LOVE OR HATE: DIFFERENTIAL EXPRESSION OF SYNAPTIC RECEPTORS FOLLOWING ODOR PREFERENCE VERSUS AVERSIVE LEARNING IN RAT PUPS?

By

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A Thesis submitted to the

School of Graduate Studies

In partial fulfillment of the requirements for the degree of

Master of Science, Medicine

Faculty of Medicine

Memorial University of Newfoundland

Octobwe 2016

St. John's Newfoundland Canada

ABSTRACT

Odor plus 0.5mA shock conditioning paradoxically induces odor preference in rat pups (≤PD10), while a strong 1.2mA shock results in odor aversion (Sullivan, 2001). Previous research showed that anterior piriform cortex (aPC) is activated following odor preference training with a 0.5mA shock, while posterior piriform cortex (pPC) is activated following odor aversive learning with a 1.2mA shock. The olfactory bulb (OB) is activated by both and serves as a common structure (Raineki, Shionoya, Sander, & Sullivan, 2009a). As a first step to delineate synapses involved in preference and avoidance learning, we measured expressions of glutamatergic AMPA GluR1 and NMDA NR1 receptors in the OB, aPC, & pPC using a synaptoneurosome preparation following odor+shock conditioning.

Our results show that a shock of 0.5mA and 0.1mA, produced preference 24-hours following learning indicating that aversive experiences can produce preference in neonatal rodents as previously reported by Sullivan (2001).. Moreover, our results illustrated that this shock resulted in down-regulation of NMDARs 3-hours following training but not of AMPARs in the OB. Our behavioural results did not produce odor aversion with the strong shock training and likewise there was no change in pPC, suggesting no change in total number of NMDARs and AMPARs perhaps due to an absence of odor aversion learning. Future experiments can delineate whether different paradigms will produce odor aversion to produce synaptic expression in the pPC. Additional experimental protocols can also assess if each region is engaged in synaptic trafficking of NMDAR and AMPARs within the extrasynaptic and synaptic sites.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my parents and family for their endless support in pursuing my academic career as well as my friends, Sonia Isoufi and Rowan El-Baily. for always being there to lend a helping hand and a listening ear.

I would like to take this opportunity to especially thank Sohaib Mohammad for being my pillar of support and helping me see the best in every situation. I'd also like to thank Dr. Gerald Cupchik and Dr. David Nussbaum; they have greatly contributed to my persistence in completing my master's degree and providing me with pearls of wisdom along the way.

I'd like to thank Dr. Jules Dore for his guidance and for providing me with constructive feedback. His immense knowledge on western blots and biochemical reactions has provided me with a stronger foundation to conduct experiments and given me the opportunity to grow as a novice researcher.

I'd like Dr. John McLean for his technical expertise and for providing me with feedback for improvement. I would also like to thank my co-supervisor Dr. Carolyn Harley for her collaboration in constructing my master's project.

I would like to thank Dr. Qi Yuan for affording me the opportunity to join her lab and her financial support without which my master's education would not have been possible.

I am grateful for all the experiences my master's has provided me with; it has helped me learn, grow and understand where my passion lies. Every experience has been a learning one, and as I navigate through the world, I will turn back to this experience as one that aided me in becoming the person I would like to be.

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Symbols, Nomenclature or Abbreviations

2-DG	2-deoxyglucose
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AMPAR	AMPA receptor
aPC	Anterior piriform cortex
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CREB	Cyclic adenosine monophosphate response element-binding protein
CS	Conditioned stimulus
D-APV	D-amino-5-phosphonopentanoate
DTT	Dithiothreitol
EPSC	Excitatory post-synaptic current
EPSP	Excitatory post-synaptic potential
GABA	Gamma-aminobutyric acid
HSV	Herpes simplex virus
HRP	Horseradish peroxidase
ISO	Isoproterenol
JG	Juxtaglomerular cell
LC	Locus coeruleus
LOT	Lateral olfactory tract
LTD	Long-term depression
LTP	Long-term potentiation
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
ON	Olfactory nerve
OR	Odorant receptor
PD	Postnatal day
PG	Periglomerular
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
pPC	Posterior pirifom cortex
PPR	Paired-pulse ratio
SEM	Standard error of measurement
TBS	Tris-buffered saline
TBS/T	Tris-buffered saline with tween
TTX	Tetrodotoxin
UCS	Unconditioned stimulus

Chapter 1: Introduction

Overview:

The survival of altricial infants depends on attachment to the caregiver — a process that requires infants to recognize and thereafter recall or remember their attachment figure (Sullivan & Holman, 2010). Upon birth neonatal rodents form rapid attachment to the mother, which will ensure survival of the newborn pup. Remarkably, this ability to form an attachment occurs despite the lack of vision and audition and solely rests on olfactory and somatosensory cues (Wilson & Sullivan, 1994b).

Survival-dependent learning is supported by *imprinting*, which refers to any kind of phase-sensitive learning occurring independent of the consequences of the behavior. It is also temporally limited within the sensitive period; rodents for example experience the sensitive period from birth until post-natal day (PD) 10 (Sullivan, 2003; Sullivan & Holman, 2010). Attachment formation can be viewed from a unique lens because during this period neonatal rodents are predisposed to rapidly acquire approach behaviors. This sensitive period is also a vulnerable phase, in which developmental switches can be directly observed and manipulated (Wilson & Sullivan, 1991). As such, this restructuring of behavior can be used advantageously to study learning through associative learning models to experimentally induce preference or avoidance in developing rodents to better understand early life learning using the lens of attachment (Sullivan, Landers, Yeaman, & Wilson, 2000; Sullivan & Holman, 2010).

At birth, neonatal rodents need to acquire robust approach behaviors despite being born blind and deaf; they rely on olfactory cues from the environment to locate the dam (Wilson,

2000). Researchers take advantage of this approach tendency to assess learning (preference/approach or dislike/avoidance) using associative learning models. In associative learning paradigms, a novel odor like peppermint is paired with an unconditioned stimulus (e.g. stroke: which mimics maternal care-giving behavior such as licking) eliciting a pair-bonding dynamic following conditioning (Sullivan, Wilson, Kim, & Leon, 1988; Wilson & Sullivan, 1994a). Learning is assessed by whether rodents prefer the learned odor paired with a stroke. Other associative learning models include odor-shock, which also allows direct evaluation of the approach behaviors during the sensitive period (Raineki et al., 2009a; Raineki, Cortes, Belnoue, & Sullivan, 2012; Roth & Sullivan, 2005; Sullivan & Holman, 2010; Wilson & Sullivan, 1991). The protocol for odor-shock, however, pairs a shock (mild or strong) with peppermint-scented bedding to produce associative memory. The protocol requires 30 seconds of peppermint scent exposure prior to shock delivery for 1 second, over a span of 11 trials with 1-minute inter-trial resting intervals (Wilson & Sullivan, 1991).

Paradoxically, a mild shock during the sensitive period produces preference, while a strong shock results in aversion (Raineki et al., 2009a). Moreover, the mild shock, which initially produced preference, results in odor aversion beyond PD 10 demonstrating a loss of the predisposition to acquire odor preference after the sensitive period (Raineki et al., 2009a; Sullivan, 2005). Neonatal rodents exhibit odor preference acquisition until PD 10, however not beyond that period (Woo, Coopersmith, & Leon, 1987). This behavioral switch demonstrates developmental changes in behavior following the sensitive period as well as maturation of the rat pup.

Learning induced changes in the brain following associative learning provide a window into the underlying synaptic plasticity that occurs during odor learning within the olfactory

system. Many of the higher cortical structures involved in odor learning and memory are not yet developed or matured during the sensitive period (Sullivan, Wilson, & Leon, 1989a; Sullivan et al., 1991; Woo, Wilson, Sullivan, & Leon, 1996). Therefore, the associative odor-learning model allows for direct evaluation of the role of specific olfactory structures involved in early life learning.

A myriad of processes implicated in odor preference and avoidance behaviors are still not well understood, this is even truer in terms of the cortical structure involvement and the neural connection changes supporting these behaviors. They are currently seen as the result of complex interactions between the rodent's experience, learning and synaptic re-wiring or plasticity (Sullivan, Landers, Flemming, Young, & Polan, 2003; Tempel, Bonini, Dawson, & Quinn, 1983).

My Master's project aimed to directly assess the consequence of odor preference and aversion following odor shock learning on measures of plasticity in the olfactory system, namely the olfactory bulb (OB), anterior piriform cortex (aPC) and posterior piriform cortex (pPC). Previous studies measuring 2-Deoxy-$_{D}$-glucose (2-DG) uptake have demonstrated that the aPC is activated during odor preference, whereas odor aversion primarily activates the pPC with the OB acting as a relay for both (Raineki et al., 2009a; Sevelinges, Sullivan, Messaoudi, & Mouly, 2008). However, the question of whether long-term potentiation-like phenomena and characteristics, which are considered the neural footprints of learning and memory in the brain (Hasselmo & Barkai, 1995) occur in the all the structures implicated in these behaviors has not yet been investigated. The experiments conducted during my master's work aimed to answer these fundamental questions by honing in on the consequential synaptic properties of odor learning during the sensitive period. Specifically, N-methyl-D-aspartate (NMDA) and α-Amino-3-

hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are the primary mediators of long-term potentiation (LTP)-like changes and synaptic plasticity (Brun, Ytterbo, Morris, Moser, & Moser, 2001). The aim for this project was to address: 1. Whether preference and avoidance learning could be induced, as previously reported (e.g. Raineki et al., 2009), using a whole body shock paradigm and various shock settings. For this, the effects on odor preference behavior of a mild shock of 0.1mA paired with peppermint odor, a moderate shock of 0.5mA paired with peppermint odor and a strong shock of 1.2mA paired with peppermint odor were compared to odor only rodents during the critical period (PD 6-7) and, 2. The roles of NMDA and AMPA receptor in mediating this learning were probed by measuring NMDA subunit NR1 and AMPA subunit GluR1 in both preference and avoidance paradigms in the OB, aPC and pPC through semi-quantitative immunoblot analysis of synaptoneurosome preparations (which is a tissue extraction method for detecting synaptic protein expression in the neuronal membrane) (Hollingsworth et al., 1985; Quinlan, Philpot, Huganir, & Bear, 1999)

1.1 Memory and learning

The question of how the central nervous system is able to store, retrieve and associate complex patterns through neural circuitry is not a new one. Memory and learning are closely intertwined; they loop around one another. Learning is the process of acquiring new information, while memory is the consequence of learning (Squire, 1987).

Memory is the faculty by which the mind, or brain, stores and recalls information. It is comprised of multiple specialized components that allow for mental representations of retained information about past experience. Memory also allows for the acquisition of new knowledge to

solve problems by comparing newly acquired knowledge with stored information. Neuronal plasticity permits memory encoding in the brain, as many neurons in the brain exhibit plasticity as is evident from brain lesion studies in rodents (Baddeley, 1992). Memory itself has been categorically broken into functionally distinct operations; it can be organized along dimensions that distinguish duration, permanence and the processes involved. For example, memory is considered either short- or long-term in regards to the period that the memory can be recalled after initial retention. Albeit, addressing all concerns regarding long-term memory is beyond the scope of the current project, it will be focusing specifically on associative memory. Associative memory falls under the larger umbrella of implicit memory, which refers to memory of events that cannot be consciously recalled. Implicit memory is confined to recollection using priming or unconsciously paired associations (Anderson, 2000; Baddeley, 1992).

1.12 Associative memory

Associative memory refers to the ability to learn and remember the relationship between unrelated items by storing maps of specific input representations to specific output representations (Anderson, 2000; Sommer & Wennekers, 2001). Associative memory takes the form of a filter that dynamically adapts and transforms as additional signals come in. This notion of adaption of output based on input rests on internal structure alteration of the memory system (Squire, 1987). Two forms of associative learning can be demonstrated through classical and operant conditioning paradigms. In operant conditioning a certain behavior is either reinforced or punished which results in an altered probability that the behavior will happen again, which will result in either strengthening or weakening of the association. Operant conditioning opens the door for additional variable assessment, as well as clouding the assessment of neural pathways involved in associative learning when using simpler models. Classical conditioning, or Pavlovian

conditioning as coined by Ivan Pavlov in 1901, provides a simple form of learning bearing fewer confounds. In classical conditioning, a neutral (conditioned) stimulus (CS) elicits an unconditioned (UC) response following repeated pairing of the CS with an unconditioned stimuli (UCS). This resulting attribution is referred to as an associative memory (Grant, 1964).

1.13 Classical conditioning models to study associative memory in the brain

Patterns of neural activity during associative memory formation can be examined by using associative learning models like classical conditioning. Classical conditioning animal models provide a window into the underlying mechanics of the brain using simple association models. Hebb (1949) was among the first investigators to extensively analyze possible relationships between the behavior of whole animals and the behavior of single neurons and the hippocampus was the primary focus of prevailing memory and long-term potentiation (LTP) studies (Hebb, 1949; Klopf, 1988). Synaptic changes related to memory and learning also occur in the olfactory cortex (Akalal, Yu, & Davis, 2011).

1.131 Olfactory system

The olfactory system also has the capacity to learn and remember, and it does not simply encode and discriminate odor information. Infant rodents' recognition of maternal odor depends on olfactory memory during the sensitive period in which rodents show approach responses to novel odors (Sullivan, Stackenwalt, Nasr, Lemon, & Wilson, 2000). During this time, specific learning-associated neural changes occur within the olfactory system providing researchers with a window of opportunity to experimentally manipulate odor learning and odor memory using artificial associations (Sullivan & Wilson, 1995). The role of the olfactory system is therefore

not limited to sensory processing, but in fact is involved in integrating sensory input with previously learned associative information that is then transmitted through reciprocal connections with downstream regions (Haberly, 2001).

1.2 Olfactory system

1.21 Overview of the olfactory system

The olfactory system is deemed the oldest part of the brain, perhaps due to the lack of a thalamic relay unique to this sensory modality (Berry, Krause, & Davis, 2008). The thalamic relay exists as a relay point to other cortical structures, complicating the neural pathways of newer processes in the brain (Cousens & Otto, 2003). However, olfactory connections to the forebrain and other higher cortical structures like the hypothalamus and amygdala exist and are functionally significant. For example, chemical stimuli from the environment initiate appropriate motor, visceral and emotional reactions to odorants immediately. The olfactory system abides by the same rules of other sensory modalities in that stimuli (odors) interact with receptors and result in electrical signals that transduce and encode odor information to downstream cortical structures (Acevedo, Froudarakis, Tsiorva, & Skoulakis, 2007; Do, Sullivan, & Leon, 1988; Galili, Ludke, Galizia, Szyszka, & Tanimoto, 2011)

1.22 The Olfactory system

1.221 Olfactory epithelium

Chemical stimuli in the environment, called odorants interact with cells in olfactory epithelium—in mice, olfactory epithelium contains more than 2 million sensory neurons;

individual sensory neurons express only one type of odorant receptor gene out of 1000 genes (Buck & Axel, 1991; Ressler, Sullivan, & Buck, 1993). The epithelial sheet that lines the interior of the nose contains neurons called olfactory receptor neurons (ORNs) and other support cells. A large multi-gene family of olfactory-specific G protein–coupled receptors (GPCRs) was initially identified in the rat (Buck & Axel, 1991) and belongs to what is now referred to as the OR family of odorant receptors (Mombaerts et al., 1996). A series of experiments indicated that odorant activation of olfactory receptor neurons was mediated by a G-protein-dependent pathway which leads to the activation of adenyl cyclase, which in turn increases the intracellular concentration of cyclic adenosine monophosphate (cAMP), activation of nucleotide-gated channels and eventually the depolarization of the neuron (Chang et al., 1989; Firestein, Darrow, & Shepherd, 1991; Nakamura & Gold, 1987). These experiments strongly implicated odorant receptors as G-protein-coupled receptors (GPCRs) (Zou et al., 2004).

Olfactory receptor neurons line approximately half of the nasal cavity and are small bipolar cells with unmyelinated axons (Mombaerts et al., 1996). The ionic milieu of the apical dendrites of olfactory receptor neurons called olfactory cilia is covered with a thin layer of mucus. Combined, the epithelium, the mucus layer, the supporting cells and the olfactory receptor neurons are referred to as nasal mucosa (Purves, Augustine, & Fitzpatrick, 2004). Within the nasal mucosa, the olfactory receptor neuron gains direct access to the odorant molecules. Olfactory receptor neuron axons project through the cribriform plate directly to OB neurons, which then project onto the pirifrom cortex in the temporal lobe. The bundle of olfactory receptor neurons from the nose to the OB forms the olfactory nerve, possessing first-order olfactory perception (Haberly & Bower, 1989b).

1.222 Olfactory bulb

The OB is located in the ventral anterior aspect of the forebrain, where it receives input from axons of olfactory receptors in the nasal epithelium via projections from ipsilateral olfactory receptor nerves (Purves et al., 2004). The most distinct physical feature of the OB is that it contains an array of spherical neuropil accumulations approximately 100 to 200μm in diameter, called glomeruli (Haberly & Price, 1977; Mombaerts et al., 1996). Glomeruli are formed from branching ends of axons of receptor cells and from outer dendritic branches of neurons called mitral cells. As well, tufted cells, which are smaller than mitral cells, and periglomerular cells, are other types of neurons that contribute to the formation of the glomeruli (Purves et al., 2004).

The OB is the first site of processing olfactory information following transduction by receptor cells in the nasal epithelium. Each receptor appears to express predominantly one type of receptor protein, which results in about a 1000 different types of odorant receptor cells expressed (Buck & Axel, 1991). Within each glomerulus, axons of receptor cells contact apical dendrites of mitral cells—which are the principle projection neurons of the OB. The cell bodies of mitral cells are found in a distinct layer deep to the glomeruli, with each glomerulus including the dendrites of ~25 mitral cells which receive innervation from thousands of olfactory receptor axons (Ressler, Sullivan, & Buck, 1994). Mitral cell sensitivity is enhanced by a strong degree of convergence from the thousands of innervating olfactory receptor axons. Each glomerulus is surrounded by approximately 50 tufted cells and 25 periglomerular cells, which are believed to sharpen odor sensitivity of individual glomeruli (Wilson, Fletcher, & Sullivan, 2004). The layer of the OB constitutes the granule cells, which primarily synapse on the basal dendrites of mitral cells. Granule cells are the most common inhibitory interneurons and they lack an axon—

producing local dendro-dendritic inhibition on excitatory synapses of mitral cells. They act to fine-tune the response output of mitral cells to the rest of the cortex (Okutani, Yagi, & Kaba, 1999).

