction in overall synaptic expression of the NMDAR 3 h following training as well as a significant reduction in GluN2B subunit expression at 24 h following training. *Ex vivo* examination of ON-evoked MC EPSCs

revealed significantly higher AMPA/NMDA ratios in MCs recorded from non-occluded OBs at 1-3 h following odor conditioning compared to MCs recorded from occluded OBs. However, this enhanced AMPA/NMDA ratio was not evident in MCs sampled at 24 h following training. This increased AMPA/NMDA ratio could result from enhanced AMPAR-mediated currents or a reduction of NMDAR-mediated currents, or a combination of both. In addition, we observed a significantly lower PPR at 1 h but not at 24 h in MCs recorded from non-occluded compared to occluded OBs, suggesting a transient pre-synaptic change in transmitter release may be involved in early odor preference learning.

4.2 Localization of pGluN1 staining

Early odor preference training significantly increased phosphorylation of the NMDAR within the lateral glomerular layer. This fits well with supplementary data published by Cui et al. (2011) in which Western blotting was used to show a learning-induced increase in OB pGluN1 expression within 10 min post-training. Importantly, the data presented in this thesis extend the work reported by Cui and colleagues by revealing the synaptic location of learning-induced NMDAR phosphorylation within the OB.

The lateral glomerular layer has been shown to be a peppermint-encoding region that had enhanced active 2-DG uptake following early odor preference learning (Coopersmith and Leon, 1986; Sullivan and Leon, 1986; Johnson and Leon, 1996). In addition, we specifically studied PKA-mediated phosphorylation of the NMDAR. Early

odor preference learning is a robust cAMP-PKA-CREB signalling model (McLean et al., 1999; Yuan et al., 2000; Yuan et al., 2003b; Yuan et al., 2003a) and we hypothesized PKA activation of NMDARs may mediate MC-LTP following early odor preference learning. Together, the location of enhanced pGluN1 staining within a training odor-specific region and the fact that this is mediated by PKA, support our hypothesis that the NMDAR is activated by PKA during early odor preference learning.

Within the glomerular layer, there are several cell types that express the GluN1 subunit of the NMDAR including MCs, PG cells, and glia (Giustetto et al., 1997). Immunolabelling studies have shown the majority of GluN1 labelling in the glomerular layer to occur in MC dendrites while some labelling is observed in PG and glial cell processes (Giustetto et al., 1997). We observed processes in both the glomerulus and the external plexiform layer (Figure 3) which are likely dendrites of MCs, and small cellular staining in the glomerular layer and GC layer. We did not further pursue the identity of the cells but suspect at least those in the glomerular layer are glial cells. Within the glomerular, glia encapsulate individual glomeruli and act to prevent the spread of cellular activity between glomeruli by removing extracellular potassium and excess glutamate (Amedee et al., 1997; Utsumi et al., 2001; Goriely et al., 2002; Kofuji and Newman, 2004; De Saint Jan and Westbrook, 2005). Glial activity is thought to reflect glomerular and MC activity as well as the release of glutamate from ON terminals (De Saint Jan and Westbrook, 2005). Consequently, it would not be surprising to see enhanced activation of glia with odor preference learning in a region corresponding to enhanced activity of MCs. In fact, studies investigating active glycogen phosphorylase staining following early odor

preference learning have found significantly higher levels of staining in the outer portion of the glomerular layer in learning animals (Coopersmith and Leon, 1987). As this is the portion of the glomerular layer consisting predominantly of glia, these results suggest glial activity may be increased in response to olfactory learning. In support of this, Coopersmith & Leon (1995) went on to show that NE can significantly increase glycogenolysis in OB slices, further suggesting a role for glycogen metabolism in olfactory preference learning. It is also possible that a learning-related increase in glycogen metabolism observed in glia reflects an increase in glycogen breakdown and shuttling of lactate (an alternative energy source) from glia to neurons, which has been proposed to occur during periods of heightened neuronal activity (Pellerin et al., 2007).

While we did not conduct experiments to specifically determine the cell type(s) which express the learning-induced increase in NMDAR phosphorylation observed here, there is little doubt it is learning-specific due to its odor-specific location and dramatic difference from control conditions. However, in future experiments it would be interesting to perform double-labelling of pGluN1 with neuronal and glial markers to verify the cell type(s) expressing enhanced phosphorylation of GluN1 induced by learning.

4.2.1 Significance of learning-induced pGluN1 enhancements

PKA-mediated phosphorylation of the NMDAR can significantly modify receptor function resulting in a larger EPSC amplitude and greater calcium influx (Raman et al.,

1996). As a result, learning-induced increases in PKA-mediated phosphorylation of NMDARs on odor-encoding MCs may lead to greater excitation and calcium influx, resulting in learning-dependent synaptic changes. NMDARs require both glutamate binding and membrane depolarization to become activated, remove the magnesium block, and open the ion channel. For these reasons, the NMDAR has been identified as a coincidence detector of simultaneous pre- and post-synaptic activity, a function that fits very well with associative learning (Erreger et al., 2004).

We hypothesize that on MC apical dendrites, odor-induced release of glutamate from ON terminals binds to the NMDAR, fulfilling the pre-synaptic activation requirements. Post-synaptically, NMDAR activation may occur via direct activation of β-adrenoceptors on the MC membrane (Yuan et al., 2003a), resulting in cAMP and PKA recruitment and leading to PKA-mediated phosphorylation of the NMDAR. Alternatively, as β-adrenoceptors have also been found to be expressed on PG cells (Woo and Leon, 1995; Yuan et al., 2003a), it is possible that during learning their activation on PG cells may provide further depolarization of MCs by releasing them from the inhibitory glomerular network (Lethbridge et al., submitted) and allowing further NMDAR activation. Either way, the odor-induced pre-synaptic activation and arousal-induced post-synaptic activation of NMDARs may lead to significant opening of NMDARs on the MC membrane and result in an abundant influx of calcium required to activate signalling cascades and induce synaptic plasticity changes (see summary Figure 14). Importantly, this working hypothesis suggests a convergence within MCs of β-adrenoceptor-mediated activation of AC-cAMP-PKA-CREB signalling and NMDAR-dependent calcium

activation of this signalling cascade to induce long-lasting synaptic modulation at the ON-MC synapse. This hypothesis fits well with data from *Aplysia* studies showing that pairing of a calcium-mediated CS and a G-protein-mediated UCS can activate AC leading to a critically timed peak in cAMP production and the recruitment of PKA and downstream substrates (Yovell and Abrams, 1992). The present data suggest that in early odor preference learning, the calcium-mediated CS may occur, at least in part, due to NMDAR activation and opening.

4.3 Early odor preference learning is NMDAR-dependent

To test a causal role of NMDAR activation in early odor preference learning, we infused the NMDAR antagonist D-APV (50 μM) into the OBs during odor conditioning. Within the OB, the majority of NMDAR expression occurs in MCs and GCs (Giustetto et al., 1997). Activation of the NMDAR on each cell type results in very different effects on OB circuit activity. NMDAR activation on MC primary dendrites occurs due to odor-induced release of glutamate from ON terminals (Berkowicz et al., 1994). In contrast, activation of the NMDAR on GC dendrites occurs via glutamate release from MC secondary dendrites. Once activated, GCs release GABA back onto MCs as well as onto neighbouring MCs resulting in both feedback and lateral inhibition (Schoppa et al., 1998). Therefore, experimental blockade of NMDARs at GC-MC synapses would be expected to reduce inhibition onto MCs and enhance MC activity in response to odor input. In contrast, blocking NMDARs at ON-MC synapses would be expected to block odor-induced activation of NMDARs and result in reduced MC activity in response to odor

input. As we are working under the hypothesis that learning induces critical changes at the ON-MC synapse, we established a lateral infusion protocol to apply D-APV directly to the lateral glomerular region where pGluN1 activation was observed in learning pups. Previously in our lab, immunohistochemistry using pCREB (a transcription factor thought to reflect neuronal activity) has shown that MC activity in the mid-lateral region of the OB is reduced in our lateral infusion protocol, in contrast to enhanced MC activity following a more medial infusion protocol (Lethbridge, et al., submitted). These results confirmed that our lateral infusion protocol of D-APV primarily affects ON-MC synapses.

Blockade of NMDARs at lateral glomerular synapses was shown to completely block odor preference learning, with these animals being no different from saline+odor or ISO only control animals. These data further support a critical role of the NMDAR at the ON-MC synapse in the induction of early odor preference learning.

4.3.1 Glomerular NMDAR blockade does not block odor detection

As the NMDAR is involved in the glutamatergic transmission from ON terminals to MCs, it is possible that glomerular infusion of D-APV does not block learning per se but instead affects odor detection and/or discrimination. To exclude this possibility, in another set of experiments conducted in our lab, D-APV was infused immediately before odor testing (Lethbridge et al., submitted). We found no effect of D-APV infusion before testing on either odor perception or memory retrieval. Specifically, animals who had

received intrabulbar infusion of ISO paired with odor during training on PD 6, followed by infusion of D-APV immediately before testing on PD 7, still spent significantly more time over peppermint-scented bedding compared to the non-learning control group (sham cannulae implantation surgery+odor). In addition, the percentage of time that the D-APV infusion group spent on peppermint-scented bedding was comparable to the learning control group (ISO+odor on PD 6 and no D-APV infusion before testing). If glomerular D-APV infusion did block odor perception or discrimination, animals would be expected to spend the same amount of time over unscented and peppermint-scented bedding because they would be unable to detect the difference. On the other hand, if D-APV infusion blocked memory retrieval (but not odor discrimination), we would expect D-APV infused animals to show normal mild aversion to peppermint as the non-learning control animals. Therefore, we conclude that glomerular D-APV infusion does not block odor detection or discrimination. In line with this conclusion, a report by Quinlan et al. (2004) found that an intraperitoneal injection of an NMDAR antagonist did not affect olfactory discrimination.

In further support of this, *in vitro* experiments investigating ON-evoked spiking activity of MCs in the presence of D-APV have shown that D-APV does not affect the early component of MC spiking in response to odor input, but significantly reduces the late component of MC spiking (Lethbridge et al., submitted). These experiments suggest that while NMDARs mediate a late component of synaptic potentials that lead to MC spiking, the early component is mediated by AMPARs. Based on these experiments, we propose that it is most likely that odor information is still encoded during lateral

glomerular NMDAR blockade, as glutamate binding to AMPARs on MC dendrites maintains the spatial pattern of MC spiking and detection of the odor. Meanwhile, NMDAR activation is most likely involved in the temporal pattern of MC activity and may be critical for inducing synaptic plasticity.

4.4 The relative synaptic expression of AMPA and NMDA receptors in the OB changes dramatically over an early developmental period

There is a critical postnatal period for odor preference learning, which is similar to many other forms of learning that occur most efficiently at developmentally early ages across many species. A current view relates this developmental decline in plasticity to a change in the relative synaptic strength of AMPARs vs NMDARs (Malinow and Malenka, 2002; Malenka and Bear, 2004). Here we show that over the course of only two weeks the synaptic expression of NMDA and AMPA receptors within the OB changes dramatically. During early life, NMDARs are highly expressed at OB synapses compared to expression levels two weeks later. In contrast, AMPARs are more highly expressed at OB synapses in older compared to young pups. As a result, the ratio of synaptic AMPA/NMDA receptor expression is significantly higher in older pups.

