THE ANTERIOR PIRIFORM CORTEX AS A LOCUS FOR EARLY ODOR PREFERENCE LEARNING IN RATS

by

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ABSTRACT

Early odor preference learning in rats has been localized to the physiological changes in the olfactory bulb. However, the role of the olfactory cortex (e.g. the piriform cortex) was unexplored. In this work, my experiments support a critical role of the piriform cortex in early odor preference learning. First, the anterior piriform cortex (aPCX) is a critical component of early odor preference learning circuitry. Transiently silencing cells using lidocaine or muscimol local infusions in the aPCX prevents odor learning and recall. Second, acquisition of early odor preference learning in the aPCX is dependent on N-methyl-D-aspartate (NMDA) receptor activation both *in vivo* and *in vitro*. NMDARs are highly implicated in synaptic plasticity and critical in many forms of associative learning. Pups are unable to learn following local infusion of D-amino-5-phosphonopentanoate (D-APV) prior to odor conditioning. *In vitro*, D-APV prevents LTP induction. Third, β-adrenoceptor activation in the aPCX is required and sufficient for early odor preference learning. Stroking elicits natural norepinephrine release from the locus coeruleus to the olfactory structures including PCX. Pups cannot learn following propranolol infusions before odor conditioning. If stroking is replaced by isoproterenol infusions in the aPCX during novel odor presentations, animals will learn to prefer that odor. Fourth, odor plus stroking (OS^+) training enhances phosphorylated cAMP response element-binding protein (pCREB), a molecule highly implicated in intracellular signalling pathways involved in learning, expression in the trained hemisphere. Finally,

learning induces NMDAR subunit expression changes. At 3 hr following OS^+ training, there is a significant NR1 down-regulation and at 24 hr following OS^+ training, NR1 displays a significant up-regulation from baseline expression levels.

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LIST OF ABBREVIATIONS

2-DG	2-Deoxy-D-glucose
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AMPAR	AMPA receptor
AON	Anterior olfactory neuron
aPCX	Anterior piriform cortex
$aPCX_D$	Anterior piriform cortex dorsal
$aPCX_V$	Anterior piriform cortex ventral
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
ChR2	Channelrhodopsin
cPCX	Central piriform cortex
CREB	Cyclic adenosine monophosphate response element-binding protein
DA	Dopamine
D-APV	D-amino-5-phosphonopentanoate
DNQX	6,7-Dinitroquinoxaline-2,3-dione
E	Epinephrine
EPSP	Excitatory post-synaptic potential
EPL	External plexiform layer
ET	External tufted
fEPSP	Field excitatory post-synaptic potential
GABA	Gamma-aminobutyric acid
GC	Granule cell
GCL	Granule cell layer
GL	Glomerular layer
HDB	Horizontal diagonal band of Broca
IHC	Immunohistochemistry
IPL	Internal plexiform layer
IPSP	Inhibitory postsynaptic potential
ISO	Isoproterenol
JG	Juxtaglomerular
LC	Locus coeruleus
LOT	Lateral olfactory tract
LTD	Long term depression
LTP	Long term potentiation
MC	Mitral cell
MCL	Mitral cell layer
NE	Norepinephrine

NMDA	N-methyl-d-aspartate
NMDAR	NMDA receptor
NPY	Neuropeptide Y
NTX	Naltrexone
OB	Olfactory bulb
OBFC	Orbitofrontal cortex
OD	Optic density
ON	Olfactory nerve
ONL	Olfactory nerve layer
OP	Olfactory peduncle
OR	Odor receptor
ORN	Olfactory receptor neuron
OEC	Olfactory ensheathing cell
OS^+	Odor + stroke training
OS^-	Odor only training
OSN	Olfactory sensory neuron
OT	Olfactory tubercle
PBS	Phosphate buffer solution
pCREB	Phosphorylated CREB
PCX	Piriform cortex
PKA	Protein kinase A
pPCX	Posterior piriform cortex
PD	Postnatal day
PG	Periglomerular
ROD	Relative optic density
ROI	Region of interest
SA	Short axon
SEM	Standard error of measurement
SOM	Somatostatin
TBS	Theta burst stimulation
TC	Tufted cell
VIP	Vasoactive intestinal peptide

CHAPTER 1 – INTRODUCTION

1.1 Overview

A fundamental theme in neuroscience is the pursuit for understanding the underpinnings of learning and memory. That is, elucidating what memories are and how they are formed, stored, and recalled. Learning and memory are essential for survival from the perinatal stage until the end of life. During the early days of life, the relationship between the mom and infant is the most critical for health and survival for all mammals. Hence, learning during the postnatal period is centered around the quest for physical proximity to the mother, or caregiver (Leon, 1992; Moriceau et al., 2010). This period of learning in which the pup seeks out maternal care is referred to as the sensitive period. In rats, this time lasts before postnatal (PD) 10-12 (Woo and Leon, 1987).

The early days of life for a rat pup transpire without sight and without sound (Gregory and Pfaff, 1971). Their eyes are closed and they are unable to hear thus, these altricial pups rely on olfactory (Pedersen and Blass, 1982) and somatosensory (Polan and Hofer, 1999) cues to find the nest. In doing so, pups find food and warmth provided by the dam, thus ensuring survival. This first postnatal week is critical for forming attachments while the immature central nervous system is rapidly developing. Pups readily learn to approach cues that ensure survival, such as the odors and touches

associated with maternal care. These stimuli may be positive (i.e., pleasant) (Sullivan and Hall, 1988) or negative (i.e., painful) (Camp and Rudy, 1988). This type of learning is a form of Pavlovian classical conditioning, also known as associative learning. In the real world, individuals learn to associate environmental cues with behaviorally significant outcomes. In the lab, researchers can train pups to associate a particular cue (i.e., an odor) with maternal care (i.e., tactile stimulation), thereby eliciting approach behavior. When given the choice, pups will approach the trained odor presumably in hopes of a reward (e.g., nourishment) in return. Researchers can gain insight into the cellular and molecular processes that underlie associative learning by taking advantage of this natural phenomenon.