The axons of similar odorant receptors generally converge on the same glomerulus in the OB, and then synapse on distal mitral cells, produce an olfactory map with varying inputs of specificity from excitatory inputs (G. M. Shepherd, 2004). Individual odorant receptors respond to many different odorants—resulting in widespread excitatory input to mitral cells with an array of regions in the OB (Acevedo et al., 2007; Berry et al., 2008; Canteras, 2003). This gives the OB the task of odor representations within the map that is it must optimize the various patterns of synaptic input associated with different odorants and relay that information onwards to downstream regions of the olfactory system, namely the anterior and posterior piriform cortex.

1.223 Piriform cortex

From the olfactory receptor neurons in the nasal epithelium, the glomeruli in the OB are the sole odor output to the rest of the olfactory system via axons of mitral and tufted cells (Neville & Haberly, 2003). The path from nose to the cortex is relayed by a bundle of mitral cell axons called the lateral olfactory tract (LOT), which send direct input from the OB to the anterior olfactory cortex (AOC), olfactory tubercle (OT), entorhinal cortex, agranular insula and portions of the amygdala which project to the accessory olfactory nuclei, the olfactory tubercle, the entorhinal cortex, the piriform cortex and other portions of the amygdala (Neville & Haberly, 2003). However, the main output from the OB via the LOT is the piriform cortex, which is the largest region of the olfactory system (Shipley, Ennis, & Puche, 2003). Like the OB, the

pirifrom cortex sends and receives sensory information that has not yet been processed by a thalamic relay.

The piriform cortex plays an important role in associative olfactory memory and learning as well as in odor discrimination, recognition and memory (Haberly & Bower, 1989b). The piriform is activated at multiple phases of olfactory learning such as encoding and retrieval (McCollum et al., 1991). Moreover, previous experimental evidence demonstrates that synaptic strength can be altered by experience in the piriform cortex suggesting that the piriform is also involved in associative memory processing (Jung, Larson, & Lynch, 1990; Roman, Chaillan, & Soumireu-Mourat, 1993; Stripling & Patneau, 1999). In addition, lesions in the piriform cortex produce odor discrimination and recognition deficits in humans, which further demonstrates the role of the piriform in functions of memory (Wilson, Kadohisa, & Fletcher, 2006). Therefore, piriform cortex is believed to function as a processing network that is critically involved in information processing and associative memory (Gordon H. Bower, 1994; Granger & Lynch, 1991; Haberly, 1985; Haberly & Bower, 1989a)

Unlike the OB, there is no topographic odor representation in the piriform cortex (Komiyama & Luo, 2006). *In vivo* calcium imaging studies (Fletcher et al, 2009), as well as patch clamp research (Poo & Isaacson, 2009) show that odors activate a small and dispersed number of pyramidal neurons in the piriform cortex depicting an sparse and distributed map of activity within the piriform. Despite a lack of global clarity, there do seem to be trends emerging to help unravel the firing patterns of neurons within the piriform cortex. For example, different sets of neuron ensembles are activated with each distinct odor; some responding with a strong degree of activation and others weakly (Poo & Isaacson, 2009; Stettler & Axel, 2009). Different odors do recruit some overlap in neuronal activation, suggesting neurons participate in more than

one representation in the piriform cortex (Poo & Isaacson, 2009). Collectively, piriform studies agree that odor encoding is sparse and distributed within the piriform cortex (Isaacson, 2010).

Structurally, the circuits of the piriform cortex are similar to those of other associational cortices (Gottfried, 2010). However, in contrast to the sensory neocortex, which is six-layered, the piriform cortex is a three-layered allocortex. The piriform cortex can been seen as a more primitive and simpler model to study in comparison to the more complex neocortex (Kanter & Haberly, 1990b). The morphology of the piriform cortex varies within the three layers. The first layer (layer I) is the superficial plexiform layer and it contains few GABAergic neurons (Haberly & Price, 1978). Layer I has mainly dendritic and axonal fibers, the dendrites arise from pyramidal cells in deeper layers. Layer I is further divided into layer Ia, which contains afferent fibers from the OB, and layer Ib which contains afferent fibers from other neurons with the piriform cortex. Layer II contains a large number of cell bodies described as semilunar cells. These cells are characterized by their spiny dendritic branches that reach layer I (Haberly, Hansen, Feig, & Presto, 1987). This layer also holds pyramidal cells with basal dendrites extending into layer III. Cells in layer II are predominantly glutamatergic whereas layer III consists of GABAergic small globular stellate cells (Watanabe & Kawana, 1982). There are also fewer pyramidal cells in layer III in comparison to other layers, however this layer also hosts a large size of GABAergic multipolar cells. Below layer III are additional multipolar cells, which are densely packed in the endopiriform nucleus (Haberly & Price, 1978). The location and dense interconnections of the endopiriform nucleus with the piriform cortex has led this region to be commonly referred to as layer IV (Kanter & Haberly, 1990a).

Anatomically, the piriform cortex runs along the olfactory tract, and continues on the dorsomedial aspect of the temporal lobe (Hori, Akaike, & Carpenter, 1988). The caudal aspect

of the piriform cortex fuses with the anterior cortical nucleus of the amygdala (Shionoya et al., 2006). The piriform cortex is also divided into anterior and posterior portions, with differentiating cellular structure and function in these areas (Raineki et al., 2009a). The anterior region is situated in the temporal and frontal region around the choroidal fissure, whereas the pPC lies in the temporal and posterior as well as inferior to the choroidal fissures and extends posterior near the hippocampus (Purves et al., 2004). The border between the aPC and the pPC is tentatively marked by the disappearance of the LOT on the surface of the piriform cortex (Haberly & Price, 1978). Moreover, the aPC and pPC are reported to support different functions in odor memory, as well as having distinct connectivity with neighboring regions (Calu, Roesch, Stalnaker, & Schoenbaum, 2007a; Roth & Sullivan, 2006). In humans, the anterior and posterior PC have been reported to support different olfactory processes. For example, the aPC is responsible for odor identification, whereas the pPC plays a role in odor categorization as well as processing the information of multiple odor cues (Okutani, Zhang, Otsuka, Yagi, & Kaba, 2003). Additionally, aPC and pPC connectivity can be traced from the OB to downstream regions. Specifically, the anterior olfactory nucleus (AON), which is immediately caudal to the OB, shares strong synaptic connections with the OB and associational fibers. It connects the ipsilateral and contralateral olfactory system and plays a role in memory retrieval (Johnson, Illig, Behan, & Haberly, 2000). The AOC includes the medial olfactory cortex (MOC), which consists of ventral tenia tecta and the dorsal peduncular cortex. Both of these structures are three-layered palecortical tissue similar to the piriform cortex. AOC has reciprocal connections with the anterior portion of the piriform cortex and the MOC connects with the pPC and entorhinal cortex (Haberly & Price, 1978; Kanter & Haberly, 1990a; Luskin & Price, 1983). Additional

details pertaining to each area of the piriform areas will be discussed at length in the sections to follow below.

1.2231 Anterior piriform cortex

The aPC provides a unique cortical model to study glutamatergic function and subsequently synaptic plasticity contributing to olfactory learning and memory. The aPC plays an important role in olfactory recognition and memory, as it is separated from sensory information in the environment by only 2 synaptic contacts (nasal epithelium and OB) (Hori et al., 1988). LOT fibers from the OB synapse on pyramidal (or principle) cell apical dendrites in the distal half of layer I (layer Ia) of the aPC (Price, 1973)**.** There are heavy projections from pyramidal cells in the aPC back to the ipsilateral OB (Haberly, 1985). As well, the proximal half of aPC layer I (layer Ib) receives input from associational fibers from pyramidal cells. Pyramidal cells of the aPC are glutamatergic and are morphologically similar to principle cells in cortical areas. Intrinsic projections from principle cells form associative (ASSN) connections with nearby cells. Also, axonal projections are formed vertically through the layers of the piriform cortex by the dendritic spines of principle cells. These synaptic populations (i.e., LOT and ASSN projections) express NMDAR-dependent LTP (Cherng et al., 2010; Dorman, Miller, D'Antonio, James, & Morgan, 1997; Gordon H. Bower, 1994; Haberly & Bower, 1989b; Kanter & Haberly, 1990b). However, there are differences in LTP expression, which appear to mediate cortical representations of olfactory information within each type of synaptic population (Poo & Isaacson, 2011).

In addition, studies have shown that perceptual odor information is encoded in the OB by demonstrating that odor-evoked spatial activity correlates with behavioral measures of odor similarity. This led to the implication that activity of neuronal ensembles in the OB represents odor quality. Similarly, the piriform cortex has been suggested as the location of odor encoding and classification because it receives extensive and direct input from the OB. Thereafter, the piriform cortex interconnects with amygdala, hypothalamus, the entorhinal cortex and oribitofrontal cortex. That means that the piriform cortex has access to sensory, affective, physiological and motivational features within the olfactory system for olfactory stimuli. Therefore, the assumption is that the aPC can act as associational cortex and may reflect the prominent features observed in downstream regions involved in associative processing. Studies provide evidence for this hypothesis. For example, a single-unit study demonstrated similar neuronal activity in lateral and ventrolateral orbital regions and the piriform cortex in awake rats trained to perform an 8-odor discrimination task (Schoenbaum, Setlow, Nugent, Saddoris, & Gallagher, 2003)**.** This finding shows that there is reciprocal activity in associational areas that parallels neuronal activity in the piriform cortex. Moreover, evidence suggests that learning and reversing odor discrimination problems in rats show prominent associative features in aPC neurons (Gottfried, Schoenbaum, Roesch, Stalnaker, & Takahashi, 2011; Roesch, Stalnaker, & Schoenbaum, 2007). These neurons exhibit similar firing trends to those observed in the orbitofrontal cortex (OFC) and adjacent areas including the dorsal and ventral agranular insular regions (Eichenbaum, Schoenbaum, Young, & Bunsey, 1996; Schoenbaum, Chiba, & Gallagher, 1998; Schoenbaum, Setlow, Saddoris, & Gallagher, 2003)**.** Interestingly, results from these studies provide evidence for cue-based specificity in the aPC. Specifically, aPC neurons did not switch firing from one cue to another, with an overall neuron population-response reflecting

sensory features of the cue. This apparent trend was unique to the aPC, and absent in sensory encoding in the OFC and basolateral amygdala (ABL) (Schoenbaum et al., 1998; Schoenbaum et al., 2003; Schoenbaum et al., 2003). The results from these studies suggest that although the aPC is receptive and influenced by associative input from downstream areas, it is only secondary to its primary role of sensory coding of olfactory input from the OB (Calu, Roesch, Stalnaker, & Schoenbaum, 2007b). However, this trend is not surprising since the aPC only receives input from the OFC (Fletcher, 2012).

1.2232 Posterior piriform cortex

Previous research has shown that spatially distributed ensemble activity in the human pPC coincides with perceptual ratings of odor quality in human participants. A recent study by Howard and colleagues (2009) investigated spatial ensemble coding of odor qualities and categorization in the pPC of human subjects using fMRI. This study assessed whether ensemble activity patterns represent sensory perception to infer olfactory odor experience. The results suggested that the ensemble of activity in the pPC is distributed and overlapping for odors, and that there are no obvious odor clusters for specific odors. This finding is consistent with previous data on the anatomical organization of this region (Illig & Haberly, 2003; Shepherd, 2004; Stripling & Patneau, 1999). However, an interesting finding from this study was that similar odors evoked similar ensemble activity, which indicates that perceptual information about odor quality is present in neuronal ensemble activation. Lastly, the results of this study demonstrate that odor objects categories are organized in the inferotemporal cortex, like the visual cortex, which strongly implicates the pPC in sensory (olfactory)-association.

Additionally, work from Calu and colleagues (2007b) also conceptualized the pPC as an association cortex, in that it is capable of integrating olfactory information that is incoming from

the OB and aPC with descending input from higher order association regions. The general consensus is that the piriform cortex acts like an association cortex due to its ability to integrate incoming odor signals with descending input from higher order regions like the OFC and ABL. These regions are involved in processing multimodal input and associative information, collectively bridging the function of the olfactory system of olfactory processing with associative processing (Calu et al., 2007b). Additionally, the ABL sends much stronger output to the pPC and is implicated in associative odor encoding of odor cues and outcomes (Johnson et al., 2000; Majak, Rönkkö, Kemppainen, & Pitkänen, 2004). There are also few direct contacts from the OB to the pPC, which are sparsely distributed within the pPC (Haberly & Price, 1977; Kanter & Haberly, 1990b). This shows that the pPC, unlike the aPC, is less involved in processing odor information directly from the external environment, and is more involved in associative functioning. Moreover, the posterior region of the piriform cortex receives far more associative input from layer Ib than afferent input from layer Ia (Behan & Haberly, 1999).

Furthermore, a study by Calu and colleagues (2006) proposed that input into the piriform is organized topographically with more sensory representations contained in the aPC and associative representations maintained in the pPC. This proposal was tested by recording neurons in the pPC in rodents learning and reversing novel two-odor discrimination problems. Consistent with the hypothesis, the researchers found that neural activity in the pPC was highly reactive during reversal learning which is indicative of associative processing. Additionally, population-based responses exhibited pronounced phasic activity to positive odor cues in comparison to negative odor cues. This demonstrated the significant role of associative sensory encoding of cues in the pPC. A large number of pPC neurons were also activated in anticipation of the predicted outcome following odor exposure. This observed trend was previously reported in the

OFC and ABL for the same task further exemplifying the associative features of the pPC (Schoenbaum et al., 2003)**.** Furthermore, activity in the aPC was significantly less associative in comparison to the pPC with a lower number of neurons activated during reversal learning. In summary, current studies suggest that the role of the aPC is to encode sensory cues and process information relayed from the OB, whereas the pPC takes on a role similar to associative cortices, much like the OFC and ABL.

1.3 Synaptic plasticity

During embryonic development, neural networks are built in the presence of little sensory input so as to be prepared to perceive the outside world following birth. After birth, newborns must absorb and process large quantities of information in a short period of time, which requires synaptic plasticity. Throughout life, synaptic plasticity becomes less urgent and synapses can become more or less stable. Therefore, the study of sensory experiential learning during development provides optimal conditions for the study of synaptic plasticity (Lohmann & Kessels, 2014).

Synaptic plasticity is a dynamic and complicated mechanism. Information transfer across synapses is a complex process, which essentially requires the release of neurotransmitter from the presynaptic cell to the post-synaptic, resulting in signal transduction by receptors located in the postsynaptic membrane. The integration of several synaptic responses can bring about a sequence of action potentials via voltage-gated ion channels that allow the entry of calcium and sodium in and potassium out of the cell leading to cellular depolarization. In nearly every stage of this process, activity-dependent plasticity can be observed which can be individually studied

for mechanistic action (Hempel, Hartman, Wang, Turrigiano, & Nelson, 2000; Nelson & Turrigiano, 2008; Turrigiano & Nelson, 2000).

The olfactory system is not static and it displays remarkable plasticity both during learning and during development on the neuronal and molecular level (Haberly, 1985). The development of the olfactory system from birth until adulthood reveals stark transitions in neuronal activity, as well as in receptor composition within the olfactory system. However, prior to the discussion of plasticity within the olfactory system, it is important to outline the major players active in synaptic plasticity, which include long-term potentiation and depression and the N-methyl-D-aspartate (NMDA) receptor and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Leslie, Nelson, & Turrigiano, 2001).

1.31 Long-term potentiation and long-term depression

Synaptic plasticity refers to the ability of neuronal synapses to either strengthen or weaken in response to increases or decreases in their activity (Bear & Malenka, 1994; Morishita et al., 2007). Activity dependent modifications of synapses are fundamental for the storage of information in the brain (Abraham & Bear, 1996; Bear, 1995). These cortical representations are not fixed entities, but rather are dynamic and ever changing with incoming environmental input throughout the duration of life (Buonomano & Merzenich, 1998). The cortex reorganizes its synaptic connections between neurons affecting local connectivity and response following peripheral or central alterations of inputs as well as in response to behavior (i.e. learning). Terje Lomo and Tim Bliss were the first to introduce the concept of synaptic plasticity in 1966, while studying long-term potentiation (LTP) in rabbit hippocampi (Bliss & Lomo, 1973). LTP is one of the underlying phenomena of synaptic plasticity, and refers to a long-lasting enhancement in

signal transmission between two neurons, the pre-synaptic and post-synaptic, that results from stimulating them synchronously (Bliss & Lomo, 1973).

LTP and LTD induction is critically dependent on NMDA activation and calcium influx. A significant amount of calcium entry, following depolarization of NMDA receptors, permits LTP induction. Whereas moderate amounts may contribute to LTD induction. Once LTP is induced, AMPAR insertion into the synaptic cleft from extra-synaptic regions, results in the maintenance of synaptic transmission (Abraham & Bear, 1996; Malenka & Bear, 2004).More specifically, glutamate binds to AMPARs and NMDARs, the AMPAR channels open immediately allowing sodium influx into the postsynaptic cell. Ample sodium influx results in cellular depolarization, which lifts the magnesium voltage-dependent block of NMDARs. NMDARs, unlike AMPARs, are permeable to both sodium and calcium. Calcium entry triggers downstream cellular pathways that activate synaptic trafficking of AMPARs to the synaptic site. This AMPAR up-regulation results in a long-lasting increase in EPSP size, which underlies LTP (Abbott & Nelson, 2000).

Following the induction of LTP, most synapses use (AMPA)-type glutamate receptors for fast transmission of signals (Lohmann & Kessels, 2014). AMPARs are non-selective receptors, which permit potassium efflux and sodium influx. The number of AMPARs at any given postsynaptic site predicts the efficacy of signal transmission across that synapse. LTP and LTD synaptic strength is therefore expressed by the addition or removal of synaptic AMPARs, respectively (J. D. Shepherd & Huganir, 2007). Thus, AMPAR insertion into the synapse is regarded as the mechanism for learning and plasticity expression, whereas NMDARs are considered the critical initiators of learning and plasticity (Jerome, Hou, & Yuan, 2012; J. D. Shepherd & Huganir, 2007).