During early life, rat pups are completely dependent on somatosensory and olfactory stimuli to navigate their environment (Wilson and Sullivan, 1994). In particular, they must learn to associate the odors of the dam, nest, and littermates with care, food, and protection, making olfactory learning and plasticity at this age essential to survival.

During a critical developmental period, NMDAR expression is essential for synapse formation and plasticity necessary for survival (Rebola et al.; Katz and Shatz, 1996; Yashiro and Philpot, 2008). However, as development progresses, plasticity within the OB becomes less important for survival and olfactory learning comes to involve predominantly higher cortical structures. Critical imprinting-like associations have already formed during the early critical period and maintenance may now become more important. This may help explain the developmental switch from predominantly NMDAR expression to AMPAR expression observed at OB synapses, which parallels developmental patterns observed in many cortical areas (Durand et al., 1996; Isaac et al., 1997; Mierau et al., 2004). While the AMPAR is considered to be essential for basal synaptic transmission, the NMDAR is considered to be critical for associative plasticity (Malinow and Malenka, 2002; Malenka and Bear, 2004). Correspondingly the observed NMDAR to AMPAR switch parallels a heightened need for plasticity during early development compared to later life.

4.5 Early odor preference learning involves a significant but transient decrease in NMDAR expression at OB synapses

Recent research has also implicated a change in the AMPA/NMDA receptor ratio in learning and plasticity. Specifically, the widely held belief is that synaptic plasticity involves NMDAR-dependent insertion of AMPARs into the synaptic membrane (Malinow and Malenka, 2002). But whether this AMPAR insertion occurs coincidentally

with removal of NMDARs from the synapse remains unknown. We examined whether during a critical developmental period, odor learning could involve changes in synaptic AMPA and NMDA receptor expression beyond the normal developmental pattern.

A transient and reversible reduction in synaptic expression of the obligatory GluN1 subunit of the NMDAR was observed in learning animals at 3 h following training that was not present at 24 h following training. This learning-induced reduction in synaptic NMDAR expression is thought to result in reduced plasticity at affected synapses. This reduced synaptic plasticity may help promote stabilization of the newly forming memory by temporarily preventing further synaptic strengthening and thus allowing learning specific synaptic changes to occur. This is in line with other studies suggesting the importance of plasticity-induced molecular mechanisms in preventing runaway synaptic enhancement and maintaining memory (Barkai et al., 1994; Quinlan et al., 2004). There is little doubt that the observed decrease in synaptic GluN1 expression is learning-specific as it was only observed in learning animals. Since immunohistochemistry experiments aimed to determine the synaptic locus of GluN1 changes were not successful, we cannot confirm the synaptic location of NMDAR downregulation. However, based on our pGluN1 immunohistochemical analysis, showing enhanced activation of NMDARs at lateral glomerular synapses, we hypothesize that changes in NMDAR expression may occur at the ON-MC synapse.

It should be noted that in our experimental design, the saline+odor group was used as control. It would be good to include a group of naive animals in the data sets in the future to test if odor exposure itself results in any cellular changes in the olfactory bulb.

4.6 Lack of learning-induced changes in synaptic AMPAR expression

We did not observe significant changes in the GluA1 subunit of the AMPAR at either 3 h or 24 h following learning using Western blotting of synaptoneurosomes. This is in contrast to recent data reporting a significant increase in synaptic GluA1 expression at 24 h following learning using a similar protocol (Cui et al., 2011). This discrepancy may be due, at least in part, to the use of different antibodies probing for GluA1 expression. The antibody we used was monoclonal (Millipore) while Cui et al. (2011) used a polyclonal GluA1 antibody (Chemicon). This is an important difference, as monoclonal antibodies are highly specific and recognize a single epitope on the targeted antigen. In comparison, polyclonal antibodies recognize multiple epitopes on the targeted antigen and are less specific. Furthermore, polyclonal antibodies tend to be more tolerant of minor changes in the antigen induced by sample preparation and processing while monoclonal antibodies tend to be much more sensitive to chemical processing and protein denaturation. As a result, polyclonal antibodies are often used for detecting denatured proteins. In this case, it is highly possible that changes in synaptic GluA1 expression were not observed here because a potentially small change in protein expression coupled with a high degree of chemical processing and protein denaturation with the synaptoneurosome protocol superseded the ability of the applied monoclonal antibody to detect differences in GluA1 expression.

Alternatively, it is possible that the sensitivity of our antibody is quite high, such that we used an inappropriate concentration for Western blotting. Indeed, we applied GluA1 antibody at a 1:1000 dilution while Cui et al. (2011) applied a 1:4000 dilution. It is

possible that the concentration of antibody we applied was so high that it saturated at the basal level of expression and thus we were unable to pull out differences between groups. Furthermore, the discrepancy between our GluA1 results and those reported by Cui et al. (2011) may reflect differences in synaptoneurosome extraction protocols. Differences between the protocols include differences in homogenization buffer composition, centrifugation, and pellet re-suspension. Depending on the quantity and quality of the extracted synaptic membranes, as well as the specificity of the antibody, probing for GluA1 may have been very different between the two studies.

Finally, it is possible that within the OB, learning-induced changes occur only at a small subset of synapses such that a bulk preparation such as Western blotting does not reveal significant changes in overall expression. However, we were able to detect significant changes in NMDAR subunit expression indicating that either changes in GluN1 and GluN2B subunit expression are more dramatic than changes in GluA1 subunit expression, or that our methods of investigating changes in GluA1 expression were not appropriate. However, the finding that interruption of GluA1 insertion within the OB completely prevented odor preference learning (Cui et al., 2011) supports a role for the AMPAR in supporting long-term memory in this learning model

4.7 NMDAR subunit expression at OB synapses undergo significant changes during development

Besides changes in NMDAR total expression level measured by GluN1, we also examined changes of NMDAR subunit composition of GluN2A and GluN2B. We show that expression of both the GluN2A and GluN2B subunits increases from PD 6 to PD 21. While the GluN2B subunit showed only a modest increase in expression, expression of the GluN2A subunit more than doubled over the course of only two weeks. This is in line with other studies investigating changes in these subunits in several cortical areas (Sheng et al., 1994; Wenzel et al., 1997; Chen et al., 2000a; Cull-Candy et al., 2001; Liu et al., 2004b). Such studies suggest GluN2B expression increases from day of birth to reach a peak at 3 weeks of life, after which time expression declines. Meanwhile, GluN2A expression has been reported to be very low at birth but begins to increase dramatically within the first few postnatal days, similarly reaching a peak at 3 weeks that is equivalent to adult expression levels (Wenzel et al., 1997). Our results showing a modest increase in OB GluN2B expression concurrently with a dramatic increase in GluN2A expression between 1-3 weeks of life, are similar to the developmental expression patterns reported by others. Further studies investigating synaptic expression of the subunits at a later time point, perhaps at >1 month of age, would likely show a greater difference in GluN2A and GluN2B expression and a significantly higher GluN2A/GluN2B ratio compared to younger time points.

It is unclear why synaptic expression of both GluN2 subunits increases while GluN1 expression decreases over the observed developmental window. It is possible that

this reflects the formation of heterotrimeric receptors at OB synapses, consisting of a single GluN1 subunit together with GluN2A and GluN2B subunits. It is thought that at least one GluN1 subunit is required for a functional receptor (Erreger et al., 2005). Indeed, Al-Hallaq et al. (2007) reported that approximately one third of NMDARs in the hippocampus are triheteromeric in subunit composition (GluN1/GluN2A/GluN2B), confirming that these subunits can assemble to form functional synaptic receptors with properties intermediate between diheteromeric GluN1/GluN2B and GluN1/GluN2A receptors. Future electrophysiology experiments investigating the GluN2B- and GluN2A-mediated contributions to the NMDAR component of ON-evoked MC EPSCs may provide a clearer understanding of developmental changes in NMDAR expression and subunit composition within the OB.

4.8 Reduced synaptic GluN2B subunit expression may help maintain odor preference memory

While decreased overall expression of the NMDAR may play a role in maintaining odor preference memory at 3 h post-training, at 24 h post-training significant removal of the highly plastic GluN2B subunit from synaptic NMDARs may serve a similar role. So even though learning and control animals do not seem to differ in overall expression of NMDARs at OB synapses at 24 h post-training, the receptors present at the synapse seem to differ significantly in their subunit composition. The reduced expression of the GluN2B subunit in learning animals compared to controls is expected to result in

reduced synaptic plasticity at affected synapses, which may play a role in maintaining the memory by preventing further synaptic strengthening, as proposed earlier for GluN1 changes (Barkai et al., 1994; Quinlan et al., 2004).

It would be interesting to determine whether GluN2B expression remains low at OB synapses at time points when the memory is no longer expressed. One would expect that if reduced GluN2B expression was mediating a learning-induced decrease in plasticity at affected synapses, then expression of the GluN2B subunit would return to levels similar to control animals at time points when the memory is no longer behaviourally expressed. Indeed, Quinlan et al. (2004) reported a similar shift in GluN2A/GluN2B subunit expression within the piriform cortex following olfactory discrimination learning that returned to basal levels at a time point when the memory was no longer expressed.

Here, the observed decrease in GluN2B subunit expression was probed using a whole bulb protocol which did not allow for specific synaptic localization of these changes. Therefore, it would be interesting to attempt immunohistochemical examination of changes in GluN2B subunit expression to verify their synaptic location. Alternatively, electrophysiological examination of GluN2B-mediated components of ON-evoked MC EPSCs could also help to determine the synaptic location of GluN2B expression changes. Based on our previous data showing significant learning-induced changes within the lateral GL in addition to our electrophysiological data suggesting potential changes in NMDAR components of MC EPSCs, it is hypothesized that the learning-induced changes

in GluN2B subunit expression occur within post-synaptic NMDARs at the ON-MC synapse.

4.9 Lack of reciprocal change in GluN2A subunit expression following learning

While we observed a developmental increase in synaptic GluN2A expression, we did not observe a significant learning-induced change in its expression. Several reports suggest a GluN2B to GluN2A switch during development or following learning (Yashiro and Philpot, 2008). However, our data does not support this direction of changes. This may be a result of the quality of the antibody we used. Poor antibody specificity and binding may have led to the failure of detecting changes in GluN2A. In fact, the antibody we used showed poor binding and high background, as did a second GluN2A antibody we tried. It is possible that a combination of factors including potentially a small change in protein expression, coupled with poor antibody binding and specificity in a whole bulb preparation (low signal to noise ratio) may be to blame for the lack of GluN2A expression change observed after learning. As a result, in future experiments this question may be better addressed using immunohistochemistry, to look at differences in GluN2A expression after learning, or electrophysiology to examine differences in GluN2A-mediated components of MC EPSCs after learning. Furthermore, as several studies suggest synaptic changes in GluN2A expression involve increased translation of the protein (Hoffmann et al., 2000), reverse transcription polymerase chain reaction (RT-

PCR) may be a more sensitive approach that would allow quantification of GluN2A mRNA levels at various time points following learning.