Early experiments by Wilson and colleagues (Wilson et al., 1987) examined preference learning and changes in olfactory bulb (OB) neurons output firing patterns using a multi-day training paradigm, where pups underwent odor conditioning from PD 1-18. It was found that pups displayed a preference for the odor, and also that neuronal output patterns in the OB were modified following learning. Another study by Sullivan and Hall (1988) investigated 20-trial, hour-long training protocols, where they trained pups on PD6. Upon testing, which occurred 4 hours following training, pups were capable of developing a preference toward a naturally aversive odor following training. From these experiments, 10 min training protocols ensued. Sullivan and colleagues (Sullivan and Leon, 1987; Sullivan and Hall, 1988) were successful in showing associative learning after a single training session of pairing either milk infusions or tactile stimulation with a novel odor, cedar. Other researchers have employed this early

odor preference learning model and have consistently shown that memories are retained for at least 24 h following a single, 10 min training session (McLean et al., 1993; McLean et al., 1999; Yuan et al., 2003). This sensitive period, where pups show an augmented ability to learn approach behaviors and a reduced ability to learn avoidance behaviors, serves to ensure that the pup forms attachments with a specific caregiver (Moriceau et al., 2010). This approach behavior, however, does not extend past PD 12 (Woo and Leon, 1987), which is when pups are able to see and hear, and display more exploratory behavior. It has been suggested that at this age, the amygdala is starting to develop in response to corticosterone release during stress. Sullivan and colleagues (Sullivan et al., 2000a) used a shock conditioning paradigm to study associative learning in pups either before the sensitive period which paradoxically results in odor preference, or after the sensitive period which produces odor aversion. A major neurobiological difference in this model at the two periods is the involvement of the amygdala following the critical period, which has known involvement in fear conditioning and fear memories. In addition, labeled 2-deoxyglucose (2-DG) uptake is often used as a marker for neuronal activity, therefore Sullivan et al. (2000a) examined 2-DG uptake in the amygdala following odor plus shock learning in PD 8 and PD 12 pups. PD 12 pups displayed an avoidance behavior combined with an enhanced 2-DG uptake, while there was no difference in 2-DG uptake in pups who display an approach behavior following shock conditioning. These results show that both appetitive and avoidance behaviors involve distinct neural pathways that develop, modify, and are refined with age and experience.

Early odor preference learning has been extensively studied over the years, with the olfactory bulb (OB) as a primary structure of interest (Sullivan and Leon, 1987; Guthrie et al., 1993; Coopersmith and Leon, 1995; Johnson et al., 1995; McLean et al., 1999). Research has shown that odor learning, not mere odor presentation, leads to bulbar modifications, like enhanced 2-DG uptake (Sullivan and Leon, 1987; Coopersmith and Leon, 1995), increases in bulbar CREB phosphorylation (McLean et al., 1999), or changes in OB output firing patterns (Wilson et al., 1987). In addition, manipulations of adrenoceptors in the OB disrupt learning processes (Sullivan et al., 2000b). Thus, it is clear that the OB is highly implicated in early odor preference learning, which leads to the question of whether the OB is the sole locus for learning, or are there other critical areas involved in this learning circuitry?

Odor information reaches the piriform cortex (PCX) directly via axons of OB output neurons, thus it is one of the primary structures in odor processing (Neville and Haberly, 2004). Previous work suggests that the PCX may serve a role in early odor preference learning. Roth and Sullivan (Roth and Sullivan, 2005) showed increased c-fos expression in the OB and the anterior PCX (aPCX) following an odor shock training paradigm. However, the exact role of the PCX remains, in many ways, a *terra incognita*. That is, we are unsure to what extent the PCX is critical for early odor preference learning.

The present project sought to explore some of the open issues concerning cortical involvement during early odor preference learning. Particularly, 1) What is the role of the

piriform cortex in early odor preference learning? Is it necessary and sufficient to produce learning independent of the olfactory bulb? 2) What are the synaptic mechanisms occurring in the piriform cortex that may support learning? Are both pre- and post-synaptic mechanisms involved? Is learning NMDA receptor-dependent as in other associative forms of learning?

In the following sessions, I provide relevant background information on 1) the olfactory system (with a focus on the anatomy and neuromodulations in the PCX); 2) an early odor preference learning model; 3) synaptic plasticity and LTP mechanisms in the PCX that may contribute to early odor preference learning. Finally the objectives of the project are summarized.

1.2 Olfactory system overview

The olfactory system is an attractive system for studying the underpinnings of some of the most fundamental neural processes. It is unusual compared to other modality systems because of its unique processing pathways. Unlike visual and auditory systems, olfactory information can reach cortical structures via direct synaptic relay from receptors, without thalamic relays. That is, chemical signals from the nostrils are first processed in the olfactory bulb, where they evoke a spatio-temporal pattern of activity that is further projected to the olfactory cortex, from which thalamic relay ensues. The

convergence of odor information from the OB to the PCX is necessary for odor perception (Wilson and Sullivan, 2011).

1.2.1 Olfactory bulb

The olfactory bulb (OB) is the first stop in odor processing after chemical compounds reach olfactory receptors. The sensory organ of the olfactory system is the olfactory epithelium, where volatile compounds made up of odorants bind to the first order neurons, olfactory receptor neurons (ORNs). There are over a thousand types of distinct receptor cells (Buck and Axel, 1991), all of which respond to specific odor molecules with varying affinities (Krautwurst et al., 1998). Olfactory information is transduced by ORN axons, making up the olfactory nerve (ON), which synaptically terminate in the glomeruli of the OB. Similar receptor cells converge onto the same glomerulus, creating an odographic map for each odor (Mombaerts et al., 1996).