1.311 AMPA & NMDA receptors

Glutamate receptor (GluR) channels act as crucial mediators of excitatory synaptic transmission (Mori & Mishina, 1995). GluRs are classified into three major groups, AMPAR, kainate receptor and NMDAR. The occurrence of LTP is dependent on depolarization, the presence of the neurotransmitter glutamate and the glutamate receptor subtype, NMDAR. The propagation and maintenance of LTP is dependent on AMPAR. During action potentials, glutamate is released resulting in the activation of NMDARs and AMPARs on the postsynaptic membrane that eventually generates excitatory postsynaptic potentials (EPSPs). NMDARs are ionotropic glutamate receptors with a non-selective cation channel. NMDARs are slow acting, with a rise time of 10-50ms, whereas AMPARs are fast acting with a rise time of 0.2-0.4ms. However, unlike AMPARs, NMDARs express longer activation times (50-500ms) allowing a more substantial influx of calcium in comparison to non-NMDARs (~2ms). AMPARs thus provide a rapid response to neurotransmitter release leading to depolarization, while NMDARs provide co-incidence detection, long-lasting synaptic current and calcium influx (Cull-Candy, Brickley, & Farrant, 2001; Mori & Mishina, 1995).

1.3111 NMDA

As mentioned, like the AMPAR, the NMDAR is also an ionotropic glutamate receptor (Cull-Candy et al., 2001). Three families of subunits for NMDAR have been identified: NR1, NR2 (A, B, C and D), and NR3 (A and B). NMDARs are heterotetramers composed of two obligatory NR1 subunits and two NR2 subunits. The NR3 subunits typically do not form functional synapses alone, and will usually combine with a NR2 subunit in order to function (Mori & Mishina, 1995).

Each NMDAR subunit is comprised of an intracellular carboxyl-terminus domain of variable length, three hydrophobic transmembrane segments, a pore-forming domain, and a long extracellular N-terminus domain. The C-terminal region is responsible for the activation of the channel when it is phosphorylated by PKC. NR2 subunits typically have larger carboxyl-terminal regions in comparison to other NMDAR subunits. The three transmembrane domains are located in the middle of the molecule that consists of a re-entrant selectivity filter loop (Mori & Mishina, 1995). Moreover, all subunits of NMDAR possess asparagine at the position corresponding to the glutamate pore region. This channel pore region determines permeability to calcium and holds the voltage-dependent magnesium block. The extracellular N-terminus domain of the NR1 subunit contains the binding site for glycine whereas glutamate binds to the extracellular N-terminus domain of the NR2 subunit. The extracellular N-terminus region of NR2 also contains the binding sites for allosteric modulators (Mayer, 2005).

Activation of NMDARs is voltage-dependent and ligand-gated, requiring postsynaptic depolarization as well as glutamate and either D-serine or glycine. D-serine and glycine, along with glutamate are essential co-agonists that bind subunits of the receptor allowing maximal activation. Although NMDARs show high affinity for glutamate, there is no evidence of saturation during synaptic transmission. NMDARs can act as coincidence detectors for pre- and postsynaptic activity due to the requirement of simultaneous activation through glutamate and depolarization to relieve the magnesium block. Following the removal of the magnesium block, NMDARs permit sodium and calcium influx and potassium efflux (Mayer, 2005).

NMDARs function by coupling electrical to biochemical signals by mediating calcium influx in response to synaptic activity (Zito, Scheuss, Knott, Hill, & Svoboda, 2009). Aside from serving an important role in electrical neurotransmission, NMDA receptors also contribute to the

amplitude of excitatory postsynaptic currents (EPSCs) by facilitating temporal summation and enhancing the computational power of neurons though synchronous activity. NMDAR function can also be enhanced by phosphorylation; a fundamental mechanism that regulates NMDA receptor trafficking and can alter receptor properties. NMDARs can be phosphorylated by serine or threonine kinases: protein kinase C (PKC), protein kinase A (PKA) and calcium/calmodulin-dependent protein kinase II (CamKII) (Mayer, 2005). Phosphorylation of NMDARs by PKC reduces magnesium affinity resulting in longer open times for calcium influx. Additionally, calcium influx can potentiate PKC phosphorylation resulting in an amplified response (Mayer, 2005).

The role of NMDA is rooted in calcium influx, which acts as a second messenger leading to a series of signaling pathways. Calcium influx plays a crucial role leading to LTP and LTD. Calcium also activates cyclic adenosine monophosphate response element binding protein (CREB), a transcription factor resulting in the activation of gene expression (Mori & Mishina, 1995). Calcium influx results in long-term changes in synaptic strength and synaptic structure, which is the basis for NMDARs role in synaptic plasticity (Mori & Mishina, 1995). Moreover, synapsesNMDAR-only "silent" synapses, however there is a gradual loss of these receptors following sensory experience and development (Franks & Isaacson, 2005; O'Brien, Isaacson, & Berger, 1997). NMDAR-only synapses are replaced with AMPARs in an LTP-dependent manner. LTP induction results in NMDAR-dependent excocytosis of AMPARs, whereas the endocytosis of AMPARs out of synapses occurs during LTD (Malenka & Bear, 2004).

A study assessing the role of NMDA receptors in spatial learning found that when D-APV, a NMDAR antagonist, was injected into the ventricles of rats they presented impaired spatial learning. Additionally, another study demonstrated that intraventricular infusion of an

NMDAR antagonist resulted in an inability to perform the Morris water maze task, which is indicative of impaired spatial learning (Morris & Yoon, 1989). Another study conducted with knockout mice lacking NMDAR subunits in the hippocampus provided similar evidence for a role of NMDAR in learning and memory (Collingridge, Kehl, & McLennan, 1983). Therefore, taken together evidence suggests that blockage of NMDARs prevents LTP induction as well as blocks learning and memory.

1.3111 NMDARs in the Brain

An *in situ* hybridization analysis revealed that the NMDAR channel subunit NR1 mRNA was ubiquitously distributed in the brain of rodents. The NR2A subunit mRNA, however showed a characteristic distribution. This subunit was expressed in the brain, but was widely found in the cerebral cortex, the hippocampal formation and cerebral granule cells. Whereas, NR2B was selectively expressed in forebrain regions with high levels also present in the cerebral cortex, the hippocampal formation, septum, caudate and putamen and the olfactory bulb and thalamus. The subunit NR2C was predominantly found in the cerebellum and NR2D was expressed primarily thalamus, brainstem and olfactory bulb. However, during development the expression of NR2 subunit mRNA was found to be differentially regulated in particular regions of the brain (Cull-Candy et al., 2001).

1.3112 AMPA

Like all other ionotropic glutamate receptors (GluRs), AMPARs are ligand-gated ion channels that open once an agonist binds to the soluble ligand-binding core. GluRs are composed

of four subunits with four discrete regions (Lampinen, Pentikäinen, Johnson, & Keinänen, 1998). AMPARs are tetrameric in structure, consisting of four subunits (GluR1, GluR2, GluR3, and GluR4) with four different genes that encode each receptor respectively, Gria1, Gria2, Gria3 and Gria4. AMPARs are heterotetrameric composed of 'dimers of dimers' of GluR2 and either GluR1, GluR3 or GluR4. Each subunit consists of a transmembrane ion pore, three transmembrane domains, N-terminus and C-terminus. When all four subunits of the AMPA receptor tetramers come together, they form an ion-permeable pore. AMPAR subunits differ significantly in C-terminal sequence length. The C-terminus domain determines their interaction with scaffolding proteins. Moreover, these subunits also differ based on the PDZ-binding domain they utilize. The PDZ-domains are structural domains comprised of 80-90 amino acids. For example, GluR1 interacts with the PDZ-domain regions of SAP97, which belongs to a family of proteins that interact with NMDARs (Malinow & Malenka, 2002). PDZ is an abbreviation made from the first letters of 'postsynaptic density protein' (PSD95), 'Drosophila disc large tumor suppressor' (D1g1), and 'zonula occluden-1 protein' (Zo-1). The PDZ-domain is bound to the C-terminus region of AMPARs (Lohmann & Kessels, 2014).

Each of the four subunits contain a binding site for their agonist, glutamate, located at the N-tail and transmembrane domains. When an agonist binds to two sites on the N-terminus of the subunits, the transmembrane loops shift inward forming an opening (or pore). An open pore allows rapid desensitization, which stops the EPSC, resulting in a short open-close window (Malinow & Malenka, 2002).

AMPAR channel conductance and permeability is also dependent on receptor subunit composition. The GluR2 subunit governs calcium, sodium and potassium permeability. The presence of this subunit renders it impermeable to calcium. AMPAR subunit composition also

determines receptor modulation. For example, receptors lacking the GluR2 subunit are susceptible to blockade by voltage-dependent polyamines. Polyamines are molecules with two or more primary amino groups (-NH2). Polyamines are capable of blocking AMPARs following membrane depolarization by preventing potassium flux through the channel pore. On the other hand, the presence of subunit GluR1 is an essential component of LTP and LTP in the hippocampus, and as a result the focus for the study of synaptic plasticity and learning and memory (Lampinen et al., 1998). Specifically, AMPARs containing subunit GluR1 are trafficked to recently active synapses following LTP induction to strengthen the synapse (Malinow & Malenka, 2002). Moreover, GluR1 phosphorylation has been reported as necessary for synaptic plasticity in the brain (Cull-Candy & Leszkiewicz, 2004). A study by Lee and colleagues (2003) generated a knockout strain with a mutation in GluR1 phosphorylation sites, and they discovered that there were defects in LTP induction in the hippocampus. Moreover, rodents demonstrated deficits in spatial learning tasks (Lee et al., 2003).

AMPAR function is also modulated by phosphorylation of its subunits by PKC, CaMKII and PKA. Once phosphorylated or turned on, they regulate channel localization, conductance and probability of permeability. AMPARs, and not NMDARs, conduct currents at resting membrane potential. AMPAR channels are permeable to monovalent cations; primarily sodium and potassium. These cations provide the inward current generating synaptic responses at the resting membrane potential. If AMPARs are absent, the synapse is “silent.” Silent synapses are referred to as synapses that lack EPSCs at resting membrane potential and only become active during depolarization. These silent synapses reflect the functional presence of NMDARs, which only exhibit activity following depolarization. AMPAR activity is dependent on phosphorylation by PKC and CaMKII both of which are essential to induce LTP (Ma & Lowe, 2007).

As previously mentioned, following LTP, additional AMPARs are delivered from the extra-synaptic membrane and inserted into the active synapse to increase signal transmission and channel conductance. AMPARs are rapidly transported to and from active synapses sites to strengthen or weaken their function. AMPARs are transported to postsynaptic densities (PSD) through PKA phosphorylating activity. Trafficking of AMPARs can also occur by lateral movement of AMPARs from extrasynaptic sites, as well as by exocytosis during LTP (Malinow & Malenka, 2002). Exocytosis refers the cells ability to direct secretory vesicle release into extracellular space allowing the contents to be taken to alternative locations**.** A two-process hypothesis has been proposed for AMPAR trafficking through PKA activity. AMPAR trafficking into synapses is widely accepted as the mechanism underlying learning and memory (Seebohm et al., 2012).

1.31121 AMPARs in the Brain

A study conducted by Higuchi (Higuchi et al., 1993) assessed the regional, cellular and subcellular distribution of AMPARs using antibodies that recognized the C-terminus domain of individual AMPAR subunits. The immunocytochemistry results indicated that AMPARs were distributed abundantly as well as differentially within cell bodies and processes in the cerebral cortex, basal ganglia, limbic system, thalamus, cerebellum and brainstem. High levels of AMPAR subunits were also reported by Hampson (1999) in the striatum, pyramidal and radiatum layers of the hippocampus and the molecular layer of the cerebellum. Specifically, western blot analysis of the GluR1 subunit revealed that the subunit was selectively expressed in non-pyramidal, calcium-binding neurons in the cerebral cortex. AMPA GluR1 subunits were also localized in dopaminergic neurons of the substania nigra and striatum. Interestingly, *in situ* hybridization mRNA coding for AMPAR subunits depicted higher mRNA expression for

subunits than the distribution of AMPA binding sites. This suggests that AMPAR locations may possess binding sites but remain inactive (Higuchi et al., 1993).

1.32 Developmental Switch of Receptors

AMPARs and NMDARs also contribute to synapse maturation during early life development in areas that are functionally significant to learning and memory (ie, hippocampus, cortex, striatum and the olfactory system). Receptors exhibit characteristic subunit composition corresponding to increased neuronal activity during development or following learning (Sullivan, 2012).

1.321 AMPA Receptors

A recent study reported that the AMPAR subunit GluR4 was expressed before birth and in the first postnatal week in excitatory neurons of the hippocampus and cerebral cortex (Lohmann & Kessels, 2014)**.** GluR4 trafficking into synapses is dependent on spontaneous, low level activity**.** However, following birth the expression of GluR4 decreases as neuronal activity spikes. The rise of neuronal activity coincides with an increase in GluR2 expression. AMPAR subunit GluR2 mediates synaptic strengthening during development, as synaptic activity is high (Lohmann & Kessels, 2014). These findings fall in line with a previous finding that reports low levels of GluR2 in mature hippocampi (Kolleker et al., 2003)**.**

From PD 6 onwards, there is a rise in AMPA GluR1, GluR2 and GluR3 expression until the third postnatal week of development. GluR2 is a relatively unstable subunit due to a positively charged arginine molecule located in the transmembrane region. To reduce instability, the subunit forms heteromers with other subunits (Greger, Khatri, Kong, & Ziff, 2003; Sommer & Wennekers, 2001). At maturity, the hippocampus and cortex contain AMPARs composed of

GluR1-GluR2 heteromers and GluR2-GluR3 heteromers, and a small number of GluR1 homomers. The estimated ratio of GluR1, GluR2, and GluR3 subunits is 1:2:1, indicating GluR1-GluR2 heteromers are equally abundant as GluR2-GluR3 heteromers (Kessels & Malinow, 2009). Analysis of AMPAR currents during development indicates that there is a nearly an 80% decrease in currents when GluR1 is deleted and only a 15% decrease in currents when GluR3 is deleted during the first weeks of development. These results demonstrate that synaptic AMPAR subunit expression is predominantly comprised of GluR1-GluR2 heteromers (Kessels & Malinow, 2009).

Neurons lacking AMPAR GluR1 subunits display an impairment of LTP induction resulting in a decrease in synaptic strength (Greger et al., 2003). Interestingly, LTP and synaptic strength is not impaired in GluR3-deficient neurons (Kessels & Malinow, 2009). GluR1 homomer-containing neurons increase GluR1 expression following high levels of neural activity. GluR2-lacking AMPAR GluR1 homomers are highly permeable to calcium, allowing increased calcium influx thereby lowering the threshold of synaptic plasticity through synaptic trafficking of GluR1 containing AMPARs into the synapse (Lohmann & Kessels, 2014).

A study conducted by Martin, Furuta & Blackstone (1998) tested the regional, cellular and subcellular expression of the AMPAR subunit GluR1 during development. Their Western blot results revealed that GluR1 was detected in the whole brain as early as embryonic day 15.5 (E15.5), with progressive increases toward late embryonic periods. Following birth, postnatal assessment in the cerebral cortex and striatum depicted differential maturational patterns. Specifically, immunoblot results of the cerebral cortex indicated increasing levels of GluR1 through postnatal development. On the other hand, there was a decrease in AMPA GluR1 expression with maturation in the striatum. The study reported that cerebral cortex AMPA GluR1

expression reached adult level after PD26, whereas GluR1 in the striatum increased from PD1 to PD10, and then steadily declined (Martin et al., 1998)

Moreover, immunocytochemistry of the hippocampus demonstrated developmental changes in GluR1 localization. Adult level expression of GluR1 was achieved at PD19-21 in the hippocampus. The CA1 region was highly enriched at this time in comparison to CA3. However, the CA3/CA2 regions expressed high enrichment between PD0-5. *In situ* hybridization also detected higher levels of GluR1 mRNA in the CA3 region compared to CA1 in the early days of life in rodent hippocampi. Additionally, immunocytochemistry of the basal ganglia were consistent with immunoblots trends in the striatum. The results revealed that newborn basal ganglia were more enriched than adult basal ganglia. The results of these studies demonstrate that GluR1 expression is regulated differentially throughout development in different regions of the brain and therefore expression is region dependent (Martin et al., 1998).

1.322 NMDA Receptors

Early in life, cortical structures express high levels of NMDARs, resulting in synaptic plasticity of synapses to establish functional synapses and cortical circuits. NMDARs also undergo developmental changes during neuronal differentiation and synaptogenesis. NMDAR subunit composition varies at distinct synapses at different developmental stages. As previously mentioned, NR1 is an essential subunit derived from a single gene and it is found ubiquitously throughout the brain. The expression of NR1 subunits determines the number of NMDARs found at a particular synapse and can indicate up-regulation or down-regulation of NMDARs in an age-dependent and experience-dependent manner. Whereas NR2 subunits have multiple genes and are expressed differentially throughout the brain during development (M. Watanabe, Mishina, & Inoue, 1994). NR2B and NR2C are abundantly expressed in the early stages of development, and

NR2A and NR2D are added later in development (Köhr, 2006). Like NR1, NR2A subunits are expressed ubiquitously in the brain, whereas NR2B are predominantly localized in the forebrain and NR2C are found in the cerebellum. NR2A and NR2B subunits exhibit dramatic change in expression in the brain during development and are therefore focused on for their role in NMDAR-mediated plasticity (Köhr, 2006).

NMDAR subunit composition alters throughout development in distinct regions of the brain. The NMDAR NR2 subunit displays characteristic functionality in its kinetics, magnesium sensitivity, ion conductance and molecular interactions (Niculescu & Lohmann, 2013). During development, NR2B subunit levels are high before birth and remain relatively high for the duration of the first two weeks following birth. Thereafter, the expression of NR2B declines and the expression of NR2A increases steadily throughout the first month (Sans et al., 2000). The ratio of NR2B and NR2A increases in an activity dependent manner during development. Functionally, NR2B subunits express longer open times, and have a higher affinity for glutamate than NR2A subunits. As well, NR2B subunits also express slower voltage-dependent gating (Niculescu & Lohmann, 2013).