Alternatively, it is possible that changes in synaptic GluN2A expression were not observed because they are not involved in olfactory learning-induced plasticity. Perhaps GluN2B is replaced with GluN2C, another subunit critically involved in cerebellar plasticity, or even with GluN3 subunits (Cull-Candy et al., 2001; Prybylowski and Wenthold, 2004). However, the expression level of these subunits is quite low in the OB (Wenzel et al., 1997), making them less likely candidates for OB plasticity. In support of a role for GluN2A in olfactory plasticity are experiments analyzing the sequence in the GluN2A encoding gene. These experiments have revealed a CRE element in the promoter region of the gene that is essential in evoking the developmental increase in GluN2A expression observed in cortical neurons (Desai et al., 2002). This suggests that an increase in GluN2A expression could be mediated by a cAMP-PKA-CREB signalling pathway (Yashiro and Philpot, 2008) and lends further support to the hypothesis that GluN2A expression may be involved in early odor preference learning (although not observed here) as this is a robust cAMP-PKA-CREB model (McLean et al., 1999; Yuan et al., 2000; Yuan et al., 2003b; Yuan et al., 2003a).

4.10 Mechanism of NMDAR subunit removal from the synapse

Early odor preference learning induced a decrease in synaptic expression of the obligatory GluN1 subunit and the GluN2B subunit at different time points post-training.

Removal of synaptic receptor subunits has been proposed to occur via internalization of the receptor, lateral diffusion of the receptor to extrasynaptic sites, or proteasomal degradation of the receptor (Roche et al., 2001; Ehlers, 2003; Lau and Zukin, 2007). NMDAR internalization has been shown to occur via clathrin-mediated endocytosis (Roche et al., 2001). Specifically, internalization of the receptor is dependent on tyrosine phosphorylation of the GluN2B subunit and is prevented by post-synaptic density (PSD)-95 anchoring of the receptor to the synapse. Interestingly, NMDAR internalization occurs predominantly during early life and does not occur in mature neurons. Correspondingly, GluN2B expression decreases and PSD-95 expression increases over the course of development, suggesting that receptor trafficking and internalization play an important role in NMDAR-mediated plasticity during an early developmental period, after which NMDARs become strongly anchored to the synapse (Roche et al., 2001). This supports a role of NMDAR activation, phosphorylation, and potentially internalization in early odor preference learning.

As endocytotic regions usually occur tangential to the synapse, it has been suggested that lateral movement of synaptic receptors helps target them for endocytotic internalization (Lau and Zukin, 2007). Phosphorylation of the NMDAR by protein kinase C (PKC) has been shown to induce lateral movement of the receptor to extrasynaptic sites in cultured hippocampal neurons (Fong et al., 2002). Furthermore, lateral movement of GluN2B containing receptors occurs faster and more efficiently than lateral movement of GluN2A containing receptors, suggesting a general decrease in lateral mobility with development (Groc et al., 2006). In fact, studies have shown the subunit composition of

extrasynaptic receptors to be different from those located at the synapse, with extrasynaptic receptors containing a higher proportion of the GluN2B subunit while synaptic receptors contain a higher proportion of the GluN2A subunit (Li et al., 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999). This further supports the idea that synaptic plasticity involves changes in NMDAR subunit expression at the synapse and supports a role for lateral mobility in these changes. Therefore, it is possible that the learning-induced decrease in synaptic expression of GluN1 and GluN2B occur via lateral diffusion of the receptors to extrasynaptic endocytotic regions, followed by endocytotic internalization of the receptors. In addition, the fact that internalization and lateral diffusion of the NMDAR occurs predominantly during the early developmental period parallels the nature of this learning model as well as the developmental pattern of receptor expression observed at this time.

While lateral diffusion of the NMDAR has been shown to be induced by PKC-mediated phosphorylation (Fong et al., 2002; Groc et al., 2004), a role for PKC in early odor preference learning has yet to be determined. In fact, PKC activation is most often associated with activation of the α1-adrenoceptor (Koshimizu et al., 2002, review). While β-adrenoceptor activation has been shown to be necessary and sufficient for early odor preference learning (Sullivan et al., 2000), it is possible that α1-adrenoceptor activation and subsequent PKC recruitment may also play a role in learning (Harley et al., 2006). Future experiments could investigate whether PKC is involved in this learning model as a first step to verifying whether lateral diffusion is involved in the learning-induced decrease in NMDAR subunit expression at olfactory synapses.

Alternatively, there is evidence that NMDAR subunits may be removed from the synapse via proteosomal degradation. Ehlers (2003) reported that addition of proteasome inhibitors to cultured hippocampal neurons could dramatically attenuate an activity-dependent increase in the synaptic GluN2A/GluN2B ratio. More recently, Jurd and colleagues (2008) identified a specific E3 ligase that targets the GluN2B subunit for proteasomal degradation in an activity-dependent manner. While hippocampal LTP has long been known to require the synthesis of new proteins, it was recently shown to also require ubiquitin-mediated protein degradation (Fonseca et al., 2006). These reports suggest that proteasomal degradation of NMDAR subunits may be important in mediating LTP expression and suggest the possibility that a similar mechanism may be involved in OB plasticity.

4.11 Timing differences in NMDAR subunit alterations

Whether activity-dependent alterations in NMDAR trafficking are regulated in a subunit-specific manner is unknown (Yashiro and Philpot, 2008). It has been suggested that the increased synaptic GluN2A expression occurs via an activity-dependent increase in translation and protein synthesis (Quinlan et al., 1999b; Hoffmann et al., 2000), while the activity-dependent decrease in synaptic GluN2B and GluN1 expression is thought to reflect either endocytosis, lateral movement and/or degradation (Roche et al., 2001; Ehlers, 2003; Lau and Zukin, 2007). As a result, it seems straightforward to think that changes in the relative expression of these subunits may occur at different rates as the mechanisms underlying their synaptic alterations may be quite different. In this way,

there is no "switch" in subunits as changes in the relative expression levels of each subunit occur at different time points because they are mediated by different mechanisms.

In support of this idea, studies investigating the effects of dark-rearing on subunit composition in the visual cortex have reported a slower change in synaptic GluN2A expression compared to changes in GluN2B subunit expression (Chen and Bear, 2007). As well, studies investigating sleep deprivation have shown that depriving animals of sleep significantly reduces hippocampal LTP and this reduction is associated with a decrease in synaptic GluN1 expression, but no changes in GluN2A or GluN2B expression (Chen et al., 2006).

In summary, it seems that regulation of the subunit composition of NMDARs at the synapse may occur in a subunit-specific manner. It seems that each subunit may be trafficked to or from the synapse via several different mechanisms and that this trafficking may not occur at the same rate. The fact that synaptic changes in GluN1, GluN2A and GluN2B expression are not symmetrical suggests that receptors can exist at the synapse in forms other than tetromeric complexes, although are unlikely to be functional. Therefore, the observed decrease in synaptic GluN1 at 3 h post-training, followed by decreased synaptic expression of GluN2B at 24 h reported here may involve specific NMDARs at the same synapse that undergo transient and reversible subunit removal and insertion via different mechanisms. Even so, these subunit changes both are expected to reduce synaptic plasticity at the affected synapse to help stabilize and maintain the memory.

4.12 An increase in the AMPA/NMDA ratio of ON-evoked MC EPSCs is involved in early odor preference learning

The AMPAR- and NMDAR-mediated components of ON-evoked MC EPSCs were measured and compared between occluded and non-occluded OBs of animals who had received unilateral nasal occlusion before undergoing odor preference training. Unilateral nasal occlusion was performed to provide an intra-animal control condition, however with the difficulties of whole cell recording it was not always possible to record MCs from both the occluded and non-occluded OBs of each animal and still remain within an accurate time period post-training during which cells remained healthy. Therefore, ratios recorded from healthy MCs of all non-occluded OBs were analyzed together and compared to those recorded from MCs of all occluded OBs as a measure of learning versus control, respectively. This is considered to be an accurate comparison between the learning condition and the control condition as non-occluded OBs received β-adrenoceptor activation paired with odor, while occluded OBs received only β-adrenoceptor activation. Unilateral nasal occlusion is thought to completely restrict odor stimulation of the ipsilateral OB and cortex (Korol and Brunjes, 1990; Brunjes, 1994, review), allowing each individual OB to be treated independently.

Only MCs within the lateral glomerular layer in both occluded and non-occluded OB slices were sampled in these experiments. Again, this was based on previous studies and our results demonstrating the lateral glomerular layer to be a critical peppermint-encoding region and to undergo significant peppermint preference learning changes (Sullivan and Leon, 1986; Johnson and Leon, 1996). Specific sampling of MCs within

this area of the OB helped to minimize sampling error in that it is less likely that non-odor-encoding MCs were sampled than if a broad selection of MCs from the entire OB had been conducted. Of course, this does not eliminate sampling error as there are likely non-odor-encoding MCs included in both occluded and non-occluded groups, but it certainly reduced sampling error so that learning-induced changes could be more easily separated. Additionally, sampling error may also have occurred in recording from MCs which may have recently been involved in learning, but not necessarily the specific peppermint-odor preference learning incited by the experimenter. Such sampling errors are equally as likely to have occurred in both learning and control groups and it was expected that a large number of cells would have to be sampled to eliminate such variability. The fact that a clear learning-related difference was observed in the number of cells sampled is indicative of the dramatic nature and degree of this change in AMPA/NMDA ratio.

The transient nature of the observed increase in MC AMPA/NMDA ratio within the learning condition compared to the control condition parallels the transient synaptic NMDAR expression pattern observed using Western blotting. However, it remains to be tested whether the change in the ratio of AMPAR/NMDAR mediated currents is mainly due to an increase in the AMPAR-mediated current or a reduction in the NMDAR-mediated current, or a combination of both. We attempted to measure AMPA mEPSCs from MCs to examine the involvement of AMPARs and/or pre-synaptic changes, as discussed below. Alternatively, constructing an input-output relationship by measuring pre-synaptic volleys and post-synaptic AMPA EPSC peak amplitudes at various

stimulation intensities may help us to determine whether a relatively greater AMPAR response is presented in the learning group. From immunohistochemistry and Western blot data, it appears that both AMPAR subunit insertion (Cui, et al., 2011) and NMDAR subunit removal (our data) could be involved in changes of MC properties following early odor preference learning.

4.13 Absence of mEPSCs in MCs

Investigation of mEPSC amplitude and frequency are often used in physiological plasticity models to determine changes in pre- and post-synaptic expression mechanisms. Here we attempted to measure mEPSCs in MCs from the odor-encoding region of occluded and non-occluded OBs to help determine whether early odor preference learning is mediated by pre- and/or post-synaptic mechanisms. Unfortunately, we observed that MC mEPSCs occur very infrequently (<0.1 Hz) and are very irregular in nature, at least in this acute slice setup. Similarly, Hsia et al. (1999) were also unable to record mEPSCs from MCs *in vitro*. In fact, the only report of mEPSCs recorded from MCs was conducted in cultured cells where the likely source of mEPSCs were MCs themselves and is not a realistic impression of *in vivo* circuitry connections (Schoppa and Westbrook, 1997). Therefore, we can conclude that this method of investigation is not appropriate for studying MC potentiation mechanisms in this model.

4.14 Transient decrease in the PPR of ON-evoked MC EPSCs suggests a pre-synaptic mechanism in early odor preference learning

While the PPR of ON-evoked MC EPSCs was not significantly different between cells from the learning condition and control condition at either 1-3 h or 24 h post-training, when cells collected at 1 h post-training were examined separately, the PPR of the learning condition was significantly lower than the control. In an *in vitro* study, the PPR of ON-evoked glomerular field EPSCs and PG cell EPSCs was significantly reduced at 30 min following TBS of the ON (Yuan, 2009). Together, these results suggest that pre-synaptic mechanisms (such as greater transmitter release from ON terminals in response to the trained odor) could be involved in odor preference learning, at least during the early stages of memory formation.