From the glomeruli, olfactory information is further relayed to the distal primary dendrites of mitral cells (MCs) and tufted cells (TCs), the output neurons of the OB. MC and TC axons make up the myelinated lateral olfactory tract (LOT), which will carry odor information to other parts of the brain for higher order processing. MCs and TCs are distinguished by soma locations within the OB and by differences in dendritic morphologies. The activities of MCs are regulated by local inhibitory interneurons such as periglomerular cells (PGCs) surrounding the glomerular layer, and deeper granule cells

(GCs) in the granule cell layer (GCL). These interneurons provide both feed-forward and feed-back inhibitions onto MCs, thus refine MC firing and outputs to the cortex. The OB is not merely a relay station for olfactory information, but is responsible for decoding the synaptic firing patterns coinciding with distinct odorants (Davison and Ehlers, 2011).

1.2.1.1 Projection pathway from the olfactory bulb to the cortex

By way of the LOT, MCs and TCs provide the forward projection pathways from the OB that reach a group of structures collectively referred to as olfactory cortex on the surface of the basal forebrain. These include, PCX, olfactory tubercle, agranular insula, lateral entorhinal cortex, regions of the amygdala, and the olfactory peduncle (AON, tenia tecta, and dorsal peduncular cortex) (Neville and Haberly, 2004). The LOT displays some topographical organization in the more anterior regions, and the axons than run along the lateral aspects of the LOT are notably smaller than the axons in the medial portion of the tract. It is these smaller axons that are believed to parent the axon collaterals that branch and spread tangentially across the superficial PCX (Price and Sprich, 1975). In terms of indirect OB projections that reach the PCX, the aPCX receives OB projections through the AON. Also, the $aPCX_D$ receives projections from the $aPCX_V$ (Neville and Haberly, 2004). Furthermore, the same pyramidal cell can respond to very different odors, which suggests that the receptive fields for these pyramidal cells are discontinuous (Stettler and Axel, 2009).

1.2.1.2 Backward olfactory projections from the cortex

The PCX, lateral entorhinal cortex and some amygdaloid cells project back to the OB, with the more anterior regions projecting more strongly than the posterior regions. The majority of such projections ascend from the deeper portion of Layer II (Layer IIb) and Layer III pyramidal cells (Shipley and Adamek, 1984). Back-projections from the aPCX to the OB occur both directly and indirectly (via the AON). There are weak projections from the pPCX to the OB and aPCX. These connections terminate on GABAergic GCs in the GCL, whose activation inhibits MC firing (Nicoll, 1971). Of note, the back-projections to the OB from the PCX are much stronger than the forward projections (Neville and Haberly, 2004).

1.2.2 Olfactory cortex

The olfactory cortex is a paleocortex, with the lateral entorhinal cortex as an exception, which receives direct synaptic input from the OB (Cleland and Linster, 2002; Neville and Haberly, 2004; Wilson and Sullivan, 2011). This phylogenetically ancient cortex is described in terms of three laminae that share many commonalities (e.g., predominantly pyramidal cells, expression of molecular markers, physiology, etc.), as

well as having distinct inputs and properties. Such structural simplicity can serve as a basis for model systems that can further our understanding of cortical functions.

The olfactory cortex is divided into anatomically distinct areas. The largest subregion is the piriform cortex (PCX), which is named due to the characteristic pear-like shape in many mammalian species (Loscher and Ebert, 1996). The PCX displays heterogeneity in terms of axonal connections and cytoarchitecture from the rostral to caudal portions, thus is further divided into an anterior portion, anterior piriform cortex (aPCX), and a posterior portion, posterior piriform cortex (pPCX). The laminar structure is maintained throughout the entire PCX, however the existing differences are thought to underlie differences in functions. Haberly and Price (1978) describe the boundaries of the PCX rostrally by the AON, caudally by the lateral entorhinal cortex, medially by the olfactory tubercle (OT) and amygdala cortical areas, and laterally by the neocortex.

Other cortical regions that receive monosynaptic inputs from the OB include the olfactory tubercle (OT), lateral entorhinal cortex, agranular insula, cortical areas associated with the amygdala, and olfactory peduncle (OP). The OP consists of smaller cortical areas, the anterior olfactory nucleus (AON), tenia tecta, and the dorsal peduncular cortex (Neville and Haberly, 2004). Of note, those cortical areas in the basal forebrain that receive direct innervation from the OB also receive associational input from the PCX. Such OB and PCX projections have complimentary input patterns where the bulbar is just superficial to the cortical input, with little to no overlap (Price, 1973).

1.2.2.1 Piriform cortex

1.2.2.1.1 Neuroanatomy

The PCX, like the hippocampus, is a three-layered structure. The superficial plexiform Layer I contains very few somata, thus mainly apical dendrites, with some neural fibers and interneurons. It is subdivided into a superficial portion termed Layer Ia and a deeper portion, Layer Ib. LOT fibers originating in the OB project directly onto the dendrites in Layer Ia. Layer Ib contains associational, for receiving intrinsic associational inputs, and commissural fibers (Price, 1973; Suzuki and Bekkers, 2011; Wilson and Sullivan, 2011). There are also sparse populations of GABAergic interneurons found in Layer I.

Layer II consists mainly of compact pyramidal cell bodies. The superficial portion, Layer IIa, encompasses mostly semilunar cells, while the deeper portion, Layer IIb, contains mostly superficial pyramidal cell bodies. These divisions are described in terms of low cell comprising density and high cell comprising density, respectively. Axons of pyramidal cells in Layer II extend into Layer I, while basal dendrites extend into Layer III (Haberly and Price, 1978; Suzuki and Bekkers, 2011; Wilson and Sullivan, 2011).