Moreover, neurons traffic NMDARs containing NR2B subunits more rapidly than NR2A, which, suggests that NR2B in mature neurons is more stably anchored. The C-terminus domain of NR2B subunits also has higher affinity for calcium and CaMKII, than that of NR2A. LTP induction is, therefore, facilitated in the presence of NR2B, rendering synapses containing NR2B more plastic than those containing NR2A. However, when adulthood is reach, NMDARs NR2A subunits outnumber NR2B in the hippocampus. Mature neurons increasingly contain NR1-NR2A heteromers (Quinlan et al., 1999). This phenomenon is characteristic of synapses in the

thalamus, the neocortex and the piriform cortices (Liu et al., 2004; Quinlan, Lebel, Brosh, & Barkai, 2004).

Experience-dependent changes during development alter synaptic expression of NMDAR subunit composition as well. For example, one study assessing visual experience-dependent changes found that NR2A expression was significantly lower following six weeks of light deprivation in comparison to rats accustomed to a normal 12-hour light-dark cycle. On the other hand, the expression of NR1 and NR2B was unaltered by the changes in light exposure (Quinlan et al., 1999).These findings are not surprising in light of previous data from studies on LTP and synaptic plasticity. The differential expression of NMDAR and AMPAR subunits reflect the maturation of synapses as well as of structures during development and experience-dependent learning (Niculescu & Lohmann, 2013).

1.4 Olfactory system development

1.41 Olfactory system NMDA & AMPA

In the olfactory system, experience-dependent modifications account for olfactory learning in early life. The olfactory bulb is proposed as the underlying cortical structure involved learning in early life, whereas the piriform cortex is believed to be critical for the formation of associative memories in adulthood (Philpot, 2005).

The establishment of functional synapses and learning coincide with modification in AMPAR and NMDAR expression in the olfactory system (Philpot, 2005). In a landmark study

by Franks & Isaacson (2005), electrophysiological approaches were used to examine AMPARs and NMDARs in the olfactory system during development. Their results demonstrated that AMPAR/NMDAR ratio was low in the first weeks of life (PD 0 – PD 8), however there was a significant increase in the ratio in the LOT following weeks of development (PD 8 – PD 22), but not at the ASSN fibers. Thereafter, the researchers examined the proportion of NMDA-only silent synapses and their results demonstrated that there was a developmental loss of NMDA-only silent synapses at the LOT over the course of development. The loss of NMDA-only silent synapses has also been observed in the cortex (Isaac, Crair, Nicoll, & Malenka, 1997) as well as in the visual cortex (Rumpel, Hatt, & Gottmann, 1998) in the first weeks of life.

To address whether the down-regulation of these NMDA-only silent synapses was associated with olfactory learning and maturation of synapses, the researchers used a nostril occlusion method on PD 1 (Meisami, 1976). This technique allows researchers to effectively block olfactory stimulation in a single hemisphere by blocking a single nostril while exposing rodents to odor providing an intra-animal control. They found that a lack of olfactory-driven activity in one hemisphere resulted in a low AMPAR/NMDAR ratio between the second to fourth week of development (Franks & Isaacson, 2005).

The increase in AMPAR/NMDAR ratio could potentially be due to an up-regulation of AMPARs, a down-regulation of NMDARs or both. To test whether there was an up-regulation of AMPARs, the researchers examined the quantal amplitude of AMPAR-mediated EPSCs at the LOT in the presence of strontium. The authors performed voltage-clamp recording from layer II of pyramidal cells in the piriform cortex by activating the LOT to measure AMPAR-mediated responses. They observed no change in AMPAR-mediated responses at the LOT synapses indicating that the change in the ratio was not due to a significant up-regulation of AMPARs

(Franks & Isaacson, 2005).Next, they considered whether the difference in AMPAR/NMDAR ratio at the LOT and ASSN inputs reflected a change in the expression of NMDA-only silent synapses. In this experiment, they evoked synaptic AMPAR responses from ASSN and LOT using minimal stimulation at -80mV. Then synaptic responses were measured at +40mV allowing depolarization of NMDARs and release of the magnesium block at PD 15-17. The results indicated that there was a dramatic increase in measured responses at ASSN but not at the LOT. This demonstrates a lack of NMDA-only silent synapses at the LOT, and a down-regulation of NMDARs following development. Interestingly, when this procedure was replicated in younger rodents (PD 7-8), there was a significant increase in NMDAR-mediated responses. These observations demonstrate that there is age-dependent decrease of NMDA-only silent synapses at the LOT and not at the ASSN.

The authors propose that the decrease in NMDA-only silent synapses may indicate maturation of glutamate uptake mechanisms at the LOT as the rodent matures. To test this hypothesis, they sought to determine the effect of blocking glutamate at the LOT in pups aged PD 8-9, and in older rodents aged PD 19-23. The NMDAR EPSC at the LOT was recorded at +40 mV in the presence of baclofen (inhibits NMDAR-mediated EPSCs), NBQX (non-selective non-NMDAR blocker), and picrotoxin (non-competitive receptor blocker). NMDAR-mediated EPSCs were measured at the LOT of young and mature rodents following the application of TBOA, a glutamate transport blocker. TBOA resulted in a modest increase in NMDAR EPSC amplitude in animals at PD 8-9, and no effect at PD 19-23. They determined that glutamate uptake mechanisms were non-functional early in life (PD 8-9) (Franks & Isaacson, 2005).

These results indicate that there is activity dependent modification in AMPAR and NMDAR expression in the olfactory system resulting in a modest up-regulation of AMPARs and

a down-regulation of NMDARs following odor learning and maturation. The increase in the ratio of AMPARs relative to NMDARs is associated with a decrease in NMDAR-only silent synapses due to AMPAR trafficking into recently activated synapses due to age-dependent modifications of synapses (Franks & Isaacson, 2005).

1.42 Learning induced changes of receptors in the olfactory cortex

The olfactory system also appears to be attuned to respond to learning-dependent modification during early life (Philpot, 2005) as proposed earlier. In a study conducted by Quinlan and colleagues (2004), they proposed that there are two-phases of olfactory learning induced modifications in NMDAR expression. The first phase is referred to as the NMDA-sensitive phase in which rule learning is observed when acquiring new information. Thereafter, an NMDAR-insensitive phase is exhibited in which animals learn to distinguish between distinct odors. To test this hypothesis, the researchers utilized an olfactory discrimination-learning task, in which requires rodents to rapidly associate odors with reward by navigating a maze and learning rules. During experimental training, rats were placed in a four-arm radial maze with positive and negative odors at the end of the arms. Rodents that successfully entered the arm with the positive odor received water as the reward. These researchers found that odor discrimination learning occurred rapidly, within the first two days of training. They also suggest that it indicates the two-phases of olfactory learning: first when the animal learns the strategy (rule learning) and second when the rodent learns associations between odors and reward (pair learning) (Quinlan et al., 2004).

To determine the role of NMDARs in olfactory discrimination learning, the authors administered a low dose of NMDAR antagonist MK801 prior to learning. MK801 significantly increased the initial time in which rodents learned odors, however MK801 did not affect learning

in the following training sessions once pair associations were established. Next, the researchers sought to determine whether olfactory discrimination learning induced long-term modification in synaptic function associated with LTP. In coronal slices of the piriform cortex from trained and naïve animals, high frequency stimulation was delivered to induce LTP. They demonstrated that LTP was significantly attenuated in trained animals compared to control animals, which suggests that synapses in the piriform cortex are strengthened following olfactory discrimination learning (Quinlan et al., 2004).

Moreover, alterations in NMDAR subunit composition following olfactory discrimination learning was assessed through field EPSP recordings from the piriform cortex in the presence of NR2B-specific antagonist ifenprodil. Their results revealed that fEPSPs of piriform cortex slices in trained animals were not sensitive to NR2B reduction, while recordings from naïve animals were. This suggests that olfactory discrimination learning results in a significant decrease in the expression of NR2B expression in the piriform cortex. Additional synaptoneurosome immunoblot analysis of NMDAR subunit composition revealed a significantly higher ratio of NR2A/NR2B synaptic expression in trained animals in comparison to controls. These results also indicate that the changes in synaptic expression of NMDAR subunits are olfactory region learning-dependent, as no changes in NR2A and NR2B expression were detected in the cortex or the hippocampus. Quinlan and colleagues (2004) also reported no significant difference in AMPAR GluR2 expression in the piriform cortex before and following training. Lastly, to test whether NMDAR subunit composition correlates with phases of olfactory discrimination learning, the researchers sacrificed animals at three separate time intervals of training: a) prior to rule learning, b) a day after rule learning and c) 5 days after learning with no further training. Immunoblot results depicted a significant increase in the NMDA NR2A/NR2B

ratio following training, however the ratio remained at baseline days after training. This suggests that NMDAR composition in the piriform cortex is limited to rule learning and returns to baseline once training is completed. Together, their results demonstrate NMDA-specific changes following olfactory discrimination learning (Quinlan et al., 2004).

1.421 Sensitive period in the olfactory system for odor learning

The olfactory system undergoes dramatic age-dependent changes, as well as experience dependent alteration in synaptic plasticity in order to allow for smooth transitioning from intrauterine to extrauterine life (Sullivan & Lasley, 2010). However, this reorganization of behavior and synaptic circuits is bound by time, and maturation occurs in a limited period of time during early life development. This short-lived time is referred to as the sensitive period or critical period and in rodents it lasts from birth (on PD 0) until PD 10. During the critical period, neonatal rodents express robust approach behaviors and are a model for the assessment of neural pathways in associative learning models.

Neonatal behavior is largely mediated by reflexes, suckling for example is a reflex that requires olfaction (Lohmann & Kessels, 2014). Behavioral models utilize pups' innate instinct to locate the dam through their sense of smell due to their lack of vision and audition. Natural odor cues in the environment can be readily replaced with neutral odors (ex. Peppermint) and will acquire properties of maternal odor during the critical period of development (Sullivan & Wilson, 1991). In this way, neonatal pup odor attachment learning is utilized in classical conditioning paradigms where novel odors are paired with preference inducing stimuli like stroking (McLean, Darby-King, Sullivan, & King, 1993) or milk (Sullivan, 2003) in order to

study the underlying properties of associative learning in the olfactory system. Additionally, injection or infusion of isoproterenol (ISO), a β-adrenoceptor agonist, paired with an odor is sufficient to induce odor preference learning during the critical period (Sullivan et al., 2000). Activation of β-1 adrenoceptor subtypea within the OB plays a critical role in odor preference learning, and blockage of β-adrenoceptors completely blocks odor preference learning in odor-stroking training paradigms (Sullivan, Wilson, & Leon, 1988; Sullivan & Toubas, 1998; Sullivan et al., 2000).

Paradoxically, the sensitive period is limited to preference learning, in fact during this time pups display attenuated avoidance and fear learning (Blozovski & Cudennec, 1980; Camp & Rudy, 1988a; Haroutunian & Campbell, 1979). Previous research studies report that novel odors paired with aversive stimuli like a tail pinch and 0.5mA shock induce approach behaviors despite the physical discomfort of those stimuli (Haroutunian & Campbell, 1979; Raineki et al., 2009a; Sullivan et al., 2000). Beyond the critical period, rodents express avoidance behaviors past PD 10 and during adulthood gaining locomotive ability and independence from the nest (Sullivan et al., 2000; Woods & Bolles, 1965). Interestingly, avoidance learning can also be exhibited within the critical period. For example, researchers found that aversion was induced when malaise (LiCl) was paired with odor in utero (Garcia & Koelling, 1966) and in the critical period between PD 7-8 (Raineki et al., 2009a). In contrast to adult fear and avoidance learning, which utilizes the amygdala, pups rely on the OB for aversive learning as well as for preference learning until after the cessation of the sensitive period, when the amygdala is incorporated into the olfactory circuitry (Raineki et al., 2009a; Shionoya et al., 2006).

1.5 Odor and stroking pairing induced preference learning and AMPAR synaptic plasticity

In neonatal rodents, learning associations between stimuli mimicking maternal behavior like stroking and milk, paired with an odor have been reported to be essential in producing approach behaviors (Sullivan et al., 2000). Cui and colleagues (2011) report that following preference learning, AMPARs were trafficked into synapses in the OB. Specifically, they investigated whether early odor preference learning was associated with PKA-mediated phosphorylation and insertion of AMPARs in OB synapses. Indeed, immunoblot results showed that AMPARs were phosphorylated at the PKA site (Ser-845) 1-hour following odor preference training with an ISO injection. Furthermore, to test whether phosphorylation also occurred following learning, the authors performed Western blots of OB synaptoneurosomes following odor preference learning. The authors performed odor preference training on PD 6, by injecting 2 mg/kg of ISO subcutaneously prior to odor preference learning. Learned animals showed a significantly higher expression of AMPA GluR1 subunits 24-hours after training in comparison to control animals (Cui et al., 2011).

1.51 Odor-Shock learning-associated plasticity

However, aversive stimuli like tail pinches and foot shocks have also been successful in inducing preference behavior within the critical period as well as experience- or learning-dependent changes in the olfactory system (Raineki et al., 2009a; Shionoya et al., 2006). For this reason, both models allow for the assessment of learning by testing for preference following training with odor paired the US. Odor-shock models allow direct evaluation of approach as well as aversion behaviors during the sensitive period (Raineki et al., 2009a;

Raineki et al., 2012; Roth & Sullivan, 2005; Sullivan & Holman, 2010; Wilson & Sullivan, 1991).

Roth and Sullivan (2004) examined memories of abuse (or aversive stimuli) in the olfactory system during the critical period by utilizing a classical conditioning paradigm of pairing odor with a shock. Thereafter, they used the immunohistochemical marking Fos protein to assess the resultant effects in the brain. Fos is an immediate early gene that serves as a marker for neural activity and reflects modifications in neuronal plasticity associated with learning and memory (Dragunow & Bilkey, 2002; Herrera & Robertson, 1996). During the training phase, pups were presented with 14-pairings of peppermint odor and a 0.5mA tail shock on PD 7. The results indicate that a 0.5mA tail-shock was successful in inducing odor preference in the Y-maze. As well, the researchers reported learning induced changes in the number of Fos-protein cells in the granule and mitral cell layers of the OB, the latter has been implicated in associative learning in pups (Sullivan, Wilson, & Leon, 1989b; Wilson et al., 2004; Yuan, Harley, Darby-King, Neve, & McLean, 2003). Moreover, Fos staining of the anterior piriform revealed a training effect, which was absent in the posterior piriform cortex. Their results indicate that odor-shock training results in odor preference learning-induced changes in the OB and aPC during early life (Barkai & Saar, 2001; Datiche et al, 2001).

A study conducted by Raineki and colleagues (2009) assessed the characteristic development of odor learning over the span of development by comparing three learning paradigms: LiCl, 1.2mA hind-limb shock and 0.5mA shock. In the first set of experiments, male and female pups were randomly assigned to one of three conditioning groups: 0.5mA shock with odor, 1.2mA shock with odor, or LiCl with odor at three developmental stages (PD 7-8, PD 12-13, PD 23-24). Training sessions consisted of 11 presentations of 30 second peppermint odor

exposure, followed by a one-second hindlimb shock and a four minute intertrial interval. Behavioral testing results indicated that at PD 7-8, a 0.5mA shock induced odor preference for that odor, while a 1.2mA shock and LiCl resulted in odor aversion. Beyond the critical period at PD 12-13 and PD 23-24, all conditioning procedures produced odor aversion (Raineki et al., 2009a)

Thereafter, the researchers used 2-Deoxyglucose (2-DG) autoradiography to assess the neural substrates associated with odor preference and aversion learning at distinct developmental stages. Prior to training, pups were injected with 2-DG. Following training, pups were decapitated and the brain was extracted for analysis of the aPC, pPC and the basolateral amygdala, which is associated with plasticity in adult fear conditioning and adult odor LiCL learning. At PD 7-8 the aPC showed enhanced uptake following 0.5mA shock conditioning. However, no changes were visible in the 1.2mA shock and with LiCl conditioning. Additionally, other age groups (PD 12-13 and PD 23-24) did not produce learning-induced modification of 2-DG uptake in the aPC. In the pPC, the aversion inducing conditioning at PD 7-8 (1.2mA and LiCl) produced enhanced 2-DG uptake. Whereas, odor preference inducing condition at PD 7-8 (0.5mA shock) showed no learning induced changes. Moreover, all conditioning groups at PD 12-13 and PD 23-24 exhibited learning induced modification in 2-DG expression in the pPC as well as in the basolateral amygdala.

The functional significance of suppressed avoidance learning with a mild shock versus a strong shock is the differential activation of regions in the olfactory system (Rainkei et al, 2009; Sullivan & Hollan, 2010). The authors suggest that odor-aversion learning in neonatal pups activates the OB-pPC neural circuit whereas, after the transitional period odor-aversion learning accesses the basolateral amygdala as well as the OB-pPC circuit (Raineki et al, 2009). This

study, along with a few past studies demonstrate suppressed odor aversion learning in rat pups during the critical period of attachment learning (Sullivan & Hollman, 2010; Raineki et al, 2009). The association between a novel odor (ex. Peppermint) with a 0.5mA shock or even a tail pinch resulting in odor preference has been previously reported by (Camp & Rudy, 1988b; Haroutunian & Campbell, 1979; Sullivan & Toubas, 1998)

Considerable progress has been made in delineating the neural changes associated with odor learning in neonatal pups through classical conditioning studies. Previous studies have indicated an enhanced response in the OB following odor-learning using a variety of techniques like 2-DG uptake, c-Fos immunohistochemistry, electrophysiology and others (Coopersmith et al 1986; McLean et al 1999; Roth & Sullivan, 2005; 2006; Yuan et al 2003). However, it is unclear whether odor-shock conditioning activates the same mechanisms associated with neural plasticity as odor learning using stimuli like milk or stroking (Bouwmeester et al 2002; Sullivan & Hollman 2010).