4.15 Conclusions & future directions

These experiments have shown the NMDAR to play a critical role in early odor preference learning in neonate rats. First, learning is associated with a significant increase in phosphorylation of the NMDAR at odor-specific glomerular synapses during training. More importantly, blocking the NMDAR at these glomerular synapses during training completely prevents learning. Together these results establish a causal role of the NMDAR in the *induction* of learning. Next, early odor preference learning induces a transient decrease in synaptic NMDA GluN1 expression at 3 h post-training and a slower change of synaptic GluN2B subunit expression at 24 h. Transient changes of the NMDAR

synaptic expression profile are thought to be important for *maintaining* the existing memory while reducing the plasticity for competing new memory. Finally, using *ex vivo* electrophysiology, it was shown that the AMPA/NMDA ratio of MC EPSC components was significantly higher in the learning cohort compared to the control. Together, these experiments not only highlight a critical role of the NMDAR in this associative learning model, but provide substantial support for the enhanced MC excitation model of early odor preference learning proposed by McLean's and our labs (Yuan et al., 2003a).

Experiments such as those described here are important for obtaining a deeper understanding of fundamental learning mechanisms as well as NMDAR functioning. Inappropriate or abnormal functioning of the NMDAR is involved in many neurological disease states including schizophrenia, Alzheimer's disease, drug addiction and cell death caused by stroke (Cull-Candy et al., 2001). Investigation of the fundamental properties of learning and memory, including the role of the NMDAR, is essential in uncovering the pathological processes involved in neurological disease and memory loss and is essential in translating research into novel clinical approaches in the future.

Future research directed from my thesis work should focus on further dissecting the synaptic locus of MC-LTP following learning. Such experiments should include investigation of potential pre- and post-synaptic mechanisms using *ex vivo* electrophysiological recording from trained animals. In addition, as the current experiments described here suggest the involvement of pre-synaptic mechanisms, the next step is to identify which pre-synaptic proteins are involved in this plasticity. This may be done through the use of Western blot and RT-PCR, which allows for the direct

measurement of pre-synaptic proteins and their mRNA expression following learning. Future experiments should also focus on determining the source of calcium entry during plasticity as calcium is an essential modulator of synaptic plasticity. While we have shown here that the NMDAR is involved in early odor preference learning, whether its activation is the only source of calcium entry into the cell during plasticity is unknown. It is possible that the depolarization and excitation induced by NMDAR activation could allow for the cell membrane to reach the activation threshold for voltage-gated calcium channels. In addition, future studies may focus on the role of the GluN2B subunit in early odor preference learning. For instance, whether GluN2B expression is essential for learning to occur may be tested using a conditional knock-out model whereby the subunit is knocked down at a specific time point immediately before learning. Furthermore, future experiments should aim to determine the mechanisms of NMDAR removal from the synapse, whether this occurs via internalization, lateral diffusion, or both is not known.

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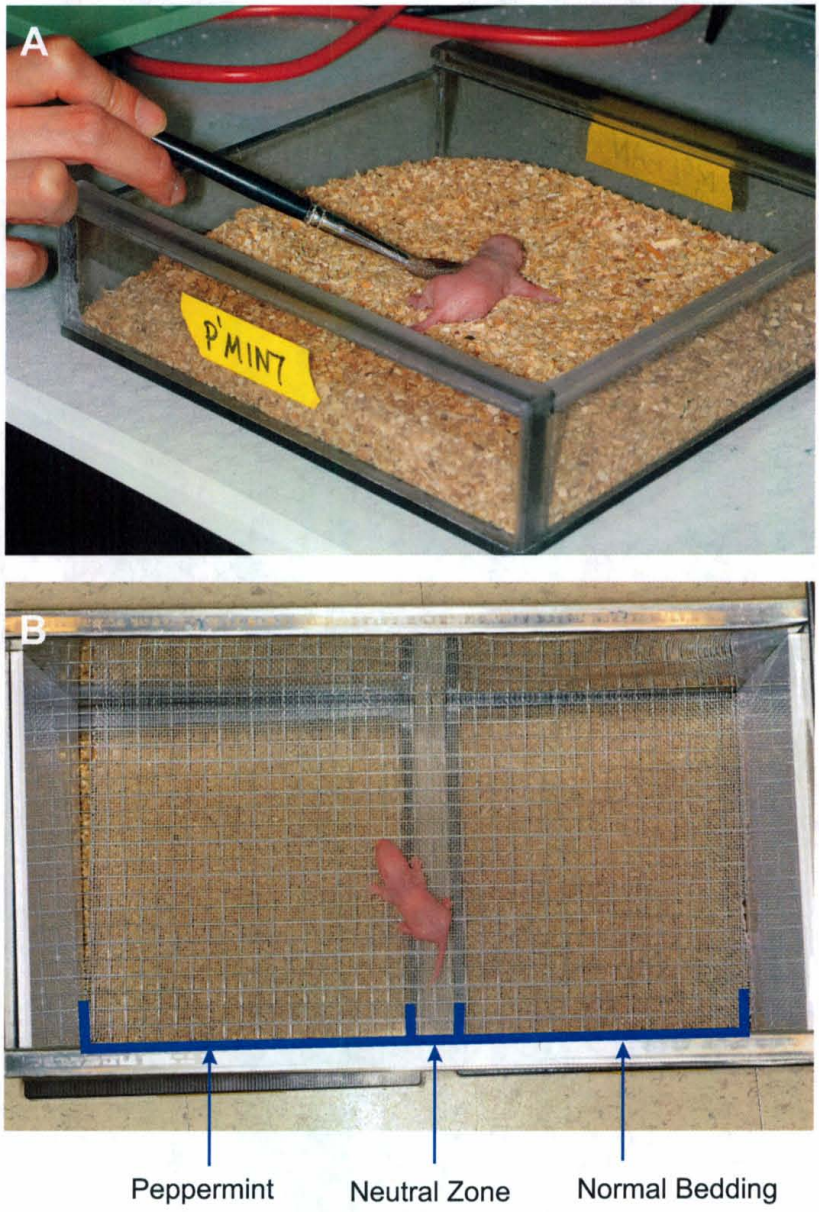


Figure 1. Early odor preference training and testing.
A. On PD 6, pups are individually removed from the nest and receive stroking on peppermint-scented bedding every other 30 sec for a 10 min period. ***B.*** On PD 7, pups undergo two-choice odor testing. They are placed in the neutral zone and allowed to move freely. Time spent over peppermint-scented bedding and unscented bedding is calculated.

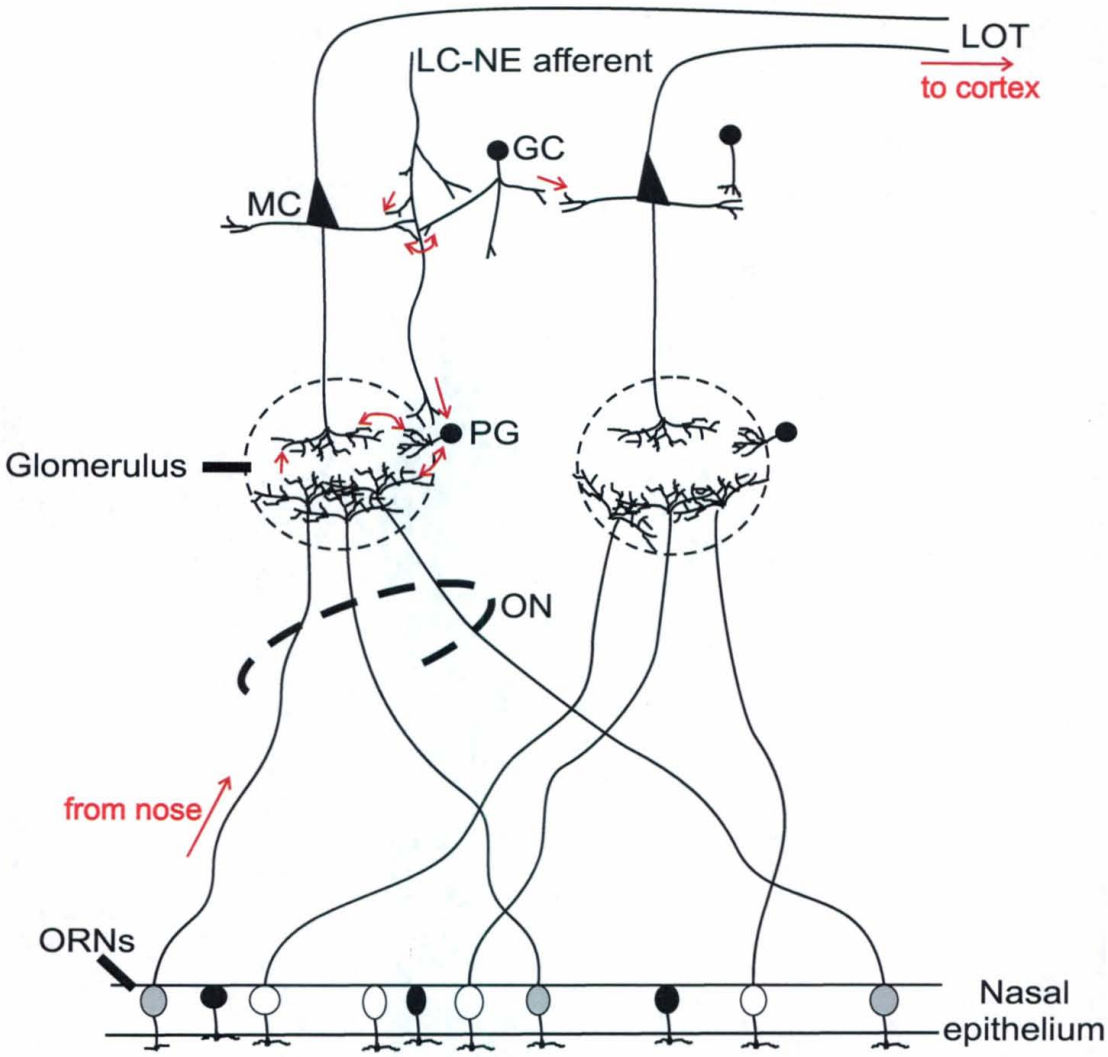
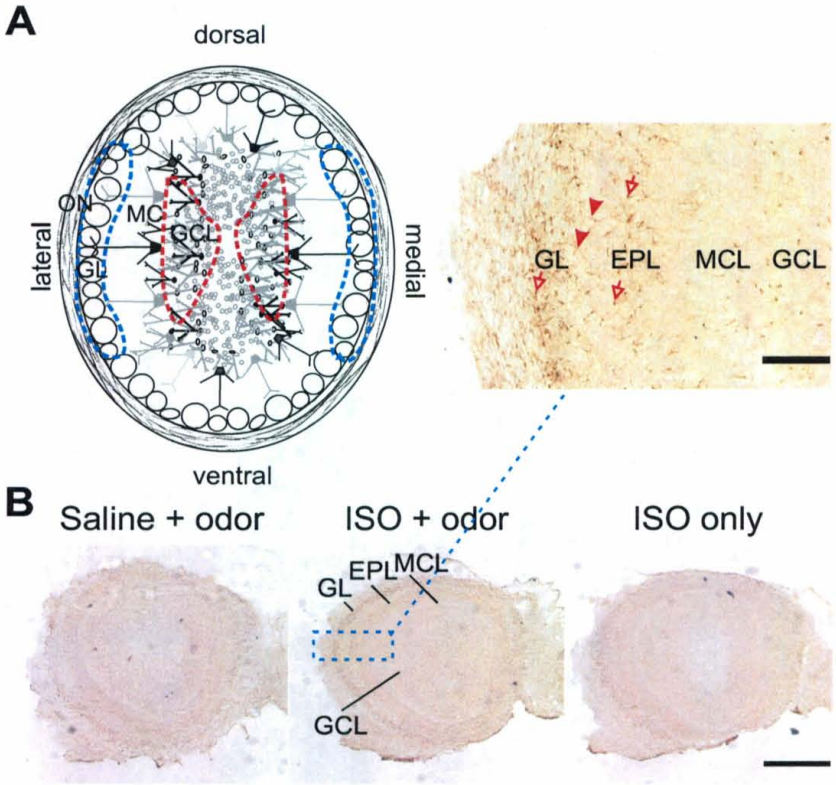