The cellular distribution in Layer III is graded from superficial to deep. Deep pyramidal cells are highly concentrated in the superficial portion, where there are less large multipolar cells. As depth increases, there are less pyramidal cells and more non-pyramidal cells. There are many associational axons synapsing on pyramidal cells in Layer III (Haberly, 1983; Wilson and Sullivan, 2011). Furthermore, there are several classes of interneurons found in Layer III.

Another layer, layer IV or the endopiriform nucleus, is not typically considered as a component of the PCX, however they are interconnected. The endopiriform nucleus, is comprised of mostly spiny multipolar neurons. The dorsal portion of the endopiriform nucleus contains a dense cluster of neurons, referred to as the dorsal endopiriform nucleus, while the ventral endopiriform nucleus contains more scattered cells (Krettek and Price, 1977). Low levels of inhibition in the endopiriform nucleus, due to few GABAergic cells, make this region highly implicated in seizure initiation and prolongation (Behan and Haberly, 1999).

As previously mentioned, the aPCX receives direct OB innervation via the LOT and is highly organized into three distinct layers, all of which have unique cell type characteristics. The aPCX can be further divided into a dorsal ($aPCX_D$) and ventral ($aPCX_V$) region. They are distinct in that the $aPCX_V$ is deep to the LOT and has a thick Layer Ia and thin Layer III, while the $aPCX_D$ is dorsal to the LOT with a cytoarchitecture different from the $aPCX_V$ (Neville and Haberly, 2004). For example, Illig (2005) demonstrates that the OBFC projects more heavily to the $aPCX_V$, especially Layer III,

and sparsely to the $aPCX_D$. This distinction is less evident in more caudal PCX regions and may indicate functional differences among the different aPCX subregions.

The pPCX is posterior to the LOT and has a well-developed Layer III. Unlike the aPCX which receives more afferent inputs and fewer associational inputs, the pPCX receives more associational inputs and fewer afferent inputs (Suzuki and Bekkers, 2011). In terms of connectivity, the pPCX is more densely connected to the amygdala (Luskin and Price, 1983). In addition, the pPCX has direct or indirect hippocampal connections by way of the septum or entorhinal cortex (Luskin and Price, 1983). Finally, unlike the aPCX, the pPCX has projections to the perirhinal cortex (Kelly and McIntyre, 1996).

There are lamination differences between the anterior portion of the PCX and the posterior portion. The one exception is Layer IIb, which is consistent from the aPCX to the pPCX. Layer III is much thicker in the pPCX as it has a higher cell packing density compared to Layer III of the aPCX (Haberly and Price, 1978). Such anatomical differences between the aPCX and may lead to differences in seizure formation between these regions (Schwabe et al., 2004).

Throughout the PCX, Layer IIa is more highly developed laterally and thinner medially. This mediolateral distinction is more pronounced in the pPCX, where there is no evidence for a medial portion of Layer IIa (Haberly and Price, 1978).

1.2.2.1.2 Cells in the piriform cortex

The PCX contains a wide variety of cells throughout all layers. There are two main divisions, glutamatergic principal neurons and GABAergic interneurons, as well as some uncategorized cell types. Below I will focus on the two main types of neurons.

1.2.2.1.2.1 Glutamatergic principal neurons

1.2.2.1.2.1.1 Pyramidal cells

The principal neurons in the PCX are pyramidal neurons that have several distinct features. A single apical dendritic tree is targeted toward the superficial surface, while a long basal tree radiates from the cell body, all of which contain a high concentration of dendritic spines. These pyramidal cells also display a deeply directed axon (Haberly, 1983). Pyramidal cells receive afferent sensory input from MCs, however they exceed, in quantity, the number of MCs by at least one order of magnitude (Neville and Haberly, 2004). Thus, each MC provides input for many pyramidal cells. This differs from the OB, where there are more receptor cells providing input for fewer principal cells.

There are two types of pyramidal cells in the PCX that are differentiated based on location and physiology. Superficial pyramidal cell somata are concentrated in Layer IIb, while deep pyramidal cell bodies are in Layer III, at higher densities in the superficial portion and gradually decreasing with depth (Haberly, 1983). These two types of

pyramidal neurons differ in morphology only by slight variations in the apical dendritic trunk. Deep pyramidal cells have longer apical dendrites compared to superficial pyramidal cells, which have short, or may altogether lack the, apical dendrites (Haberly, 1983). Between Layer I and II, apical dendrites of both superficial and deep pyramidal cells branch and extend into the afferent fiber termination zone in Layer Ia. Basal dendrites of both types of pyramidal cells are long and directed deep into the tissue. Physiologically, they differ in membrane properties and synaptic responses.

Myelinated axons of pyramidal cells give rise to many unmyelinated collaterals within a few hundred microns of their origin. This creates a high density of local synapses in Layer III, which occur on dendritic shafts of non-pyramidal neurons (Johnson et al., 2000).

1.2.2.1.2.1.2 Semilunar cells

Semilunar cells are found predominately around the superficial region of Layer IIa. These neurons resemble older versions of pyramidal cells in that they contain apical dendrites, but no basal, deep-directed dendrites. For this reason, semilunar cells are thought to be involved in receiving afferent inputs occurring in Layer Ia. In addition, they contain a much lower concentration of dendritic spines compared to pyramidal cells. Similarly to pyramidal cells in the PCX they do have deep-directed axons and they also produce associational projections. However, unlike pyramidal cells, the associational

connections of the semilunar cells do not project back to the OB, nor to the opposite hemisphere (Haberly and Price, 1978; Haberly, 1983; Suzuki and Bekkers, 2011; Wilson and Sullivan, 2011).