1.6 Objective and hypothesis

The physiological basis of learning and memory is hypothesized to be regulated by experience-dependent synaptic strengthening and weakening (Quinlan et al., 2004). Synaptic strength is accompanied by an increase in LTP threshold (Brun et al., 2001). NMDA and AMPA receptors play a vital role in initiating and maintaining long-term changes in synaptic strength (Malinow & Malenka, 2002). Previous studies have reported changes in NMDA and AMPA number at the synapse and subunit composition following learning (Sans et al 2003; Quinlan et al 1999). This current project aims to assess the synaptic composition of NMDA and AMPA receptors by conducting immunoblots of obligatory subunits in order to quantify total number of

receptors following odor preference and odor aversion learning. To do this, we adopted Sullivan and colleagues 2000 odor shock model and implement a full body shock, rather than a hindlimb shock. First, we sought to test whether preference and avoidance learning could be induced using a mild shock of 0.1mA paired with peppermint odor, a moderate shock of 0.5mA paired with peppermint odor or a strong shock of 1.2mA paired with odor compared to odor only rodents during the critical period (PD 6-7). Thereafter, we investigated changes in the NMDA subunit NR1 and AMPA subunit GluR1 in our preference and avoidance learning models in the OB, aPC and pPC through semi-quantitative immunoblot analysis of synaptoneurosome preparations.

Chapter 2: Methods and materials

2.1 Experimental subjects

Sprague Dawley rats were used for the behavioral experiments as well as protein immunoblots. The sex of the rodents was not used as a factor in these studies; rats were randomly chosen. Dams were monitored and allotted a maximum number of 12 pups (culled to 6 male and 6 female when possible). The day of birth for rat pups was PD (post-natal day) 0 and experiments were conducted on PD 6 in a temperature controlled environment maintained at approximately 26-28^{O}C. A heating pad was utilized during experiments to ensure that resting boxes were at a comfortable temperature with unscented wood shavings inside. All rodents were maintained under a reverse 12 hour dark/light cycle at 22^{O}C in polycarbonate cages with *ad libitum* access to food and water. Experiments followed guidelines set by the Canadian Council of Animal Care. All experimental procedures were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland.

2. 2 Experimental Procedure

2.21 Odor-Shock Training

On PD 6, eight rodents were randomly selected prior to each individual experiment. This protocol was adopted from the Sullivan lab (Raineki et al., 2009a; Raineki et al., 2012). Odor was paired with one of the intensities of aversive stimuli (a weak shock of 0.5mA and a strong shock of 1.2mA) to produce associative memories that could be tested for recall 24-hours

following training and compared to a control group that did not receive a shock and was only exposed to the peppermint scented bedding in the shock training chamber. The current project modified this procedure to include an additional mild shock of 0.1mA, due to initial inability to replicate the results, which was attributed to the differences in experimental equipment (see Figure 2). Therefore, an additional mild shock was added to the protocol. The four experimental conditions used were as follows: a) control group: odor only, b) mild shock: 0.1mA shock and peppermint odor, c) intermediate shock: 0.5mA shock and peppermint odor, and d) strong shock: 1.2mA shock and peppermint odor.

Pups were randomly chosen, and trained separately with a random experimental condition. Each pup was isolated from the dam for a 10-minute habituation period. Thereafter, the pup was transported to the training set-up, and placed in an odorless resting box (7 X 5 X 6 inches) that was ventilated by a medical air-tank (VitalAire) (Figure 3. A). Pups received training in a lidded and ventilated shock chamber (8 X 8 X 10 inches) which consisted of 20 shock bars. Beneath the shock bars was a shallow (2 X 8 X 10 inches) odor box with a lid (Figure 3. B). The amplitude of the shock was controlled by a switch to 0.1mA, 0.5mA or 1.2mA for each specific rodent that was undergoing training. Shocks were delivered by manually switching on a shock generator/scrambler (Muromachi Kikai Model SGS-003DX).

Training began at 0 seconds, when the pup was placed in the shock chamber while exposed to peppermint scent bedding (0.3mL/500ml bedding) from the odor box. On the 29th second, a shock was delivered for 1 second, thereafter the pup was returned to the resting box for a period of 2 minutes. Following the two minutes, they were again exposed to peppermint and received another shock and this was repeated for 11-trials over a span of 25-minutes. Following

training, pups were labeled from 1-8 to correspond with the experimental condition and returned to the dam.

2.22 Two-Odor Choice Test

24-hours post-training on post-natal day 7, pups were tested in random order for preference to peppermint scent after odor-shock training. Pups were habituated for 10 minutes away from the nest. Two sets of boxes were separately filled with peppermint scented wood shaving bedding (0.3mL/500ml bedding) and plain bedding. A Plexiglas testing chamber (30 X 20 X 18 cm) was placed over both boxes separated by a neutral area of 2cm (Figure 4. A & B). In a series of 5 trials with 1-minute inter-trial resting intervals, preference was measured by the time spent over peppermint versus unscented bedding. The criterion that qualified for preference for either peppermint or unscented bedding was the movement of the pup's nose from the neutral zone to either side: a stop watch was used to measure preference for each scent. Time spent in the neutral zone was not accounted for. During the 1-minute inter-trial resting intervals, pups were placed in a heated resting box that was ventilated with medical clean air. With the onset of every trial, the vertical position of the pup was alternated to control for possible directional preferences. The total time (in seconds) spent over peppermint scented and unscented bedding was used to calculate the mean ± SEM (total seconds over peppermint scented bedding divided by time spent over peppermint and unscented bedding). A one-way ANOVA was carried out and post-hoc Tukey and Fisher LSD pro tests were used determine whether there were significant differences between experimental conditions for odor-shock learning.

2.3 NMDA and AMPA Receptor Analysis

2.31 Sample Collection

Brain samples were collected at two separate intervals, one at 3-hours post training to test immediate changes in receptor expression and then again 24-hours post-training to assess long-term receptor changes. Pups were anesthetized prior to decapitation by being submerged in ice for 5-10 minutes. To safeguard against frostbite due to exposure to the ice, pups were wrapped in nitrile gloves and a hole was made to permit air circulation. Pups were decapitated immediately after undergoing anesthesia using a sharp scissors. The OB, aPC and pPC were extracted and flash frozen on dry ice. Thereafter samples were transferred to pre-labeled tubes and samples were stored in 80^{O}C. All animal extractions were performed with the utmost precision and speed to reduce pain and ensure accuracy of the sample extractions.

2.32 Protein assay of synaptoneurosomes

Receptors at synaptic sites have provided valuable information about molecular mechanisms underlying neuronal activity. Synaptoneurosome isolation during protein extraction has been previously reported to show an enriched quantity of synaptic proteins (Quinlan et al., 1999). To achieve these enriched neuronal markers, which contain both the presynaptic and postsynaptic vesicular component, namely, the synatoneurosome, several studies reported the use of the crude particulate fraction (Hollingsworth et al., 1985; Quinlan et al., 1999). This method was used to extract synaptic receptors from the OB, aPC and pPC that would be used to assess AMPA and NMDA receptor content following training.

Previously collected samples (n=8 x 3; OB, aPC, pPC) from neonates following 3- and 24-hour training sessions were retrieved from -80°C storage and kept on dry ice. Each region of interest was processed separately to avoid cross contamination and mix-up. Briefly, samples from the aPC, for example, were homogenized using Teflon-glass tissue homogenizers for a 10-minute period (Thomas Scientific: NJ, USA) in ice-cold HEPES buffer. The buffer mixture containing HEPES (1.0M, pH 7.4), NaCL (1.0M), NaHCO3 (0.5M), MgCl2 (100mM), CaCl2 (100mM), KCl (100mM), KH2PO4 (100mM), glucose (100mM), phosphatase inhibitor cocktail (10X) (Roche: Laval, QU) mixed with protease inhibitor (10X) (Roche: Laval, QU), EDTA (10mM), PMSF (200mM), and dH2O saturated with 95% O2 and 5% CO2 on ice for 5-minutes, was freshly prepared prior to the protein assay. Thereafter, the samples were incubated on ice for 10-minutes before being collected by syringes to be passed through syringe filter holders (Millipore: Dermstadt, Germany) which contained two 100 μm nylon filters (Small Parts Inc.: IN, USA) and a 5 μm filter (Millipore: Darmstadt, Germany). Following a second incubation on ice for 10-minutes, the samples were centrifuged for 20 minutes at 4000 x g at 4°C. Next, the supernatant was discarded and the pellet was re-suspended in 75 μl of HEPES buffer. A BCA protein assay kit (Pierce: MA, USA) was utilized to determine protein concentrations. Cell lysate dilutions for samples were 1/10 with 48.6 μl of dH_2O, 5.4 μl of lysate for a total of 54 μl, 20 μl duplicates of which would be added to wells of a 96 well plate with 200 μl of BSA solution. Standards were premade and mixed in the well with BSA reagents for 6 duplicates. The 96 well-plate was incubated for 30 minutes at 37°C. Following incubation the plate was read at 540nm on a BIO-RAD Model 3550 Microplate Reader. Protein concentrations of 30 μg were calculated using the values obtained for the samples and graphed on a regression curve based on the values of the standards using the $y=m\chi + b$ equation.

2.33 Western Blot

2.331 Gel Electrophoresis

Following the protein assay and determination process, samples were assessed for receptor content. This technique separates proteins based on molecular weight through gel electrophoresis, thus allowing for a semi-quantitative assessment for protein content (Cohen, Blomberg, Berzins, & Siekevitz, 1977; Hollingsworth et al., 1985; Quinlan et al., 1999). For this particular project, an SDS-PAGE gel composed of acrylamide/bis-acrelamide 30% 29:1, TRIS, 20% SDS, 1.5% APS and TEMED was utilized for protein separation. Both the 7.50% running gel and 4.0% stacking gel were prepared for gel electrophoresis prior to sample preparation.

Samples containing 30 µg of protein were prepared by adding pre-determined lysate volume, 6 µl of 5X sample buffer (0.3 M TRIS-HCl, 10% SDS, glycerol, 0.5M dithiothreitol (DTT), and dH20 to bring the volume to 30 µl. Following a 5 minute heat block, 32 µl samples and 5 µl protein ladder (Millipore: Darmstadt, Germany) were loaded into lanes to be separated by SDS-PAGE (approximately 1.5 hours).

2.332 Sample Transfer

Following gel electrophoresis, samples were transferred from the gel onto a nitrocellulose membrane (Amersham) in cold 1X transfer buffer [(250 mM TRIS base, 1.92 M glycine, dH20) and methanol] at 4 °C using a constant voltage of 100 V for 1 hour.

2.333 Sample Detection

Once samples were transferred, the membrane was cut horizontally at 72 kDa and washed in TBS/T. To detect whether samples successfully transferred onto the nitrocellulose membrane, the blot was stained with 1X Ponceau Red for 5-minutes. Successful transfer is marked by distinct vertical columns. Also, the colored ladder itself indicates successful transfer of proteins from the gel.

2.334 Antibody Application and Exposure

For the purpose of consistency, all top portions of membranes were first probed with a rabbit antibody recognizing NR1 (i.e., GluN1) (1:2000 Millipore: Darmstadt, Germany) with 4 % milk + TBS/T. The following day, the membrane was stripped using Restore buffer (ThermoScientific) then probed with a monoclonal rabbit antibody recognizing GluR1 (i.e., GluA1) (1:1000 Millipore: Darmstadt, Germany) with 4 % milk + TBT/T. The bottom portions were probed with a rabbit antibody recognizing β-Actin (1:10,000 Millipore: Darmstadt, Germany) with 4 % milk + TBS/T. Membranes were agitated with primary antibodies overnight at 4 °C. Antibodies were detected using an anti-rabbit secondary antibody, horseradish peroxidase conjugate (HRP) in 4 % milk + TBS/T (1:20,000 Pierce: MA, USA). Super West Pico Chemilumeniscent Substrate (Pierce: MA, USA) (ECL) was used for precise detection of the proteins. Thereafter, the blots were aligned based on the cut and developed on X-ray film (AGFA) in a dark room for visualization.

As previously mentioned, all top portions membranes were first probed with NR1 overnight, following exposure the membranes were stripped and incubated overnight with GluR1

antibody. The antibody application and exposure protocol was repeated to achieve accuracy and consistency for the second antibody.

2.335 Blot Analysis

Once films were developed, they were scanned (CanoScan LiDE 200) onto the computer for analysis using ImageJ software. To determine the levels of the proteins of interest, the optical density of each band must be determined and normalized to the β-Actin control bands for each particular sample. To do that, a small box is drawn around the first band, and dragged onto the other bands of a particular antibody of interest (NR1 samples from one blot for example) to produce density curves. The same is done for β-Actin bands of that same blot, and divided to give a ratio of normalized relative densities for each sample. Mean optical densities were obtained for each ROI and assessed for differences and outliers.

Thereafter, the means for each antibody in every experimental condition (mild, intermediate, and strong shock groups) were evaluated using one-way ANOVA and subsequently post-hoc Tukey and Fisher LSD Pro tests were used to evaluate whether changes in receptor expression following odor-shock training occurred at both 3 hours and a day later in comparison to control (3-hour and 24-hour odor-only) groups for all three ROIs. Results were reported as Mean±SEM.

Chapter 3: Results

3.1 Mild to moderate shocks result in odor preference learning in neonatal rodents

In order to assess associative odor memories in the brain, a behavioral model was required that produced odor learning behaviorally in neonates. An odor shock paradigm was adopted and utilized for the assessment of associative memory formation (Aceves-Pina & Quinn, 1979; Raineki et al., 2009a; Sullivan et al., 2000). However, an additional experimental condition (0.1mA shock group) was added because of the difference from previous models that utilized hind-limb shock, rather than the full body shock employed in the current protocol (Figure 2b) (Raineki et al., 2009a).

Our behavioral investigation yielded similar results to those previously published (Raineki et al., 2009a; Sullivan et al., 2000). A one-way ANOVA revealed a statistically significant difference among behavioral groups ($F_{(3, 58)} = 11.29$, $p = 0.04$). Prior reports were of peppermint odor preference in neonatal rodents when they received a 0.5mA hindlimb shock while exposed to peppermint odor on PD 6 and were tested for odor preference in a two-choice odor test on PD 7. Consistent with previous data, neonatal rodents that received a 0.5mA body shock on PD6 expressed peppermint odor preference on PD 7 (48.14 ± 11.30, n = 16) compared to odor only control rodents that did not receive a shock (35. 80 ± 13.31, n = 17). The post hoc comparisons using Fisher least significant difference testing demonstrated a statistically significant difference between the 0.5mA condition and the control odor-only group ($p = 0.04$).

The milder shock of 0.1mA shock paired with peppermint odor (56.36 ± 7.32, n = 11; $F_{(3, 58)} = 11.29$, $p < 0.001$) also resulted in odor preference on PD 7 compared to the odor only

group (35. 80 ± 13.31, n = 17). The post hoc Fisher least significant difference testing comparisons revealed that there was a statistically significant difference between groups that received a mild shock of 0.1mA while being exposed to peppermint in comparison to controls (p = 0.0001). However, unlike previous findings, the strong shock of 1.2mA paired with peppermint odor (30. 85 ± 16.28, n = 18; $F_{(3, 58)} = 11.29$) was not significantly different than the control group of odor-only (35. 80 ± 13.31, n = 17). Post Hoc Tukey and Fisher test results do not show a significant difference between the two groups (p = 0.64). Multiple comparisons were used (Tukey and Fisher) to assess the statistical significance of differences between means using a set of confidence intervals. Fisher LSD allows for constracts of the individual confidence intervals.

3.2 Analysis of the olfactory system AMPARs and NMDARs following exposure to an odor paired with a shock

Immunoblots were carried out to investigate the changes of AMPAR and NMDARs associated with odor-shock memories in three regions of the olfactory system, namely OB, aPC and pPC at two time points following learning. This provided profiles for short-term (3-hours) and long-term (24-hours) changes associated with learning-induced changes in receptor expressions and subunit receptor composition; however it would be meaningful to include a 3-hou memory test in future behavioural experiments to account for short-term memory. Synaptoneurosome Western blots examined the NR1 subunit of the NMDAR and the GluR1 subunit of the AMPAR to assess total synaptic number of receptors. Previous research on NMDA and AMPA receptors show that these receptors play a strong role in the induction and maintenance of LTP and involved in learning

and memory (Barr et al., 2009; Jerome et al., 2012; Landers & Sullivan, 2012; Lethbridge, Hou, Harley, & Yuan, 2012).

3.21 Olfactory bulb

First, the patterns of expression of these receptors at OB synapses 3-hours and 24-hours following odor-shock training were investigated. These levels were then compared to the respective control group (odor-only group). A one-way ANOVA indicated a general group effect in NMDA NR1 synaptic expression in the OB ($F_{(7, 53)} = 2.69$, $p = 0.01$); Figure 5). Synaptic expression of the obligatory NR1 subunit of NMDAR was statistically significantly down-regulated 3-hours following odor-shock training with a 0.5mA (PD 6: 0.66 ± 0.38; n = 7, p = 0.03) compared to control animals that were only exposed to peppermint scent (PD 6: 1.21 ± 0.87, n = 8). This down-regulation has been previously reported in work conducted in our lab indicating a down-regulation of NMDA subunit NR1 3-hours after training in a stroking preference model, which exposes neonatal rodents at PD 6 to peppermint scented bedding while being stroked (mimicking maternal behavior) (Lethbridge et al., 2012). However, it is important to note that this down-regulation only occurs transiently at the 3-hour time-point with the 0.5mA shock, but is not seen not at the 24-hour mark for the 0.5mA shock (0.84 ± 0.57, n = 8, p = 0.22). No other experimental group illustrated significant changes following odor-shock learning, which may suggest a dramatic decrease in synaptic NMDAR expression only transiently in the OB with odor-preference learning with a 0.5mA shock.