Figure 2. Olfactory bulb circuitry.
ORN, olfactory receptor neuron. ON, olfactory nerve. PG, periglomerular cell. MC, mitral cell. GC, granule cell. LC, locus coeruleus. NE, norepinephrine. LOT, lateral olfactory tract.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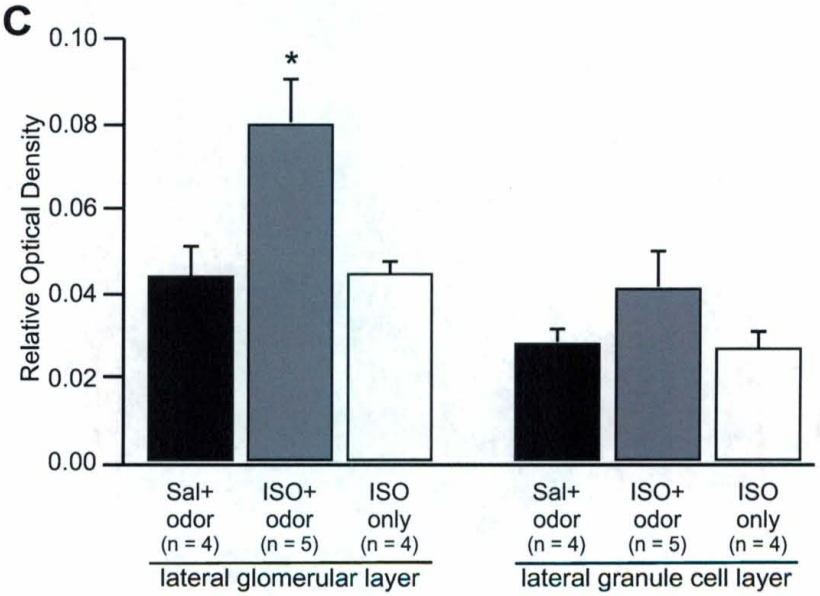
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Figure 3. Early odor preference learning induces PKA-mediated phosphorylation of the obligatory NMDA GluN1 subunit.
A. The regions of the lateral and medial glomerular layer (blue) and granule cell layer (red) that were analyzed to obtain a relative optical density measurement of pGluN1 staining. ON, olfactory nerve. GL, glomerular layer. MCL, mitral cell layer. EPL, external plexiform layer. GCL, granule cell layer. ***B.*** pGluN1 immunohistochemistry. Inset shows higher magnification of lateral olfactory bulb. Arrow heads indicate processes (possibly dendritic or glial) while hollow arrows indicate small cellular staining. ***C.*** Analysis of relative optical density of pGluN1 staining in lateral glomerular and granule cell layers. $*p < 0.05$.

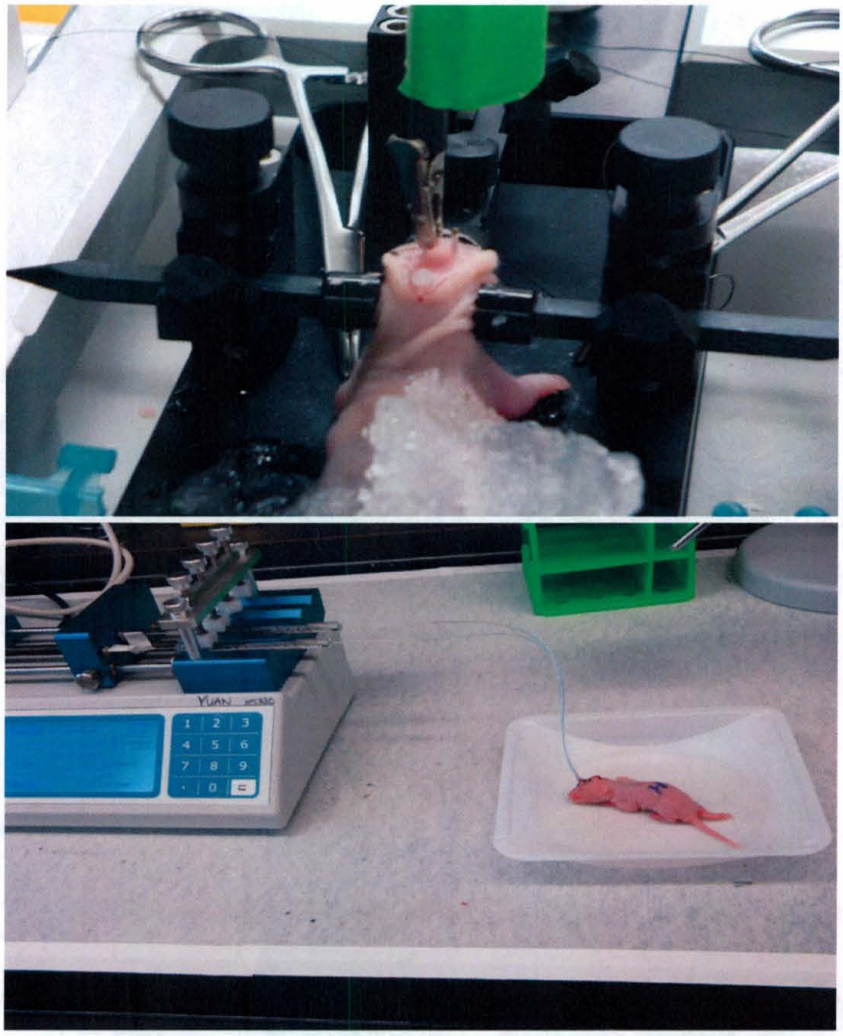


Figure 4. Cannula implantation surgery and infusion.
A. On PD 5, pups are anaesthetized via hypothermia and undergo cannula implantation surgery. ***B.*** Pups receive bilateral infusion of either aCSF or the drug of interest during training on PD 6, or methylene blue dye infusion after testing on PD 7 to verify cannula placement.

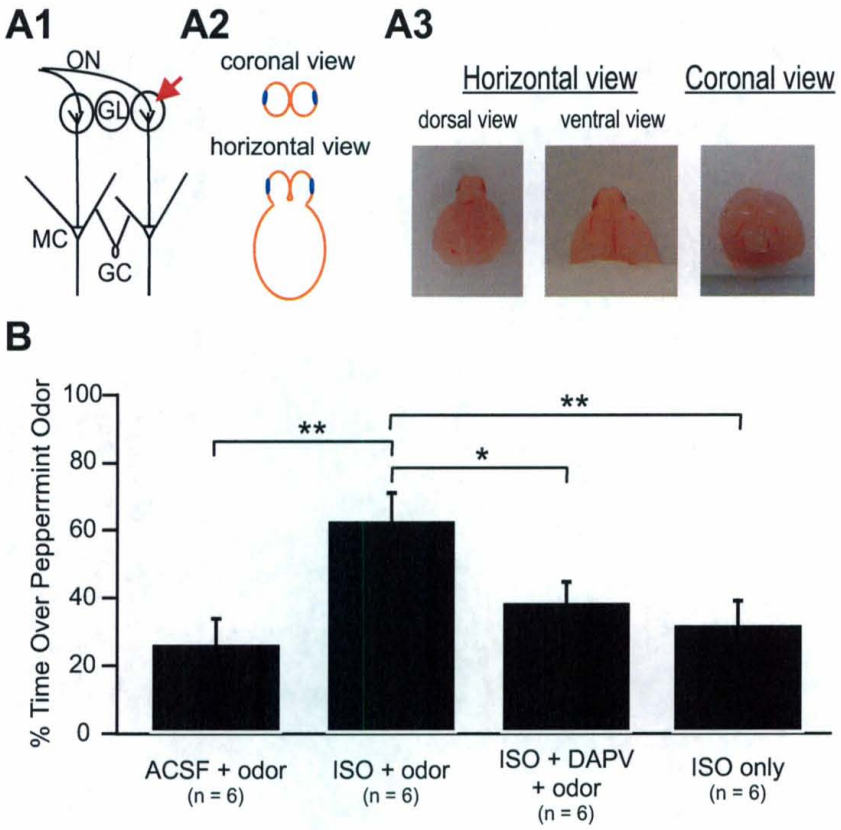


Figure 5. Intrabulbar infusion of the NMDAR antagonist D-APV completely prevents neonatal odor preference learning.
A1. Schematic of olfactory bulb circuitry. Red arrows indicates the olfactory nerve-mitral cell (ON-MC) synapse which was targeted using the lateral infusion protocol. ***A2-A3.*** Schematic and photos of lateral infusion site in a coronal and horizontal view following methylene blue dye infusion. ***B.*** Intrabulbar infusion of the NMDAR antagonist, D-APV, completely prevents early odor preference learning. Bars show the percentage of time spent over the peppermint-scented side of a two-choice odor test box. $^{**}p < 0.01$, $^{*}p < 0.05$.

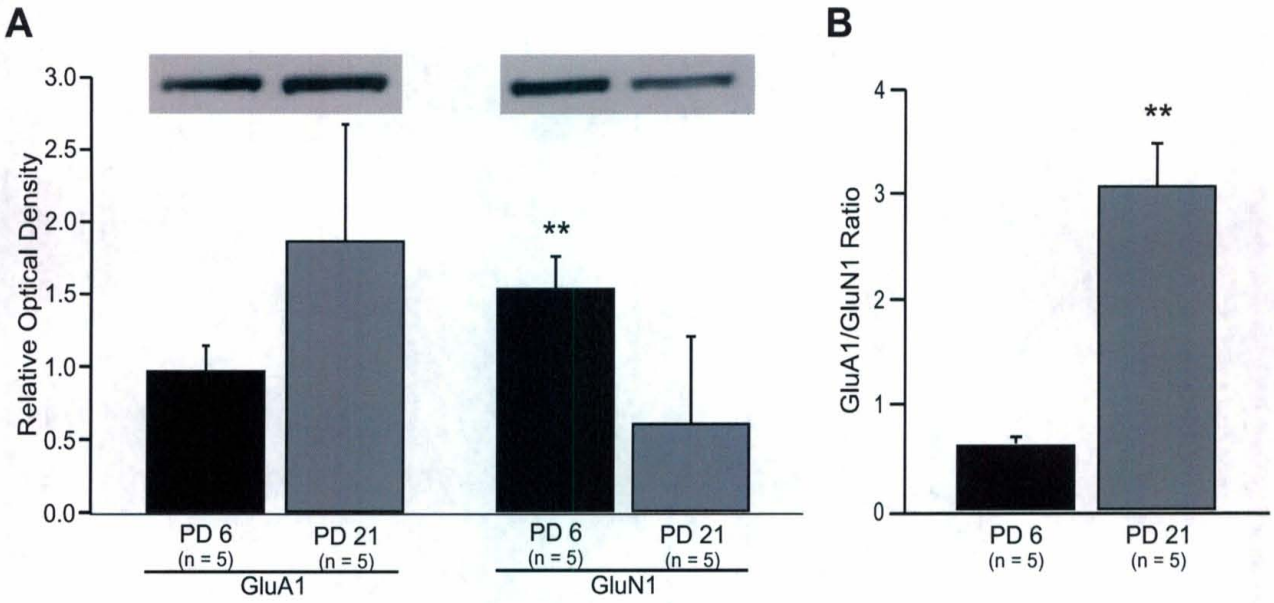


Figure 6. Developmental profile of synaptic GluA1 and GluN1 expression in the olfactory bulb. ***A.*** GluA1 subunit expression of the AMPAR shows a moderate but variable increase in expression from PD 6-21. Expression of the GluN1 subunit of the NMDAR decreases at olfactory bulb synapses from PD 6-21. $^{**}p < 0.01$. ***B.*** The relative synaptic expression of GluA1/GluN1 at olfactory bulb synapses is significantly higher in older animals. $^{**}p < 0.01$.