1.2.2.1.2.2 GABAergic interneurons in the PCX

There are several main classes of GABAergic cells in the PCX, horizontal cells, bipolar cells, fast-spiking multipolar cells, regular-spiking multipolar cells, neurogliaform cells, and complex appendage cells. Interneurons are key regulators for incoming and outgoing cellular activities of the principal cells in the PCX. The actions of interneurons in the PCX drive many necessary phenomena, such as electrical oscillations and the phasic inhibition that occurs during olfaction (Neville and Haberly, 2004). Odor-evoked electrical oscillations may serve to augment synchronous synaptic activity of coactive neurons (Wilson and Sullivan, 2011).

Inhibitory GABAergic neurons exist in two forms in the aPCX, feed-forward and feed-back interneurons (Stokes and Isaacson, 2010; Suzuki and Bekkers, 2010). Briefly, feed-forward inhibition occurs in the following sequence: LOT, Layer Ia interneuron, principal cell. The most likely interneurons in this form of inhibition are neurogliaform cells or horizontal cells. Feed-back inhibition occurs as follows: LOT, principal cell, Layer Ib, II, or III interneuron, principal neuron. Bipolar cells, fast- and regular-spiking

multipolar neurons are likely the interneurons involved in feed-back inhibition (Suzuki and Bekkers, 2010).

Some researchers prefer to categorize interneurons in terms of axonal projection patterns. This results in two main varieties, perisoma- and dendrite-targeting. There is a group of GABAergic cells in Layers II and III in the PCX which synapse on pyramidal cell soma in Layer II (Suzuki and Bekkers, 2007).

Functionally, inhibitory neurons modulate and refine the firing patterns of the excitatory pyramidal neurons. Poo and Isaacson (2009) used patch clamping techniques *in vivo* to demonstrate the distributed odor response patterns of inhibitory and excitatory signals on pyramidal cells in the PCX. They found that inhibitory signalling is much more global, while excitation is scarcer. This is due to the fact that, at the neuronal level, excitation is specific for particular odors, while inhibition is indiscriminate. Odor-evoked activity in the PCX is refined and fine-tuned by electrical oscillations caused by inhibitory processes.

1.2.2.1.3 Piriform cortex projection pathways

1.2.2.1.3.1 Input into the PCX

The major input source of the PCX is provided by the MCs in the OB. TCs heavily project to the olfactory peduncle and the olfactory tubercle and a small portion of the $aPCX_V$. As previously mentioned, the OB displays an orderly, stereotypic map in response to specific odorants, which is not the case in the PCX, where diffuse mapping of odors occurs. This nontopographic representation in the PCX allows for the greatest chances of converging afferent input from glomeruli that are spatially dispersed within the OB (Illig and Haberly, 2003; Litaudon et al., 2003). In addition, MCs and TCs respond differently to odors, and project to different areas in the PCX (Nagayama et al., 2010; Igarashi et al., 2012).

Afferent and associational inputs target both superficial and deep pyramidal cells, but synapse at different areas on the pyramidal cell apical dendrites. Afferent axons arriving in the LOT terminate exclusively on synapses on distal segments of apical dendrites, or in Layer Ia, while intrinsic associational fiber systems from the PCX terminate in Layer IIb and III. Associational, or corticocortical, projections to the PCX from other cortical areas extend across the Layer Ia-Ib border or terminate in Layer II, thereby exciting the same pyramidal cells through synapses on proximal and middle dendritic segments (Haberly and Bower, 1984). The same afferent and associational fibers also synapse on GABAergic interneurons in Layer I and III (Neville and Haberly, 2004).

Unlike the precision of depth processing in the PCX, the horizontal inputs have no topographical organization. With respect to afferent inputs, the axons of one MC will

synapse on multiple pyramidal cells throughout the PCX, resulting in inputs onto rather large, sometimes overlapping patches of pyramidal cells (Neville and Haberly, 2004).

Functional specialization has been demonstrated in the PCX between subregions during odor coding. The aPCX and pPCX differ in terms of afferent and associational inputs, physiology, and cellular patterns, and the type of processing that occurs in each region reflects these differences (Haberly, 2001). For example, during odor discrimination tasks, after repeated exposure to the same odors, neurons in the aPCX are better able to separate odorant identity and discriminate mixtures from components (Wilson, 2000). More anterior PCX regions are involved in processing the structural identity of odorants. That is, more familiar odors are more easily distinguished as the aPCX helps to refine the encoding patterns for familiar odors. Posterior regions, on the other hand, mediate perceptual odorant object quality (e.g., spicy), which is reminiscent of higher-order processing structures (Kadohisa and Wilson, 2006a; Wilson and Sullivan, 2011). In spite of these differences, neither aPCX nor the pPCX neurons display differences in response magnitudes to odorants. However, both regions display an experience-dependent modification following odor conditioning (Kadohisa and Wilson, 2006a).

The primary source of descending input to the PCX, specifically the aPCX, is from the orbitofrontal cortex (OBFC) (Illig, 2005; Cohen et al., 2008). This is a monosynaptic connection which synapses on Layer II pyramidal cells on their apical dendrites in Layer Ib of the aPCX. This is especially evident in odor discrimination tasks,

where learning odors is associated with a reward. It is suggested that the OBFC is activated in response to the odor valence and not simply the identity of the odor. This ensures that PCX neurons fire in an appropriate behavioral context (Schoenbaum and Eichenbaum, 1995). It is also suggested that OBFC neurons may initiate PCX neuronal firing in the absence of olfactory input during memory recall (Cohen et al., 2008).

1.2.2.1.3.2 Efferent projections from the PCX

Major efferent projections occur via pyramidal cells to other cortical areas. Each cortical target is reflective of the complex function in which it is involved. Projections from the PCX reach areas implicated in initiating appropriate behaviors, evaluating emotional importance, or memory-related structures. Such cortical regions are the prefrontal cortex, amygdala, and the entorhinal cortex, respectively (Luskin and Price, 1983). More specifically, prefrontal cortical regions involved in mediating complex processing and behaviors, such as the OBFC and insular cortex, are other PCX efferent targets. Finally, the PCX also projects to the hypothalamus and indirectly to other autonomic and endocrine structures.