Unexpectedly, pups that exhibited robust odor preferences behaviorally 24-hours following training with a mild 0.1mA shock paired with peppermint odor did not show a significant change in synaptic NMDAR expression 3-hours later compared to its respective control group (PD 6: 1.21 ± 0.86, n = 8; Figure 5) in post-hoc analyses (p = 0.21). Similarly, 24-hours following training the

0.1mA shock group (PD 7: 1.16, ± 0.23, n = 7) NMDAR did not differ significantly from the control group (p = 0.92). This is, perhaps due to the mildness of the shock or a delayed onset in the change of expression of NMDARs, and no conclusions can be drawn from the current data. Concurrently, AMPAR expression at OB synapses was assessed however, one-way ANOVAs did not indicate a group effect in AMPAR synaptic expression at either the 3-hour or 24-hour time points ($F_{(7, 55)} = 2.04$, $p = 0.06$); Figure 6.

3.21 Anterior piriform Cortex

Next we investigated whether a similar change occurred in the NMDAR and AMPAR expression 3-hours and 24-hours following learning in the aPC. The aPC plays an important role in early odor preference memory and learning (Motanis, Maroun, & Barkai, 2014; Raineki et al., 2009a; Truchet, Chaillan, Soumireu- Mourat, & Roman, 2002; Wilson & Stevenson, 2003). One study showed that pairing an odor with 0.5mA shock induced enhancement of 14C 2-deoxyglucose (2-DG) uptake within the aPC highlighting this as a region involved in preference learning (Raineki et al., 2009a). We investigated the role of the aPC in odor preference learning by training rodents using the odor shock model and assessing NMDAR and AMPAR expression 3-hours and 24-hours following learning. Interestingly, a similar trend was observed in our Western blot findings of the aPC (1.12 ± 0.42, n = 8). Our results showed that training with a 0.5mA shock paired with peppermint odor did not a significant change in NMDARs within the aPC ($F_{(7, 54)} = 1.82$, $p = 0.1$); Figure 8. As well, the aPC revealed an ansence of a group effect in AMPAR expression ($F_{(7, 53)} = 1.42$, $p = 0.32$; Figure 9).

3.23 Posterior piriform cortex

The relative change of NMDARs and AMPARs was also assessed in the posterior piriform cortex. This area has been included in the current project due to its previously reported

role in aversive learning in neonatal rodent during the course of development (Barr et al., 2009; Calu et al., 2007a; Canteras, Kroon, Do-Monte, Pavesi, & Carobrez, 2008; Haberly, 2001). For example, Rainenki et al. (2009) showed that neonatal rodents displayed enhanced 14C 2-deoxyglucose (2-DG) uptake within the pPC following training with a 1.2mA shock that was paired with an odor which resulted in an aversion to the odor.

We set out to see whether a similar finding could be attained through the analysis and quantification of NMDA and AMPA receptors which are implicated in LTP induction and maintenance as well as in learning and memory particularly in the pPC (Zhang et al., 2010). Despite the large number of immunoblots conducted within the posterior piriform cortex, results were negligible for both NMDARs and AMPARs for each experimental condition. For instance, although one-way ANOVA results indicated a statistically significant group effect in NMDA NR1 expression ($F_{(7, 55)} = 2.52$, $p = 0.02$), Tukey and Fisher least squares difference tests did not reveal statistically significant differences between experimental groups. AMPA GluR1 expression did not yield statistically significant group differences following a one-way ANOVA ($F_{(7, 55)} = 1.07$, $p = 0.49$).

Taken together, the current western blot experiments, within the olfactory system, show that synaptic learning changes only occur with a 0.5mA shock paired with peppermint odor in both the OB and aPC. However, the changes associated with learning and memory also require AMPA receptor change, which currently yielded insignificant results. Thus, these results suggest a lack of change in AMPAR expression, which was previously reported to occur during early development in several regions of the brain varying from the visual cortex to the hippocampus as well as the olfactory system (Baddeley, 1992; Cutsuridis & Wennekers, 2009; Lethbridge et al., 2012; Poucet, Save, & Lenck-Santini, 2000).

Chapter 4: Discussion

4.1 Behavioral results

In order to investigate the role of NMDA and AMPA receptors in early odor preference and aversion learning, we evaluated the efficacy of an adapted odor-shock model from Sullivan and colleagues (2000). Previous reports inducing odor preference and aversion learning through shock conditioning utilized a hindlimb shock at two distinct amplitudes (0.5mA and 1.2mA) during behavioral training and a Y-maze during testing (Raineki et al., 2009a; Roth & Sullivan, 2005; Sullivan et al., 1991; Sullivan et al., 2000; Sullivan, 2005). We examined odor preference and aversion by adopting a whole body shock and a two-choice odor test. In addition to the 0.5mA and 1.2mA shock paired with peppermint odor, we introduced an even milder shock of 0.1mA. The mild shock would account for potential differences in the original protocol which utilized a foot shock rather than a body shock used in these experiments. The 0.1mA shock paired with peppermint odor, as well as the 0.5mA shock paired with odor successfully produced odor preference memories 24-hours later, as neonatal pups on PD 7 spent significantly more time over peppermint scented bedding in comparison to control animals.

In fact, the mildest shock induced the highest level of preference learning, with more time spent over peppermint scented bedding in comparison to the 0.5mA shock group. To say that a mild shock may in fact be as pleasant as maternal grooming, which is often rough is a potential explanation for this result (Rother & Sullivan, 2001). The 0.5mA shock producing preference was anticipated and can be attributed to mild rough handling by the dam, also resulting in

preference learning. Neonatal rodents experience rough handling by mothers in early life as she may step on them and bite them to pick them up (Moriceau & Sullivan, 2005). Pups are pre-disposed to this rough demeanor, and exhibit approach behaviors, as the mother is the sole source of food and protection (Moriceau & Sullivan, 2005). Another hypothesis is that norepinephrine (NE) may be released in more optimal levels with less intense shocks. In fact, NE is necessary and sufficient in neonatal rodents for acquiring olfactory preference (Sullivan & Wilson, 1991). Previous studies have shown 2-DG uptake modifications in the OB following odor preference learning (Sullivan et al, 2001, Sullivan & Wilson, 1991). As such, there is a possibility that a mild shock (0.1mA) may release more optimal levels of NE and produce a much longer lasting memory.

More interestingly, the strong shock of 1.2mA paired with peppermint odor did not produce long lasting memories of aversion despite strong physical signs of pain and escape behavior exhibited by the pups. Previous studies utilizing the 1.2mA shock, reported similar findings of behavioral arousal during training with a 1.2mA shock (Sullivan & Holman, 2010). Perhaps the whole body shock was not sufficient to produce odor aversion, as the shock may have been absorbed by the whole body rather than being concentrated in one region of the body. However, the more likely answer is that 1.2mA shock was too severe to produce long-term memory. In a paper published by the Grandin (1997) in Science, testing the humaneness of electrical stunning on farm animals the authors measured the level of discomfort and physiological arousal in rodents. The authors posit that electrical stimulation results in a fear response, which activates the release of cortisol in the brain. This finding has also been previously reported in fear-conditioning models in rodents with activation of the hypothalamic axis and corticosterone release (LeDoux, 1994). Increased corticosterone levels were previously

reported as correlated with impaired spatial working memory in fear-provoking conditions (Woodson, Macintosh, Fleshner, & Diamond, 2003). Therefore, the 1.2mA shock may not have been successful in aversion learning due to high levels of corticosterone release in the brain during shock conditioning. In addition, previous reports that portray aversion learning using the 1.2mA shock were tested using a y-maze paradigm, wherein rodents must physically move to one of two arms (one with neural scented bedding and the other with peppermint scented bedding) (Roth & Sullivan, 2001. In contrast, the paradigm utilized in the present study calculated odor preference and aversion by measuring the amount of time a rodent placed its nostrils and body above peppermint scented bedding versus neutral scented bedding. It is not clear whether different results would be achieved using a y-maze, as such future testing may be helpful for aversive learning in neonatal rodents.

4.2 Semi-quantitative Analysis of Immuno Blots

Synaptoneurosome Western blot experiments were designed to investigate the role of synaptic NMDA and AMPA receptors in early odor preference and aversion learning. Recent evidence implicates NMDA-dependent insertion of AMPARs following learning. To examine whether odor-shock learning involves changes in synaptic AMPAR and NMDAR expression following conditioning, we conducted synaptoneurosome Western blots 3 hours and 24 hours to measure the change in receptor expression following learning.

4.21 NMDA NR1 Subunit Expression

We tested whether odor learning altered the expression of NMDARs in the OB, aPC and pPC by assessing obligatory subunit NR1. Immunoblotting showed that early odor preference induced by 0.5mA shock paired with peppermint odor caused a transient reduction in overall

synaptic expression of NMDA receptors 3hours following learning with a significant down-regulation if NR1 subunit expression in the OB. Previous experiments in our lab (Lethbridge et al., 2012) also found a transient decrease in NMDAR expression in the OB following odor-stroke conditioning resulting in odor preference memory. It is hypothesized that this transient reduction in synaptic NMDA receptors reflects altered plasticity in affected synapses. Reduced plasticity is associated with decreased activity in recently active synapses possibly to promote stabilization of recently formed memory, allowing learning-specific changes to occur and remain at the synapse. Previous research from our lab and other labs echo this finding suggesting that additional activity may prevent memory maintenance and memory encoding (Lethbridge et al., 2012; Quinlan et al., 2004).

The 0.5mA shock paired with peppermint odor has been previously reported to activate the OB (Raineki, Shionoya, Sander, & Sullivan, 2009b; Raineki, Moriceau, & Sullivan, 2010). Studies assessing 2-DG uptake and Fos staining have localized odor preference learning induced by a 0.5mA shock to the OB (Roth, Moriceau, & Sullivan, 2006). Roth et al (2006) showed that there was significant changes in Fos protein expression in the granule cell layer of the OB and stated that their results demonstrated that modulation of Foc protein expression reflected memory consolidation in the olfactory circuitry of neonatal brains. Similarly, we have found a transient down-regulation of NMDA NR1 3-hours following learning in the OB. Again suggesting that memory changes for odor preference occurs in both the OB following odor preference conditioning.

However, we did not observe significant changes in NR1 expression in the aPC as was reported by Roth et al (2006) in the 0.5mA shock group that produced odor preference. Moreover, other groups (0.1mA shock and 1.2mA shock) also did not yield significant changes

in NR1 expression in any region of the olfactory system (OB, aPC and pPC). The 1.2mA shock can be ruled out for NMDA receptor expression due to a lack of observed learning following odor shock behavioral conditioning. That is to say, the pups did not exhibit learning 24 hours following odor shock training, and therefore may not express learning specific synaptic changes in the olfactory system. The mild shock group receiving a 0.1mA shock paired with peppermint odor however, produced robust odor preference, but did not reveal significant changes in synaptic NMDA expression. The lack of change in total NMDA expression may in part be the reason for the absence of change in NMDA NR1 expression.

We quantified the expression of obligatory subunit NR1, however there may not have been a change in total number of NMDA receptors following 0.1mA shock training, but rather a change in NR2A/NR2B receptor expression. Previous evidence has suggested NR2A/NR2B expression exhibits dramatic change in the brain during development (Köhr, 2006). A study conducted by Quinlan and colleagues (2004) investigated the role of NR2A and NR2B in piriform cortex following learning through Western blots. There was a learning specific change in subunit expression between trained animals and naïve rodents, with a higher NR2A/NR2B ratio in the trained animals. As well, evidence from out lab also indicates a significant reduction in NR2B subunit expression at 24 h following odor-stroke training, and no change in overall NMDA expression at that time point (Lethbridge et al., 2012). These results indicate that NR2A and NR2B expression may be altered following learning, without a change in overall NMDA receptor expression. Lastly, changes in subunit expression are also reversible and transient, and an alternative explanation for the lack of receptor expression at 3 hours and 24 hours for the 0.1mA shock could be due to immediate changes in receptor expression that were restored at both those time points (Quinlan et al., 2004). However, one cannot be sure of this conclusion,

and additional experiments assessing NR2A and N2B expression are necessary.

4.22 The Lack of AMPA GluR1 Subunit Expression

We did not observe significant changes in AMPA GluR1 expression 3 hours or 24 hours following odor shock conditioning during the critical period of development. Previous reports using synaptoneurosome immunoblotting assessing GluR1 expression following odor preference learning, have reported otherwise. However, it is important to note the use of different antibodies probing for GluR1 expression utilized by Cui and colleagues (2011). In fact, we investigated the changes in GluR1 expression by using a monoclonal antibody (Millipore: Darmstadt, Germany), whereas they used a polyclonal antibody (Chemicon). Monoclonal antibodies are monospecific, which indicates that they have the same affinity for the antigen. This is due to the fact that monoclonal antibodies are comprised of identical immune cells from a single parent cell are therefore bind to and recognize a single epitope. Whereas polyclonal antibodies are comprised of diverse immune cells, and are capable of recognizing multiple epitopes on a targeted antigen (Akerstrom, Brodin, Reis, & Bjorck, 1985). Monoclonal antibodies are more sensitive to experimental conditions, if experimental conditions are kept constant, results are highly reproducible with little background staining in comparison to polyclonal antibodies. However, monoclonal antibodies are also vulnerable to loss of epitope due to experimental treatment leading to absent protein expression. Whereas, polyclonal antibodies are more tolerant of minor changes in chemical treatment and will also identify denatured proteins (Akerstrom et al., 1985). For this reason, chemical processing may account for the lack of GluR1 expression in our experiments.

4.3 Conclusion and Future Directions

The present project demonstrates that odor preference memory can be induced using both a whole body shock as well as by introducing alternative amplitudes of shock. Additionally, the synaptoneurosome Western blots show that NMDARs play a critical role in early odor preference learning with transient down-regulation of NR1 subunit in the OB and up-regulation of NR1 in the aPC. Together, these results demonstrate that total NMDA receptor expression in the olfactory system reflects associative learning changes following odor-shock conditioning.

However, many pieces of the puzzle continue to be absent in completing our understanding of the fundamental role of NMDA and AMPA receptor in synaptic plasticity associated in learning and memory. Variations in the procedures for synaptic expression can greatly contribute to the variability in the results. Future studies directly focusing on the results from my master's project, should first establish aversive learning using the odor-shock model. Thereafter, assessment of AMPA expression in the olfactory system following odor preference and aversion learning in the OB, aPC and pPC is required to provide a more complete picture of shock induced changes. Once, the role of total NMDA and AMPA can be established, extrasyanptic versus synaptic expression of these receptors should be investigated to understand the underlying mechanisms of synapse stabilization through synaptic trafficking of AMPARs and NMDARs.

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Figures

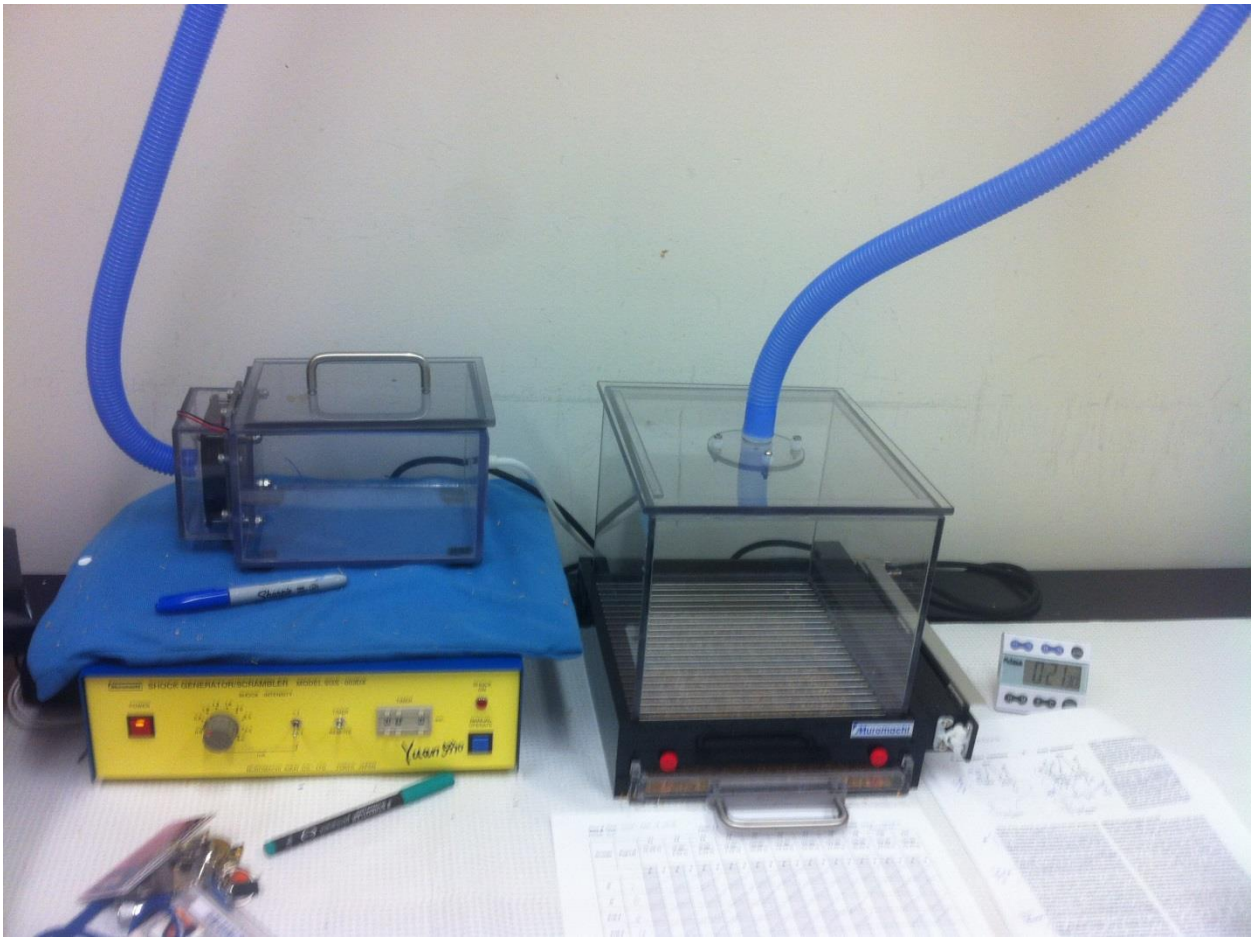

Figure 1: Odor-Shock Training Chamber. Adaptation of odor-shock paradigm with the inclusion of lidded and air-filtered resting box and shock chamber. Contact with peppermint odor was controlled by lid handle that exposed peppermint scented bedding once a pup was placed in the shock chamber.