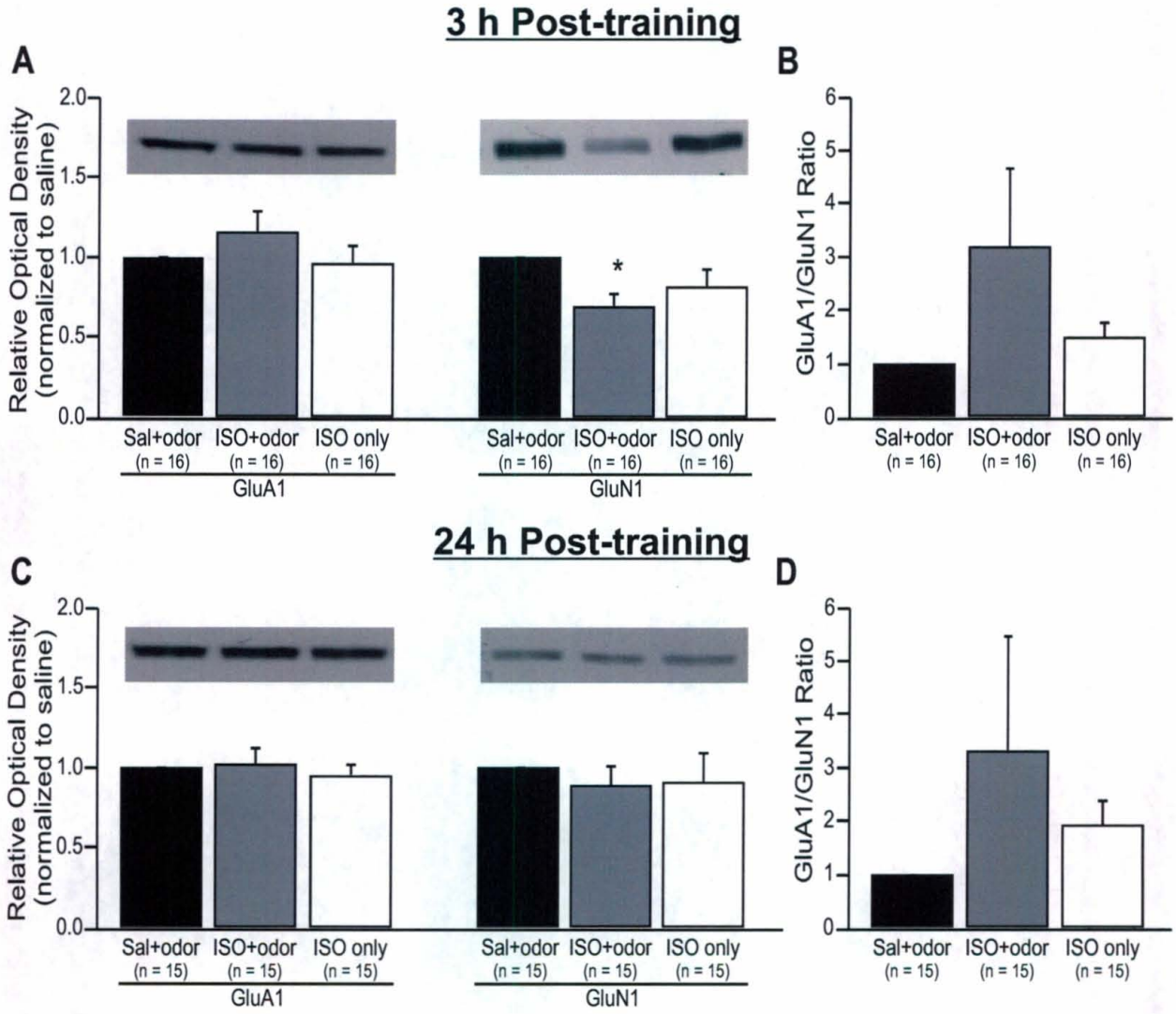


Figure 7. Early odor preference learning induces a transient and reversible removal of NMDAR GluN1 subunits from olfactory bulb synapses.

A. At 3 h following early odor preference learning there is no difference in synaptic GluA1 expression between experimental groups. Synaptic GluN1 expression is significantly reduced in learning animals compared to sal+odor controls at 3 h following training. *$p < 0.05$. ***B.*** The relative ratio of synaptic GluA1/GluN1 expression is not significantly different between groups at 3 h post-training. ***C.*** At 24 h post-training there is no difference in synaptic GluA1 or GluN1 expression between groups. ***D.*** There is no difference in the relative ratio of synaptic GluA1/GluN1 expression at 24 h following training.

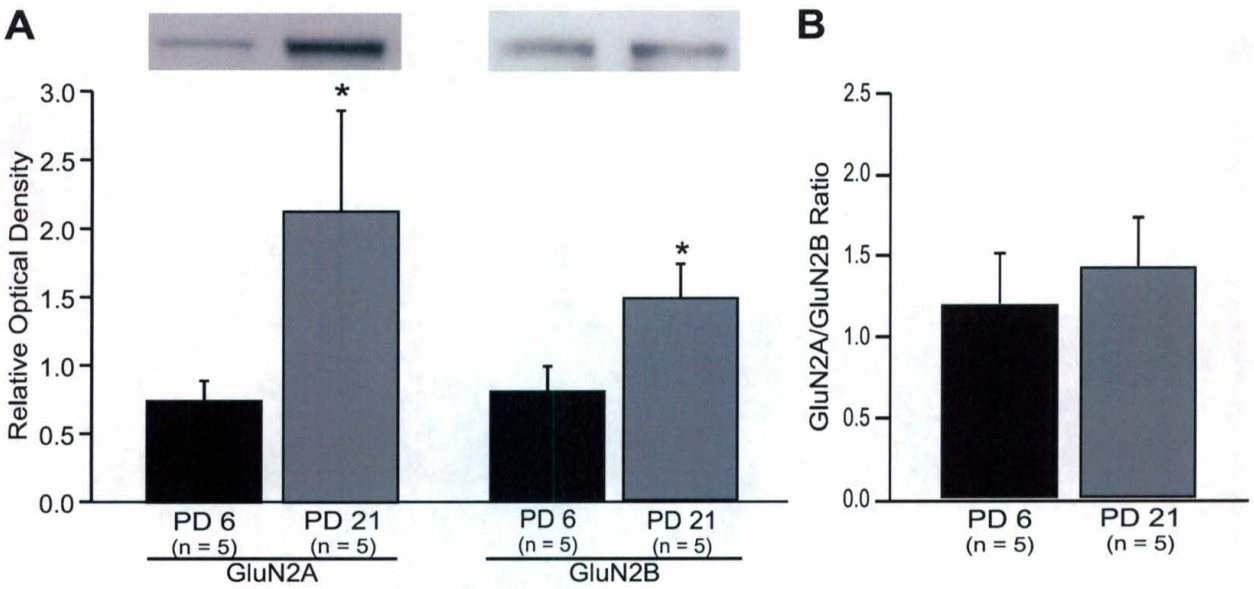


Figure 8. Developmental profile of synaptic GluN2A and GluN2B expression in the olfactory bulb. ***A.*** Synaptic GluN2A and GluN2B expression increase from PD 6-21. *$p < 0.05$. ***B.*** The synaptic ratio of GluN2A/GluN2B expression in the olfactory bulb does not change significantly from PD 6-21.