Thalamic input from olfactory areas occurs via a small number of cells that are located along the deep layers in the PCX, OT and AON (Price, 1985). The PCX projects to the mediodorsal and submedial nuclei of the thalamus, which project to the same prefrontal areas to which the PCX directly projects.

In general, rostrally directed projections of the aPCX are primarily directed toward the OB, the ventral, lateral, and dorsal AON, the aPCX itself, and the anterolateral OT. Caudally directed projections reach multiple cortical regions until the caudal entorhinal area (Schwob and Price, 1984).

1.2.2.1.3.3 Associational and parallel projections of the piriform cortex

Associational, or corticocortical, connections are ipsilateral projections of the PCX. They are mostly bidirectional, with the primary exception of the OT (ventral striatum), which only receives input from the PCX and does not project back (Neville and Haberly, 2004). Each pyramidal cell is thought to receive at least 2000 inputs from other pyramidal cells, which is much higher than the 200 afferent inputs (Davison and Ehlers, 2011). Shipley and Ennis (1996) categorize these corticocortical projections into four classes: *intrinsic*, *associational*, *extrinsic*, and *modulatory inputs*. The short intrinsic projections, also known as local connections, connect cells throughout the PCX laminae. Associational projections connect different subregions of the PCX (e.g., aPCX to pPCX), while extrinsic projections connect PCX cells with structures outside of the olfactory cortex. Finally, modulatory inputs refer to afferents terminating in the PCX from other neural systems.

1.2.2.1.3.4 Commissural connections

In the OB, OSNs project olfactory information solely to the ipsilateral OB. In contrast, olfactory cortical neurons are capable of bilateral innervation shortly after PD 10, which is when the transverse fiber tract, the anterior commissure, is fully matured. Commissural connections occur between olfactory cortical areas between contralateral hemispheres via the anterior commissure. Such connections are typically weaker, as they involve fewer areas than associational projections, however they are sufficient to activate PCX neurons from contralateral OB stimuli (Wilson, 1997). Commissural projections are initiated from pyramidal cells in Layer II of the aPCX and innervate caudal regions of the contralateral PCX as well as lateral entorhinal cortex and nuclei of the amygdala (Haberly and Price, 1978). Commissural projections of the pPCX originate in Layer III neurons and are much weaker compared to those originating in the aPCX. These projections terminate in more rostral PCX regions (Schwob and Price, 1984).

1.2.2.4 Noradrenergic neuromodulation

Both the OB and PCX receive neuromodulatory inputs from other brain regions. The PCX receives neuromodulatory innervation from the brainstem, basal forebrain, and hypothalamus (Neville and Haberly, 2004). Neuromodulators are known to play key roles in learning-related events (arousal, attention, learning, etc.). I will focus on the noradrenergic modulations in the PCX which is a topic of my thesis.

Adrenoceptors are G protein-coupled receptors that are activated by norepinephrine (NE) or epinephrine (E). The two main receptor types, α and β, have multiple subtypes, α_1, α_2, β_1, β_2, and β_3, each of which activates a specific G protein type, determining their action (i.e., excitatory or inhibitory) (Ahlquist, 1948). Thus, the postsynaptic effects of NE release are long-lasting. Both α (Young and Kuhar, 1980; Pieribone et al., 1994; Domyancic and Morilak, 1997) and β (Wanaka et al., 1989) subtypes are expressed in the PCX. Specifically, the majority of α-adrenoceptors are expressed in Layer II, with less expression in Layers I and III (Unnerstall et al., 1984).

The sole source of NE for the entire brain comes from the locus coeruleus in the dorsolateral tegmentum of the pons (Morrison et al., 1978). It is estimated that approximately 40% of noradrenergic neurons in the LC project to the OB (Shipley et al., 1985) and of all the cortices, the PCX has the highest NE concentration (Reader, 1981). LC projection to the PCX is primarily ipsilateral, where NE fibers are more prominent in Layers Ia and III compared to layer II (Mason and Corcoran, 1979; Fallon and Loughlin, 1982). NE can suppress associative synaptic transmission which may lead to an enhanced response from afferent input. An enhanced signal-to-noise ratio has been shown to enhance encoding of sensory input (Servan-Schreiber et al., 1990). In addition, *in vitro* electrophysiology studies have shown that bath-applied NE contributes to GABA-mediated IPSPs in Layer II pyramidal cells through α_1 receptors (Gellman and Aghajanian, 1993). With respect to PCX subregions, both the aPCX and the pPCX have similar LC fiber distribution (Datiche and Cattarelli, 1996) and similar proportions of

cells (approximately 60%) in both regions are affected in response to LC stimulation (Bouret and Sara, 2002).

Electrophysiological recordings examined the effects of NE application on LOT-PCX synaptic transmission. It was demonstrated that NE can both facilitate and depress excitatory transmission at LOT-Layer Ia PCX in a dose-dependent manner. Low concentrations were shown to facilitate excitatory transmission, where high concentrations depressed excitatory transmission. Such actions were inhibited by α- or β-adrenoceptor antagonists, as well as by the NMDAR antagonist, D-APV. It is suggested that at high concentrations, NE elicits the release of inhibitory GABA, therefore depressing transmission (Collins et al., 1984). *In vivo* electrophysiological experiments investigated neural responses in the aPCX and pPCX in response to LC stimulation paired with odor presentations. Both PCX regions displayed enhanced pyramidal cell responses, however the pPCX neurons showed lower activation thresholds and greater excitation than suppression compared to aPCX neurons (Bouret and Sara, 2002). The stronger suppression found in the aPCX is thought to mirror an improved temporal pattern and/or response sharpening. Such differences may be accounted for by the expression of α_1 receptors, which modulate GABAergic cells (Marek and Aghajanian, 1996) in the aPCX, which are not found in the pPCX (Pieribone et al., 1994).