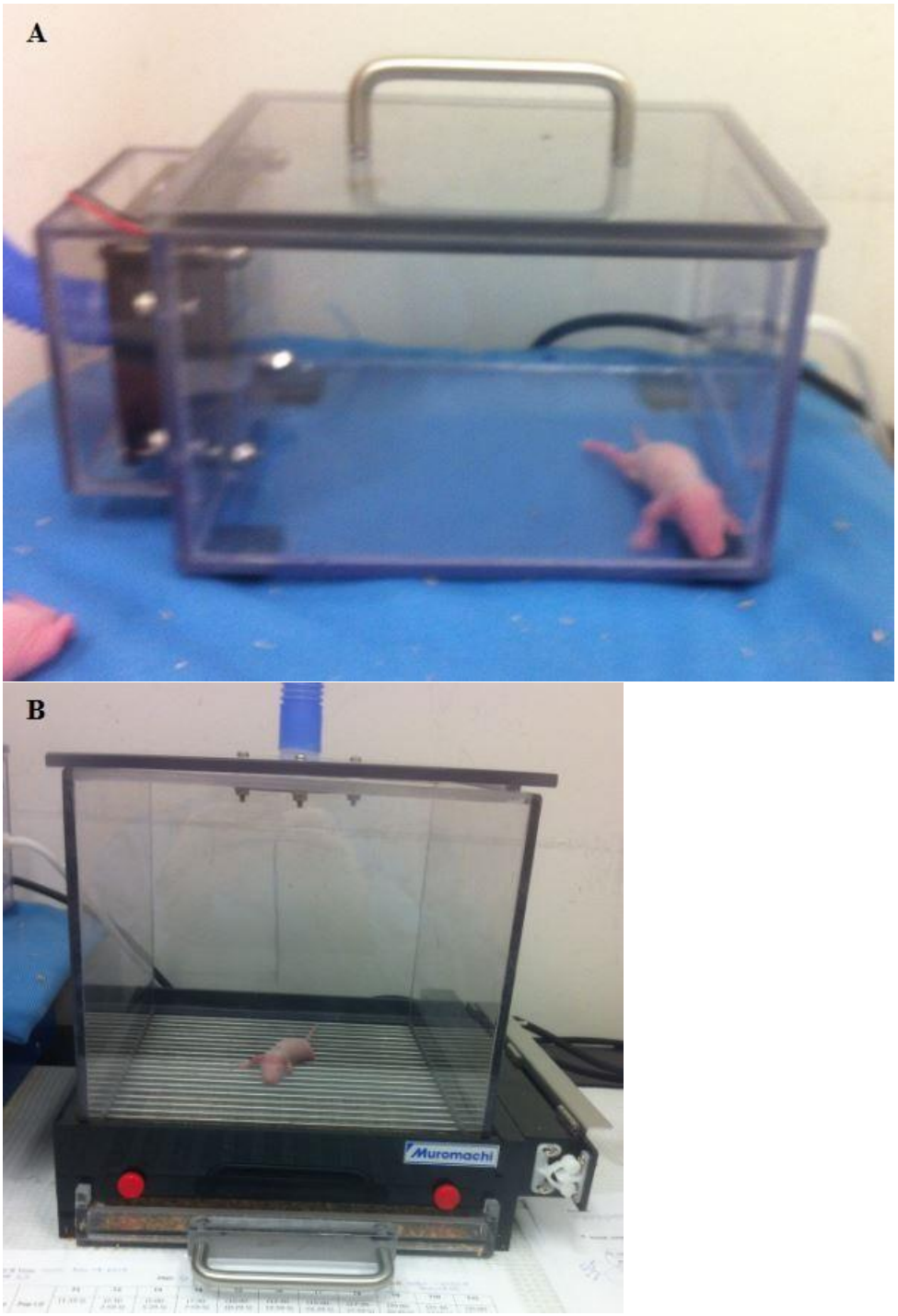

Figure 2: Odor-Shock Training paradigm. ***A.*** PD 6 pups are placed in the air-filtered resting chamber for two minute intervals between odor and shock delivery. ***B.*** Following rest, pups are exposed to peppermint scent for 30 seconds in the odor shock box and a shock is applied on the 29th second.

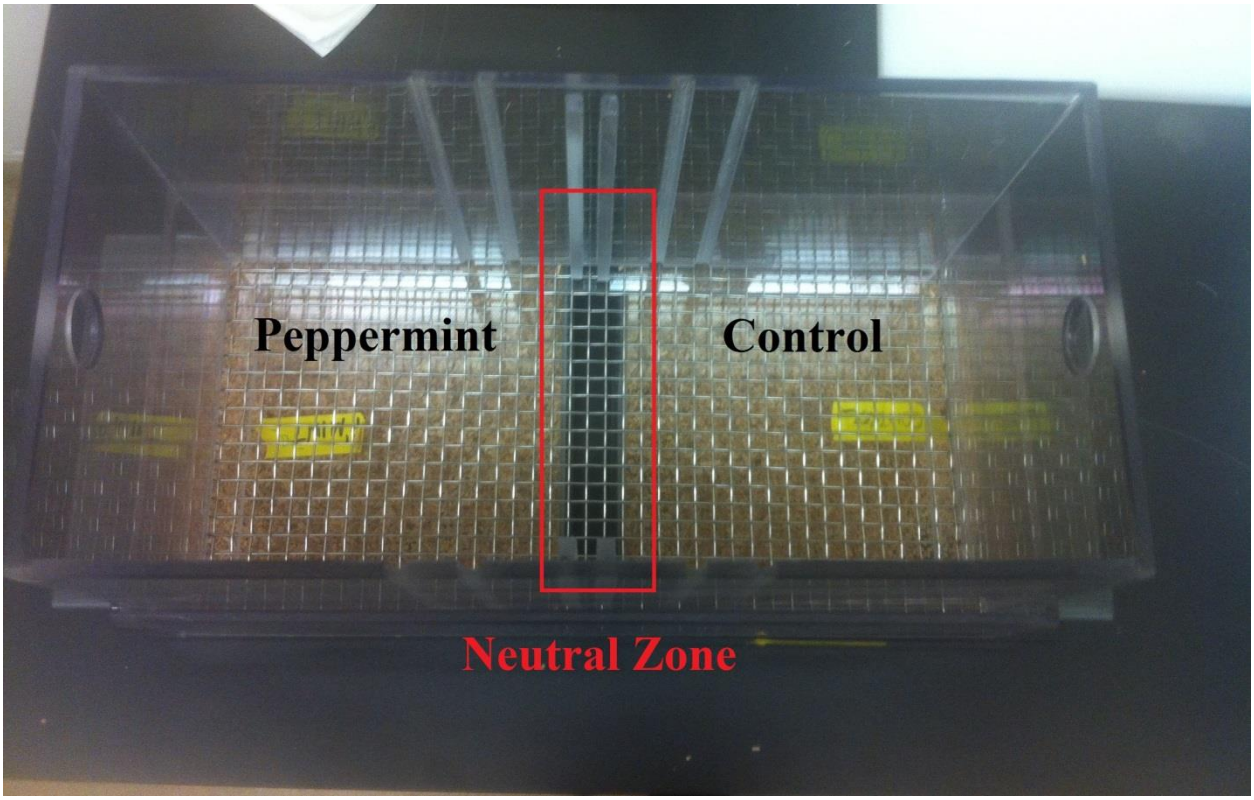

Figure 3: Two-Choice Odor Testing. Following odor-shock training, pups are tested for odor preference on PD 7. Pups are placed in the neutral zone, and freely able to move to either side for a period of 60 seconds. Over a total of five trials, the time spent over peppermint or unscented (control) bedding is calculated as an odor preference over total time with beddings.

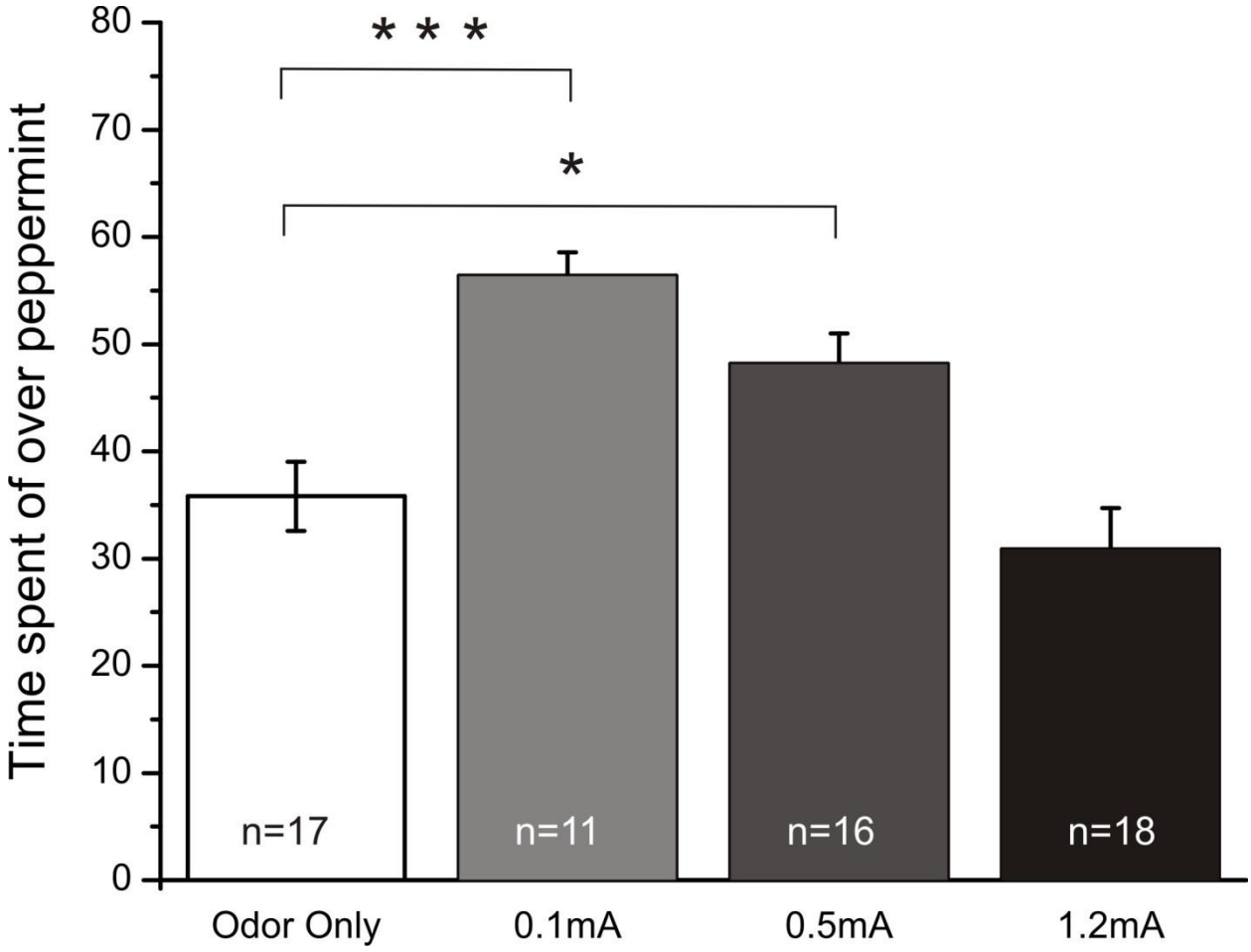

Figure 4: Mild and moderate but not strong shocks paired with peppermint odor produce preference for peppermint in rats (PD≥10). 24-hours following odor-shock training, pups that received a mild and an intermediate shock while exposed to peppermint scented bedding showed robust odor preference. Asterisk represents a significant difference from each of the other groups (*$p < 0.05$, ***$p < 0.001$).

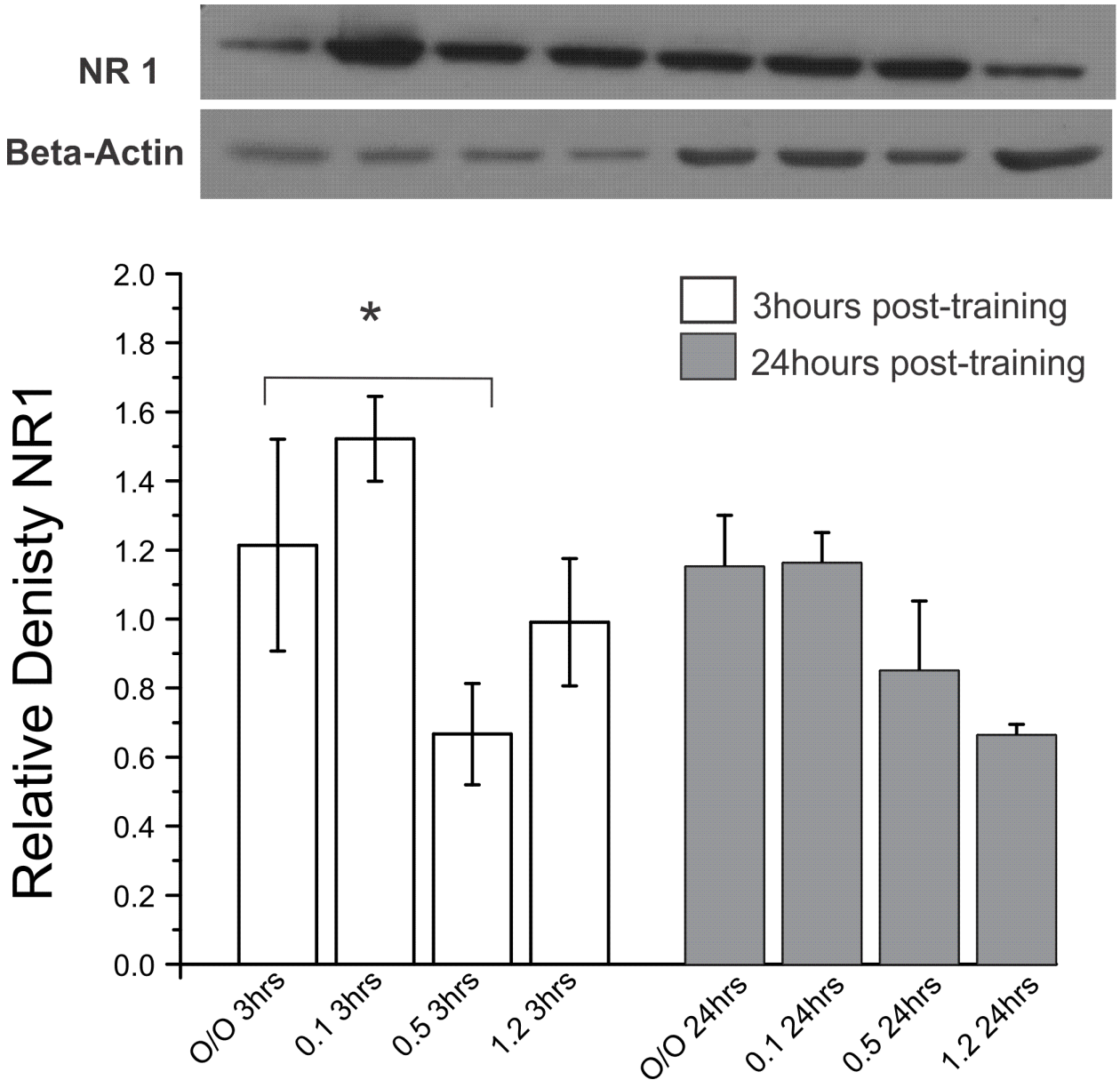

Figure 5: Transient down-regulation of NMDAR in the olfactory bulb (OB). Intermediate shock of 0.5mA produces a transient down-regulation of NMDAR subunit NR1 3hours following learning on PD6 ($p^* < 0.05$), but not after 24hours on PD7 in the OB. O/O: odor only (control), 0.1: 0.1mA shock paired with peppermint odor, 0.5: 0.5mA shock paired with peppermint odor, & 1.2: 1.2mA shock paired with peppermint odor.

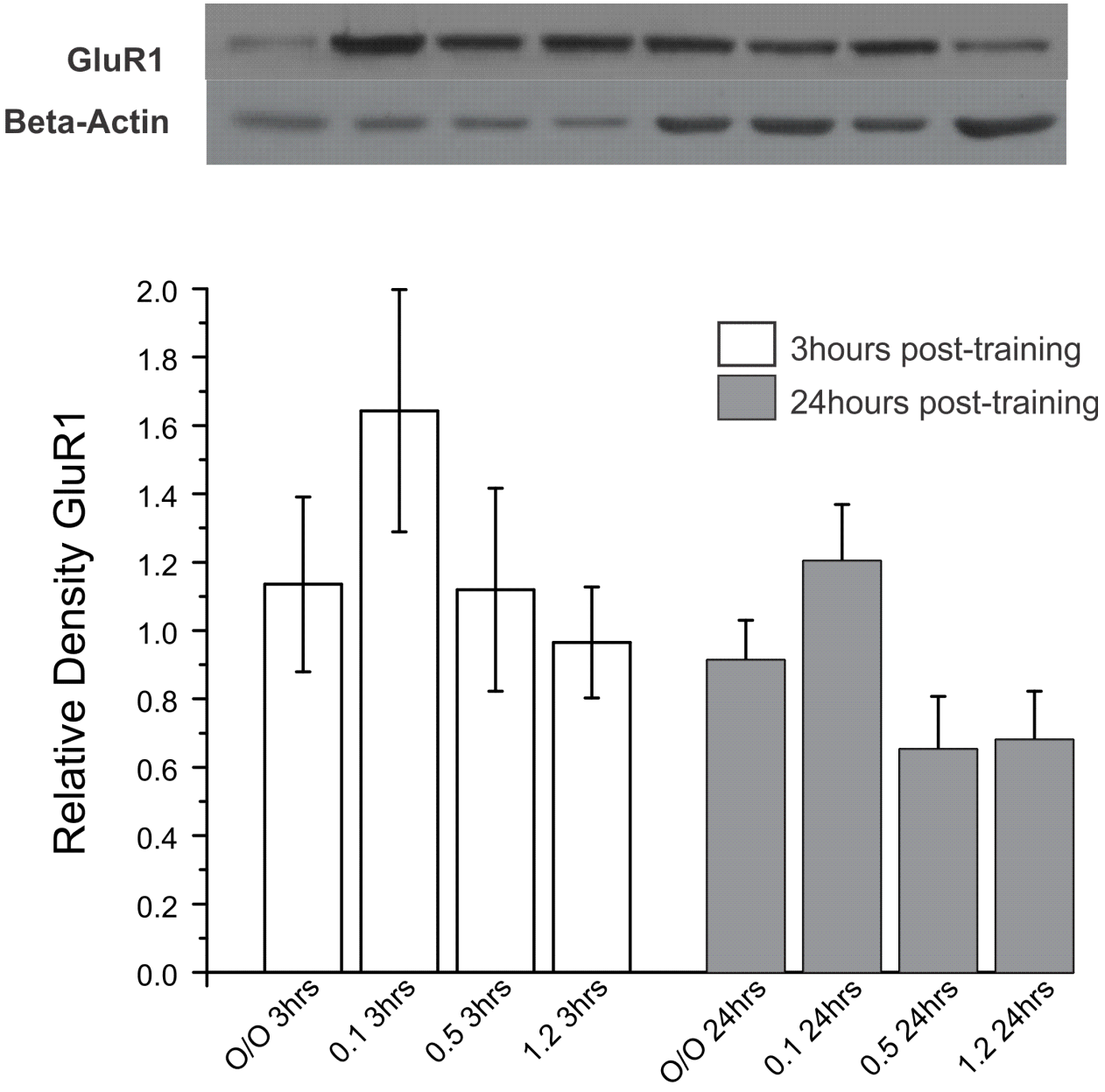

Figure 6: AMPAR subunit GluR1 in the olfactory bulb (OB). No receptor subunit changes (*$p < 0.05$). O/O: odor only (control), 0.1: 0.1mA shock paired with peppermint odor, 0.5: 0.5mA shock paired with peppermint odor, & 1.2: 1.2mA shock paired with peppermint odor.