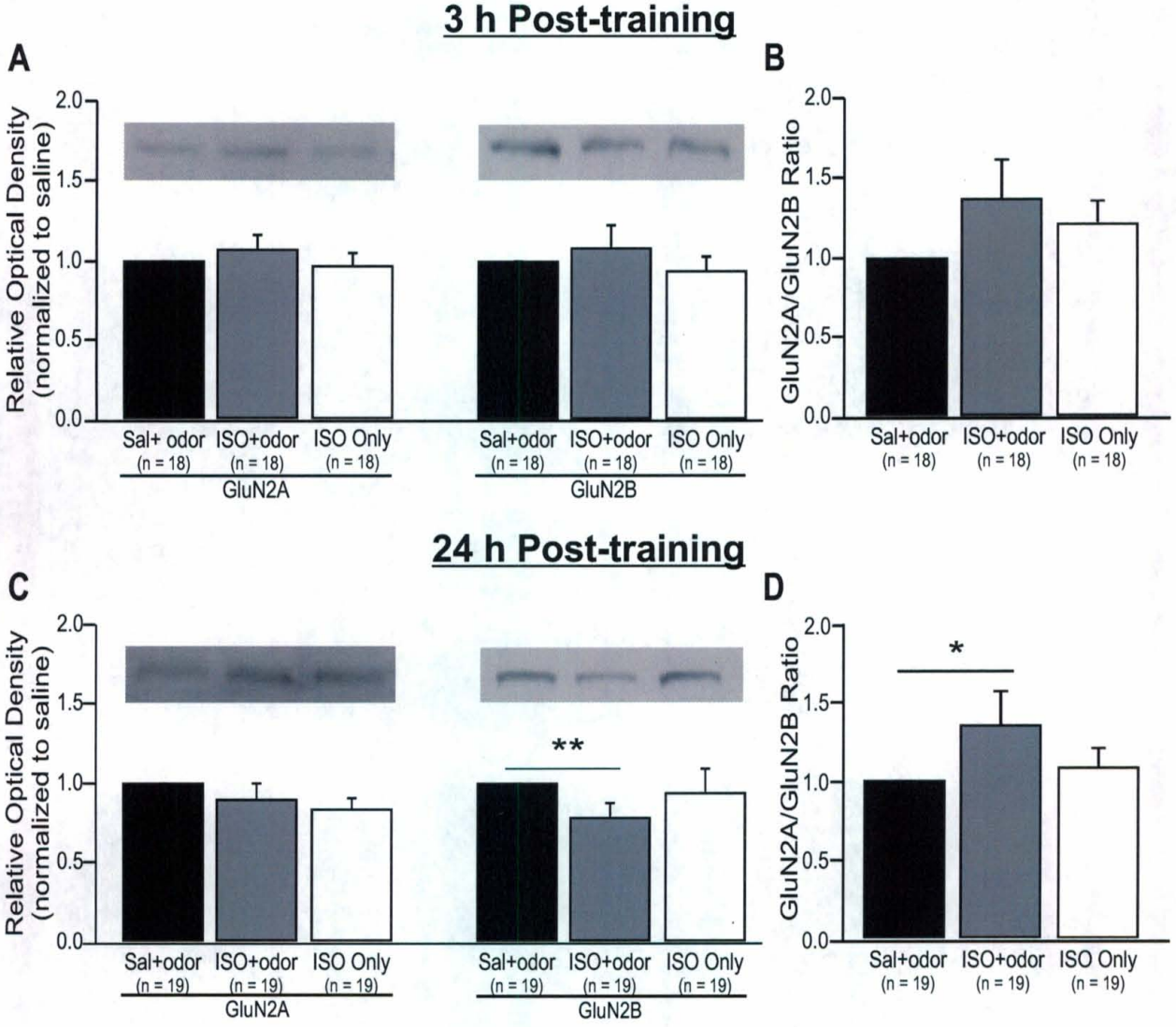
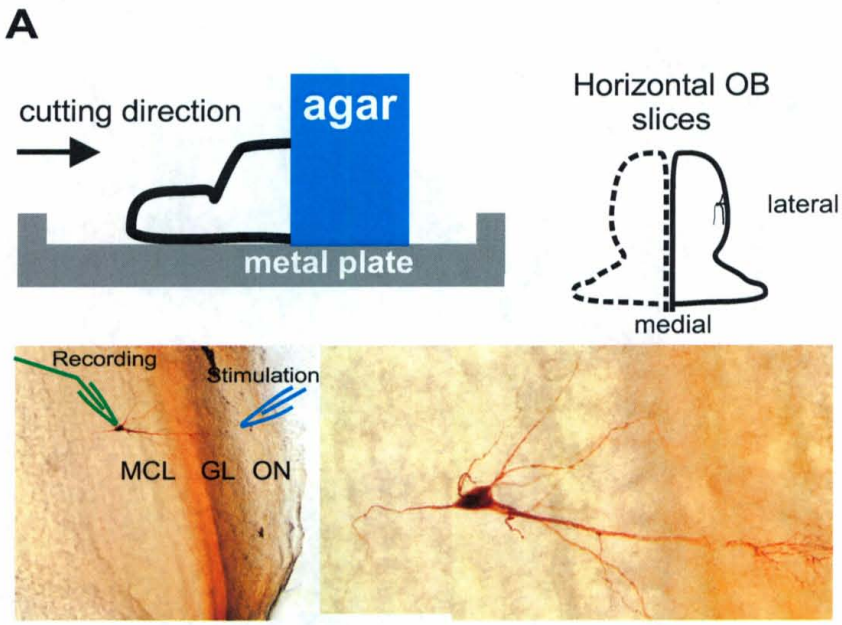
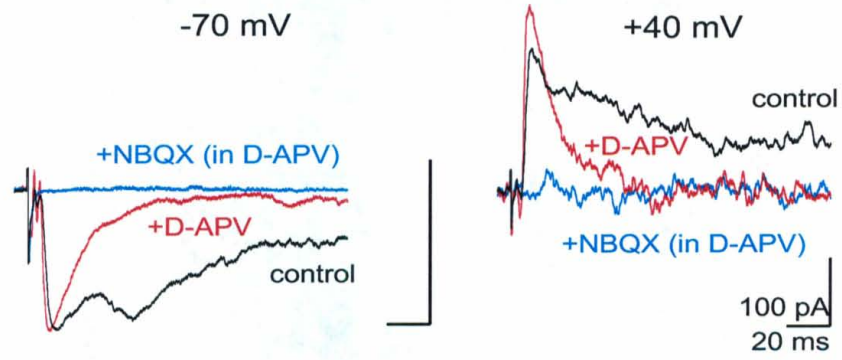


Figure 9. Early odor preference learning induces the removal of NMDA GluN2B subunits from olfactory bulb synapses.

A. At 3 h following training there is no change in synaptic GluN2A or GluN2B expression. ***B.*** The ratio of synaptic GluN2A/GluN2B expression is not significantly different between groups at 3 h post-training. ***C.*** Synaptic GluN2B expression is significantly reduced in the learning group compared to sal+odor controls at 24 h post-training. $^{**}p < 0.01$. ***D.*** The synaptic GluN2A/GluN2B ratio is significantly higher in learning animals compared to the sal+odor control group at 24 h following learning. $^{*}p = 0.05$.


A
cutting direction
agar
metal plate
Horizontal OB slices
lateral
medial
Recording
Stimulation
MCL GL ON


B Isolating AMPA and NMDA components in one cell
-70 mV
+40 mV
+NBQX (in D-APV)
+D-APV
control
control
+D-APV
+NBQX (in D-APV)
100 pA
20 ms

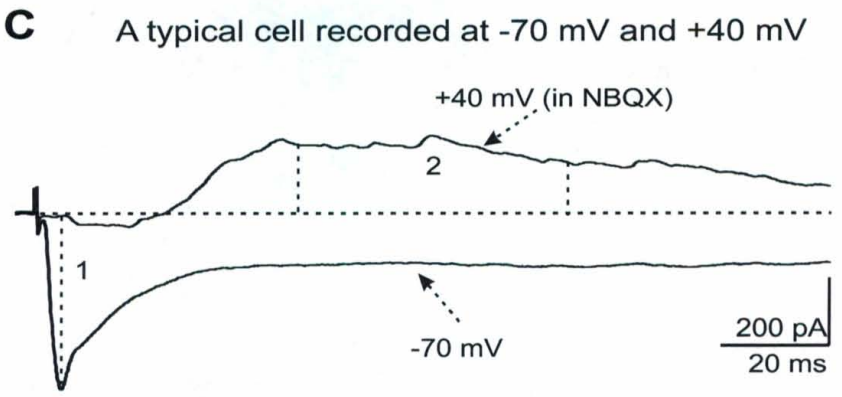

C A typical cell recorded at -70 mV and +40 mV
+40 mV (in NBQX)
2
1
-70 mV
200 pA
20 ms

Figure 10. *In vitro* electrophysiology methods and experimental setup. ***A.*** Horizontal olfactory bulb slices were obtained using a vibrating slicer. Occluded and non-occluded slices were hemisected and incubated separately. Mitral cells (MCs) were recorded from the mid-lateral region of the olfactory bulb. A bipolar stimulating electrode was placed in the olfactory nerve layer near the glomerulus where apical dendrites of the recorded MCs terminated. Biocytin staining of a patched MC shows the location of the cell in the lateral olfactory bulb and its primary and secondary dendrites. ***B.*** ON-evoked MC EPSCs recorded at VC -70 mV consist of a fast AMPA-mediated peak that is abolished by the addition of NBQX (AMPAR antagonist) to the bath. A slower, long-lasting NMDA-mediated component is abolished by the addition of D-APV (NMDAR antagonist) to the bath. ON-evoked MC EPSCs recorded at VC +40 mV similarly display AMPA and NMDA components that are abolished by bath application of NBQX and D-APV, respectively. ***C.*** The AMPA component of an ON-evoked MC EPSC was measured as the amplitude of the fast peak immediately following ON stimulation at VC -70 mV (1). The NMDA component of an ON-evoked MC EPSC was measured as the average amplitude within 50-100 ms following ON stimulation at VC +40 mV in the presence of NBQX (2).

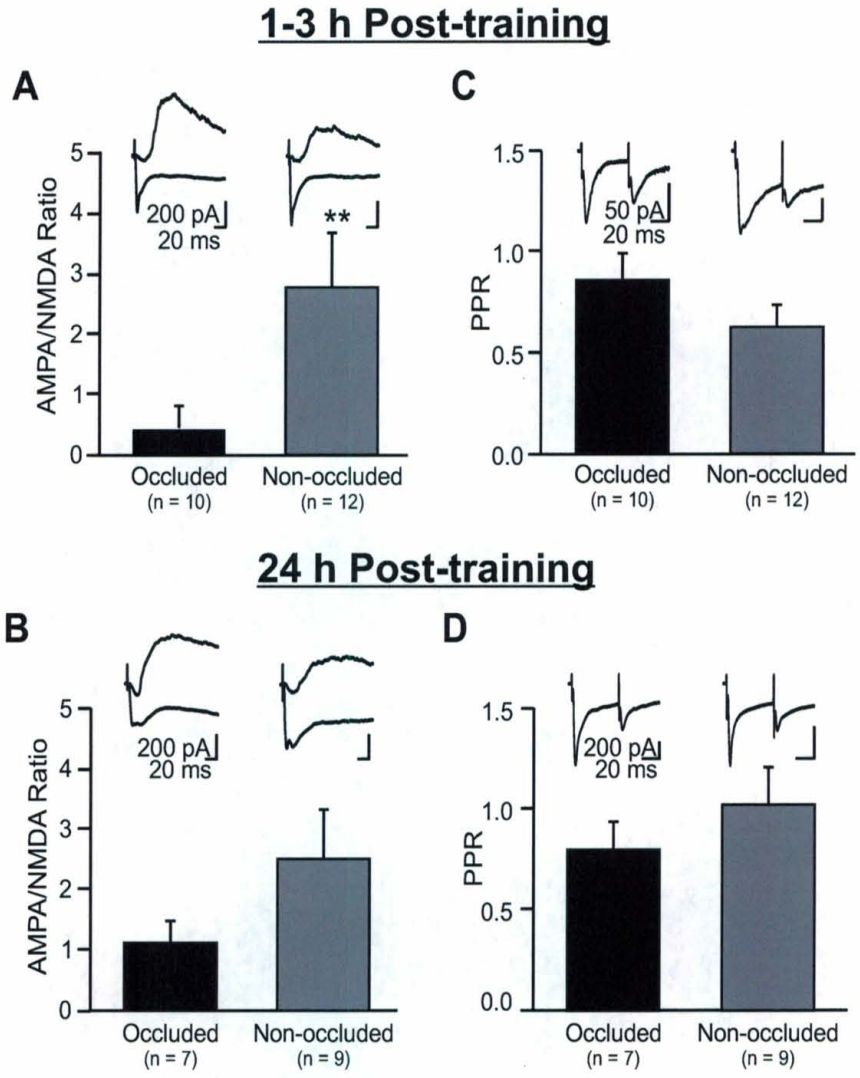


Figure 11. Early odor preference learning induces a transient decrease in the AMPA/NMDA ratio and the PPR of ON-evoked MC EPSCs within the odor-encoding region of the olfactory bulb. ***A.*** The AMPA/NMDA ratios of ON-evoked MC EPSCs recorded from non-occluded olfactory bulbs was significantly higher at 1-3 h post-training compared to occluded olfactory bulbs. $^{**}p = 0.01$. ***B.*** At 24 h post-training the AMPA/NMDA ratios of ON-evoked MC EPSCs recorded from non-occluded and occluded olfactory bulbs were not significantly different. ***C.*** At 1-3 h post-training the PPRs of ON-evoked MC EPSCs were not significantly different. ***D.*** The PPRs of ON-evoked MC EPSCs were not significantly different between occluded and non-occluded olfactory bulb slices at 24 h post-training.