1.2.3 Olfactory system as an ideal research model

The olfactory system is ideal for studying learning-related changes in the brain. As mentioned previously, olfactory processing is anatomically shallow, as it comprises a network of cortical and subcortical processing that occurs before thalamic relay. This provides a convenient site to study cellular and molecular processes underlying fundamental neuronal processes. The relatively simple trilaminar structure of the PCX and its convenient location on the ventral forebrain make it an attractive site for experimental manipulations. Also, the local circuitry and principle cell types found in the PCX resemble those of the more complex neocortex. Therefore, it may serve as a simple model on which one can build and further understand functional circuits in the brains. In terms of intra-species comparisons, many characteristics, such as physiological responses of the PCX in response to LOT stimulation, are maintained between species (Biedenbach and Stevens, 1969; Haberly, 1973; Neville and Haberly, 2004). This is convenient because findings from one species can serve as a basis for experimental designs in other species.

The distinct direct afferent and associational inputs into the aPCX allow one to detect pathway-specific changes. For example, one can isolate the afferent or associational inputs onto a single pyramidal cell by stimulating different portions of the pyramidal cell apical dendrites (Layer Ia vs. Ib) and pharmacologically blocking the associational input (Franks and Isaacson, 2005). This provides a convenient way to study experience-dependent synaptic changes associated with behavioral outcomes. In addition, both synapses display different plasticity mechanisms. That is, after the sensitive period only the associational fibers exhibit plasticity, as the afferent synapses show NMDAR

down-regulation following this period, thereby reducing synaptic plasticity (Franks and Isaacson, 2005).

Finally, relevant to the current project, olfactory information is processed unilaterally for the first 10-12 days of life in rats (Kucharski and Hall, 1987). Thus, one can confine learning to one hemisphere and compare the changes between the learning and non-learning regions. In this situation, one can achieve the ideal intra-animal control.

1.3 Early odor preference learning model

Olfaction is critical for pup survival and pups readily learn to make associations between novel odors and environmental stimuli. Researchers can take advantage of this potent reinforcement system by examining how manipulations during such tasks affect behavior. Pups simultaneously presented with a novel, naturally aversive cedar odor and milk infusions will demonstrate learning by displaying conditioned ingestive behavior, such as mouthing and increased locomotor activity, in response to the cedar odor (Johanson and Hall, 1982). To expand on this idea, pups receiving tactile stimulation with a paintbrush that mimics the grooming activity of the dam in the presence of a novel odor will show a preference for that odor in a two-choice testing paradigm (Sullivan and Leon, 1987). However, as pups rely on maternal care for survival, they have also evolved to learn an age-dependent approach behavior toward painful stimuli. Odors paired with a mild foot shock will elicit a preference in PD 6 pups, however PD 12 pups will display

avoidance behaviors in response to the odor and shock paradigm (Camp and Rudy, 1988). Interactions with the dam are sometimes painful, as pups are commonly stepped upon and bitten. These stimuli still represent proximity to the nest, therefore it is critical that pups learn to associate aversive as well as pleasant stimuli with the dam.

1.3.1 The role of the olfactory bulb

As mentioned in the Overview (1.1), early odor preference learning has been extensively studied and the olfactory bulb (OB) has been identified as a primary substrate for learning (Sullivan et al., 2000b; Yuan et al., 2003; McLean and Harley, 2004). Direct infusion of drugs that block neural plasticity into the OB prevents odor preference learning, without altering spontaneous odor responses (Sullivan et al., 1989; Lethbridge et al., 2012). Furthermore, odor preference learning itself can be induced by local OB infusion of drugs that promote plasticity when paired with odor (Sullivan et al., 2000b; Christie-Fougere et al., 2009; McLean et al., 2009; Grimes et al., 2012; Lethbridge et al., 2012). These effects suggest that the OB was both necessary and sufficient for odor preference learning (Sullivan et al., 2000b).

Early investigators reported local changes in bulbar metabolism and electrophysiology following odor preference training and correlating with odor preference memory (Wilson et al., 1987; Sullivan et al., 1989; Guthrie et al., 1993; Coopersmith and Leon, 1995; Johnson et al., 1995). More recent work from McLean and colleagues (McLean et al., 1999; Yuan et al., 2003; Cui et al., 2011; Lethbridge et al.,

2012) has revealed detailed cellular and synaptic mechanisms of early odor preference learning as reviewed in subsequent sections. The intracellular mechanisms with a focus on the CREB signalling are reviewed in the next section 1.3.1.1, whereas the synaptic mechanisms are covered in the sections within the content of synaptic plasticity (section 1.4).

1.3.1.1 Intracellular signalling and CREB phosphorylation

Second messenger pathways are critical for long-lasting cellular changes. Studying changes in these pathways that result from learning can further our understanding of the temporal characteristics of pathways involved in learning. Briefly, cAMP-dependent pathways occur as follows. Activation in adenylate cyclase inside the plasma membrane will synthesize cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). Once activated, cAMP response element binding protein (CREB), a transcription factor, can increase or decrease downstream gene transcription. It has been shown that cAMP-dependent pathways also lead to modifications in cellular activity, which occur through protein kinases (Montminy and Bilezikjian, 1987). More specifically, increasing cAMP levels recruits protein kinase A (PKA). PKA can phosphorylate numerous cellular proteins including NMDA and AMPA receptors. Once PKA reaches the nucleus, CREB becomes phosphorylated (pCREB), specifically at the Ser-133 site. As already noted this will lead to the transcription of early and late response genes, thought to be involved in long-term plasticity (Frank and Greenberg, 1994).