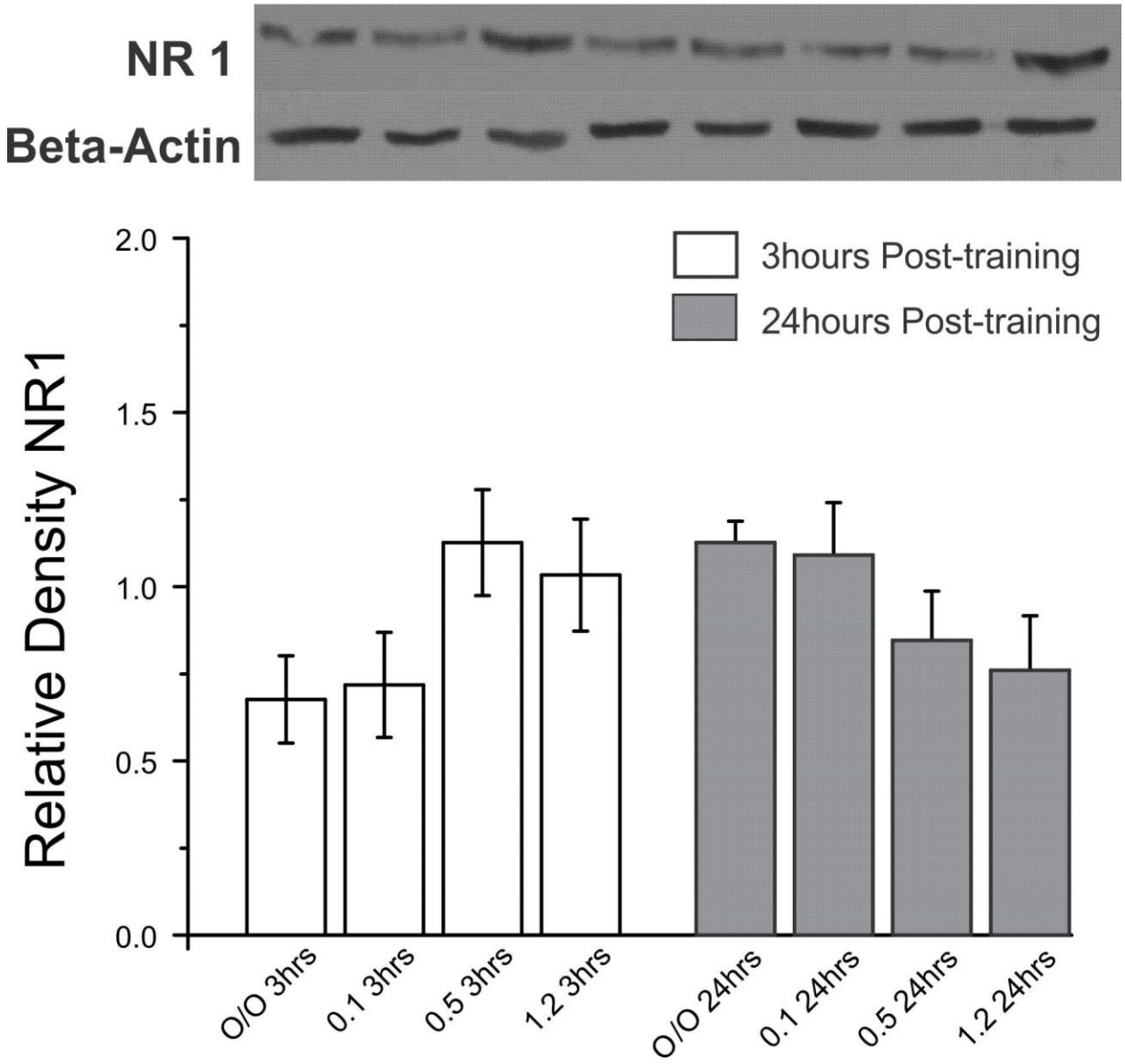

Figure 7: Intermediate shock of 0.5mA suggests up-regulation in the anterior piriform cortex (aPC). There is up-regulation 3hours following learning in the 0.5mA shock groups in the anterior piriform cortex but not 24hours following training (*$p < 0.05$). O/O: odor only (control), 0.1: 0.1mA shock paired with peppermint odor, 0.5: 0.5mA shock paired with peppermint odor, & 1.2: 1.2mA shock paired with peppermint odor.

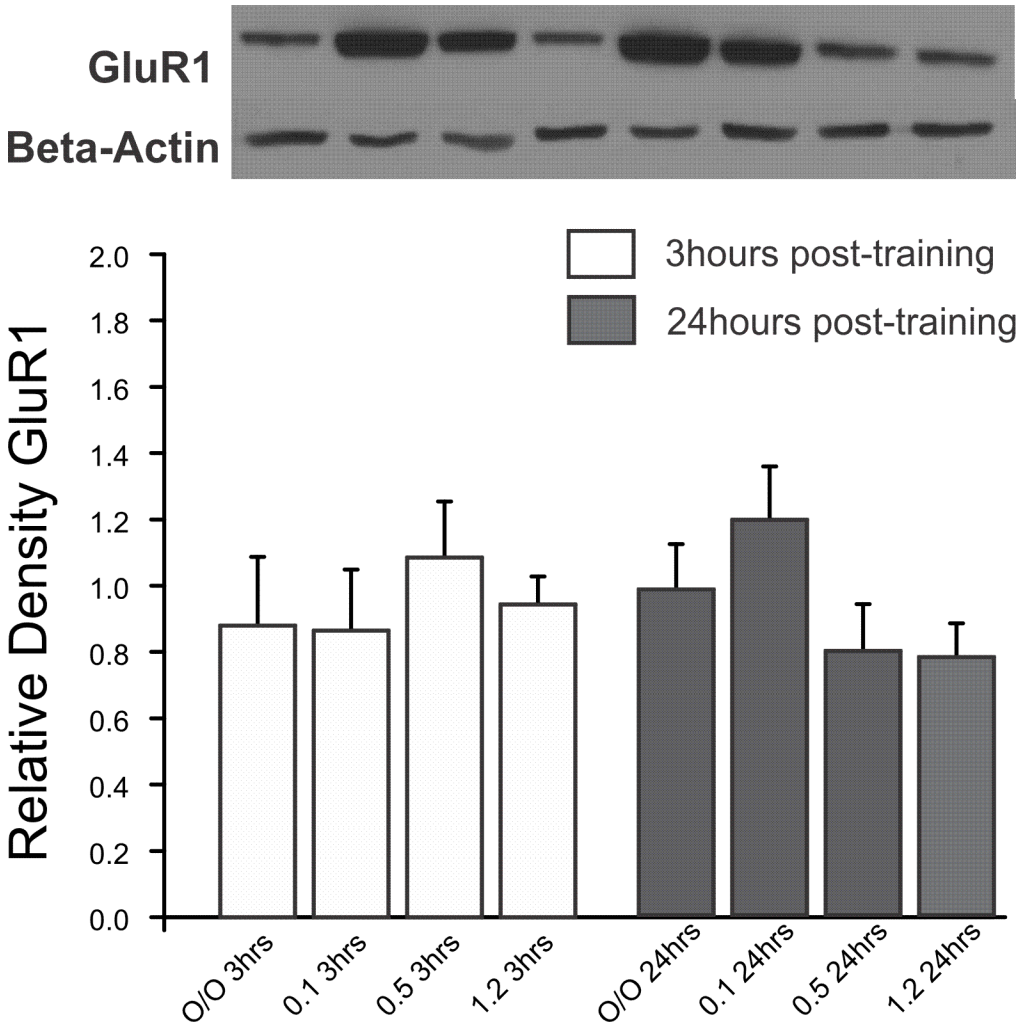

Figure 8: AMPAR subunit GluR1 in the anterior piriform cortex (aPC). No receptor subunit changes (*$p < 0.05$). O/O: odor only (control), 0.1: 0.1mA shock paired with peppermint odor, 0.5: 0.5mA shock paired with peppermint odor, & 1.2: 1.2mA shock paired with peppermint odor.

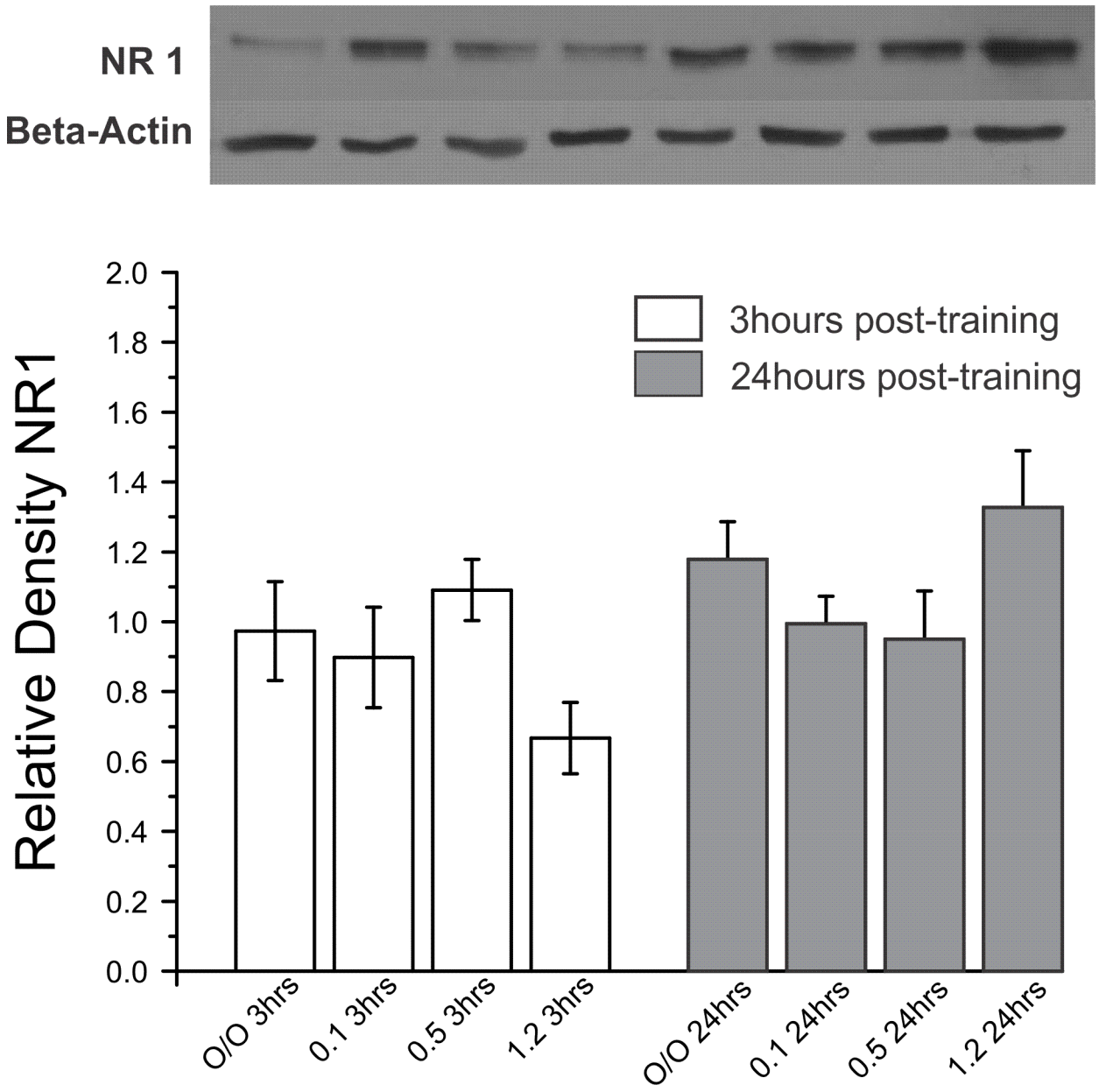

Figure 9: NMDAR subunit NR1 in the posterior piriform cortex (pPC). No receptor subunit changes (*$p < 0.05$). O/O: odor only (control), 0.1: 0.1mA shock paired with peppermint odor, 0.5: 0.5mA shock paired with peppermint odor, & 1.2: 1.2mA shock paired with peppermint odor.

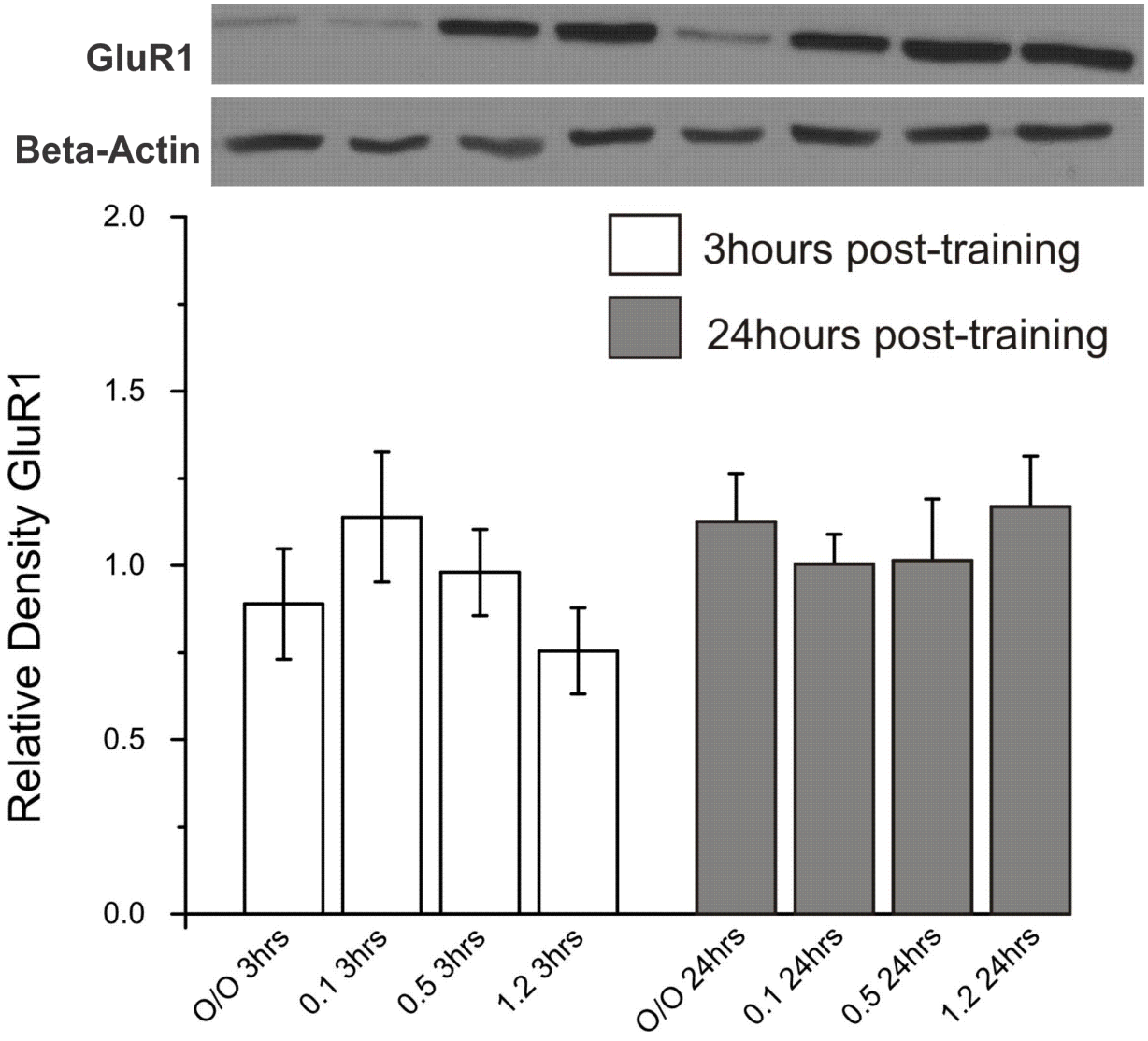

Figure 10: AMPAR subunit GluR1 in the posterior piriform cortex (pPC). No receptor subunit changes (*p<0.05). O/O: odor only (control), 0.1: 0.1mA shock paired with peppermint odor, 0.5: 0.5mA shock paired with peppermint odor, & 1.2: 1.2mA shock paired with peppermint odor.