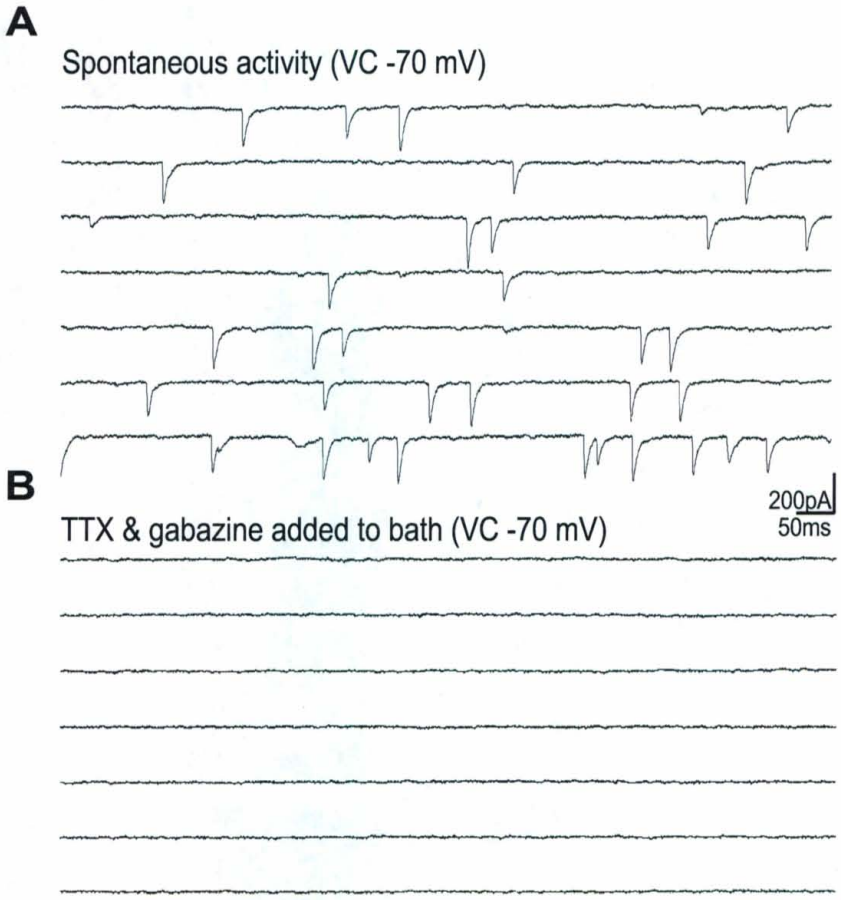


Figure 12. Absence of mEPSCs in MCs.
A. Spontaneous firing of a MC in whole-cell mode at VC -70 mV.
B. Recording of mEPSCs from the same MC held at VC -70 mV when TTX (1 μM) and gabazine (5 μM) are present.

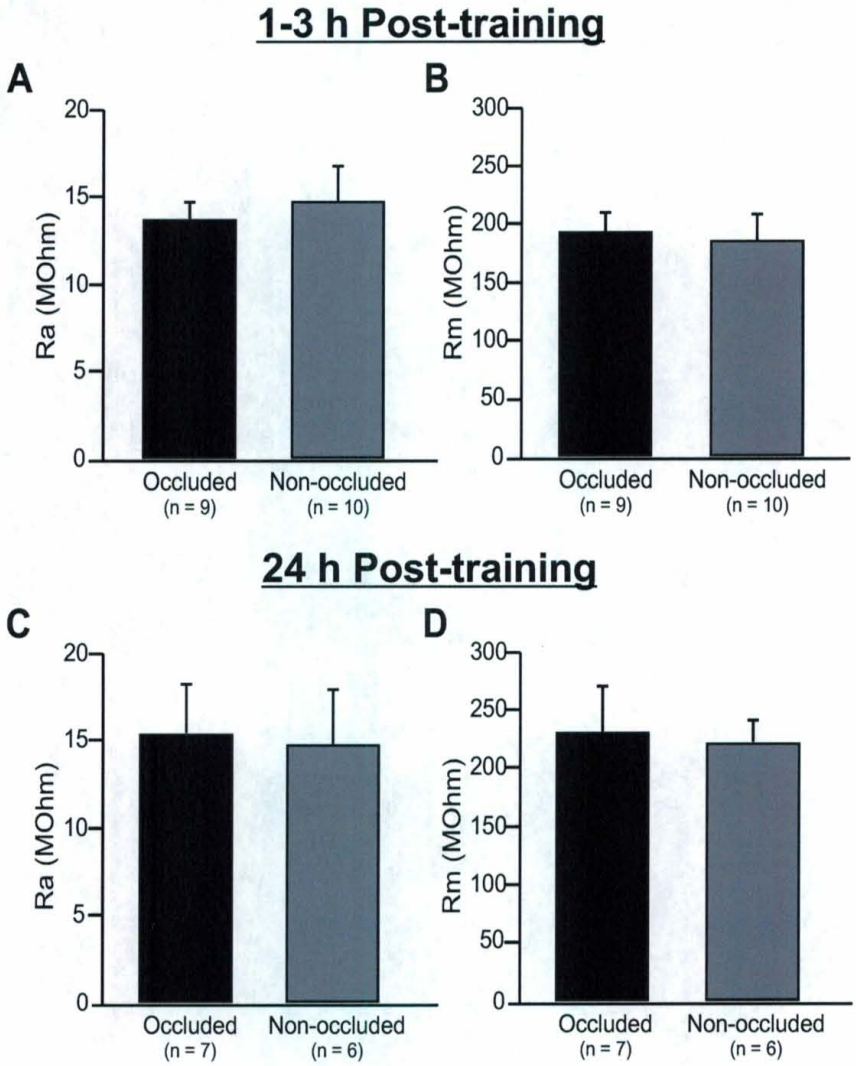


Figure 13. Access and membrane resistance values of recorded MCs. ***A.*** Access resistance values of MCs recorded from occluded and non-occluded slices at 1-3 h post-training were not significantly different. ***B.*** Membrane resistance values of MCs recorded from occluded and non-occluded slices at 1-3 h post-training were not significantly different. ***C.*** MCs recorded from occluded and non-occluded slices at 24 h post-training showed similar access resistance values. ***D.*** Membrane resistance values of MCs recorded from occluded and non-occluded slices at 24 h post-training were similar.

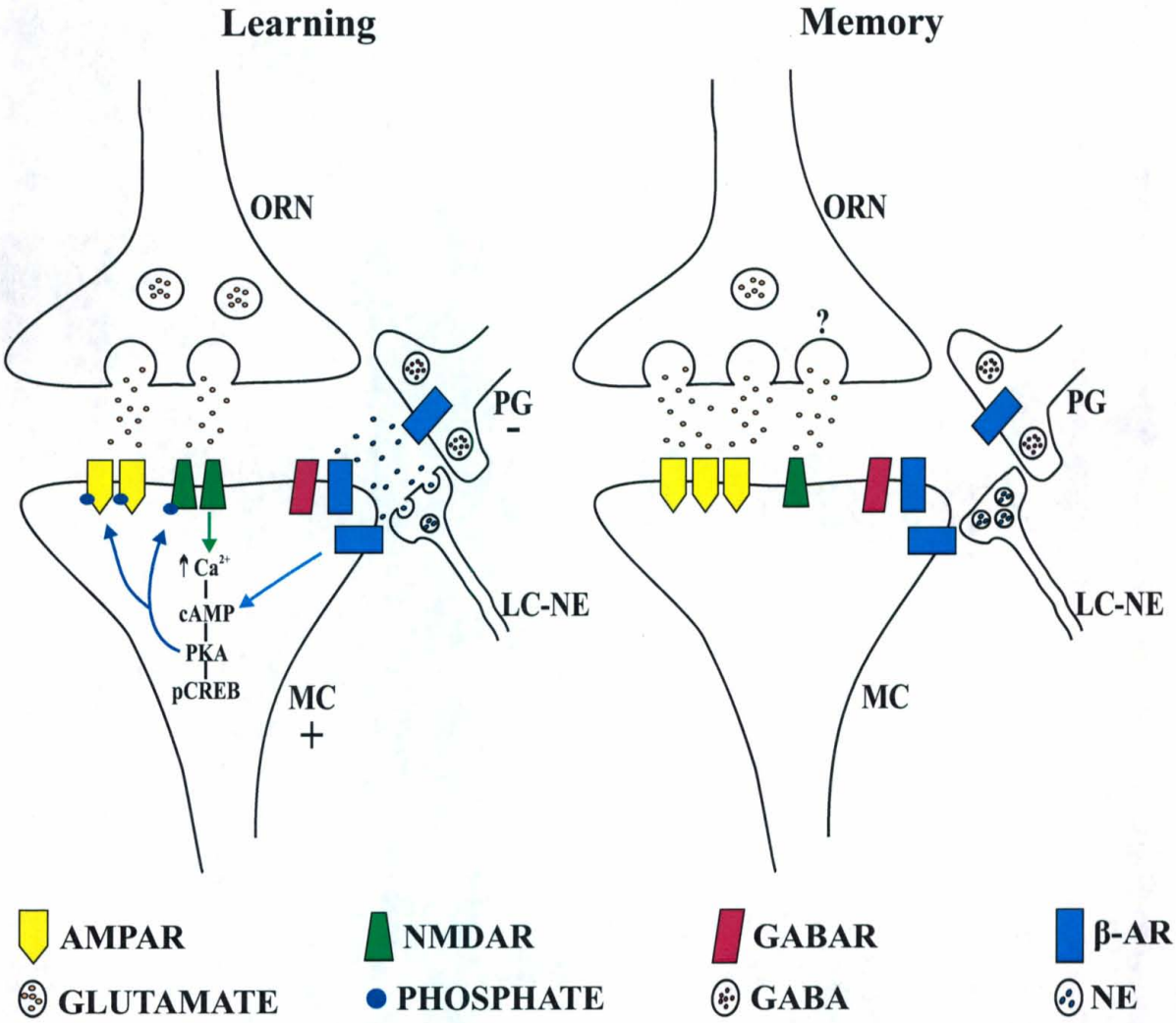

Learning
Memory
ORN
ORN
?
PG
PG
LC-NE
LC-NE
↑ Ca2+
cAMP
PKA
pCREB
MC
+
MC
AMPAR
NMDAR
GABAR
β-AR
GLUTAMATE
PHOSPHATE
GABA
NE

Figure 14. NMDAR involvement in early odor preference learning. During learning, odor mediated glutamate release from ORN terminals (CS) binds to both AMPARs and NMDARs on the post-synaptic MC membrane. Simultaneously, β-adrenoceptor activation (UCS, NE release from LC afferents) on MCs and/or PG cells (causing MC disinhibition) results in post-synaptic MC membrane depolarization. Together, the CS and UCS converge on MCs to meet activation requirements of the NMDAR, allowing long-lasting MC excitation and significant calcium influx. This results in the recruitment of intracellular signaling cascades which may lead to several downstream effects including phosphorylation of synaptic AMPARs and NMDARs as well as the transcription factor CREB. Early odor preference memory may be partly mediated by learning-induced removal of NMDARs and insertion of AMPARs into the post-synaptic MC membrane resulting in enhanced activation of odor-encoding MCs upon odor presentation. Pre-synaptic changes such as increased glutamate release from ORN terminals in response to odor presentation may also be involved in early odor preference memory.