There has been much evidence suggesting a role for CREB in learning and memory processes. Memory can be formed or prevented by altering levels of CREB in specific brain regions (Silva et al., 1998). Tactile stimulation can elicit NE release from the LC (Rangel and Leon, 1995) and when this occurs at β-adrenoceptors it leads to increases in cAMP. It has been suggested in the olfactory bulb, during odor exposure, glutamate is released from the olfactory nerve onto the mitral cells, which binds to NMDARS or voltage-gated Ca^{2+} channels, thereby allowing Ca^{2+} influx into the postsynaptic cell. Ca^{2+} acts as a second messenger by activating Ca^{2+}-dependent kinases. Coincident activations of cAMP and Ca^{2+} pathways increase pCREB (McLean et al. 1999; Yuan et al 2003) and initiate intracellular transcription involved in odor memory (McLean et al., 1999). Membrane receptor phosphorylation and insertion, new gene expression and protein synthesis, as results of these intracellular cascades, are necessary for the expression of synaptic plasticity (section 1.4).

1.3.2 The role the PCX

Following odor conditioning training, pups display region-specific neurobiological modifications. Fos protein immunohistochemistry revealed that odor-mild shock conditioning elicited a significant increase in neural activity in the OB and aPCX, not the pPCX (Roth and Sullivan, 2005). Another study expanded on this by measuring 2-DG uptake in the aPCX following odor-shock conditioning and found an

enhanced uptake in the aPCX only for young pups (i.e., PD 7-8) that underwent a mild shock (0.5 mA) (Raineki et al., 2009). Conversely, the pPCX displayed enhanced 2-DG uptake in young pups at strong shock levels (1.2 mA), and PD 12-13, as well as PD 23-24, animals for both mild and strong shocks. Thus, it appears that there is an enhanced 2-DG uptake in the aPCX in situations that evoke odor preference, whereas the pPCX is more active during odor avoidance learning.

Olfactory conditioning modifies synaptic transmission in the PCX in adult rats. Odor rule learning can enhance transmission at both the LOT-PCX and intrinsic associational fiber-PCX synapses in adult rats (Roman et al., 1987; Saar et al., 2002). In the same paradigm, learning also increases pyramidal cell inhibition (Brosh and Barkai, 2009).

Neuronal changes in response to odor conditioning show subregional differences in the PCX. In the aPCX, neuron coding becomes more refined with experience. Neurons display less overlap in response to odorants, thus enhancing discrimination abilities. In contrast, neurons in the pPCX are more broadly tuned following odor conditioning as individual components in a mixture are encoded more similarly. These characteristics may aid in recognizing common components between odorant mixtures (Kadohisa and Wilson, 2006b).

Learning may also lead to changes at the dendritic level. Experiments assessing learning-induced modifications on pyramidal cell dendritic spine structure in the PCX found an increase in spine density along associational dendritic synapses of olfactory

conditioned rats. However, the lengths of the dendrites did not change. Such morphological modifications in spine density produce more excitatory synapses that interconnect pyramidal cells in PCX following learning (Knafo et al., 2001).

1.3.3 Noradrenergic involvement in odor learning

Within the olfactory circuitry, the noradrenergic system is implicated in many types of learning, including associative learning. NE activation of β-adrenoceptors leads to a cascade of intracellular signalling that can eventually lead to *de novo* protein synthesis. β-adrenoceptor activation leads to increased levels of cAMP, which causes PKA translocation to the nucleus. From there, PKA phosphorylates CREB, which can lead to immediate-early gene transcription. The resulting transcription factors can start transcription of late genes, which are thought to be critical for long-term memory formation (Frank and Greenberg, 1994).

With respect to our early odor preference learning model, stroking the pups during training elicits NE release from the LC (Nakamura et al., 1987; Rangel and Leon, 1995). In the presence of a novel odor, which causes glutamate release from olfactory nerve terminals, pairing tactile stimulation with a novel odor can create a 24 h memory (Sullivan and Leon, 1987; McLean et al., 1999). Previous work has shown that NE and β-adrenoceptor activation are critical for early odor preference learning. For example, pups that underwent bilateral LC lesions prior to odor conditioning were unable to acquire an

odor preference for the novel odor upon testing (Sullivan et al., 1994). Also, similar results were found when pups received intrabulbar infusions of propranolol (Sullivan et al., 1989). Thus, the effects of blocking NE release or β-adrenoceptor activation in the OB demonstrates that NE acting through β-adrenoceptors is critically involved in early odor preference learning. On the other hand, pairing LC electrical stimulation or bilateral bulbar infusions of β-adrenoceptor agonists during odor presentation induces odor preference (Sullivan et al., 2000b; Lethbridge et al., 2012). Taken together, these results suggest that olfactory bulb β-adrenoceptor activation is both sufficient and necessary for odor preference acquisition. The role of the β-adrenoceptor activation in the PCX in early odor preference learning is examined in my study.

1.4 Synaptic plasticity

Hebb (1949) proposed that *neurons that fire together, wire together*. In other words, when a presynaptic cell persistently stimulates a postsynaptic cell, communication at that synapse is strengthened. This complemented then not that memories are encoded as biophysical changes to a neuronal network. Thus, experience can drive network reshaping.

Long term potentiation (LTP) is a model often used to depict Hebbian conditioning - the idea being that if a synapse can induce and express LTP then it may be capable of undergoing synaptic changes as a result of learning. In addition, learning-

induced synaptic modifications increase the threshold for further potentiation (Rioult-Pedotti et al., 2000; Brun et al., 2001), which helps maintain memories (Quinlan et al., 2004).

Synaptic plasticity is not always a potentiation, but can also be displayed as a depression. It depends on the frequency of activity at that synapse. Whether or not LTP or long-term potentiation (LTD) occurs, both are considered as mechanisms for learning. The determinant for LTP or LTD is the amount of Ca^{2+} influx through NMDARs. If synapse activation leads to shortening EPSCs, which results from an increased NR2a to NR2b ratio, the synapse will likely display LTD in lieu of LTP (Bienenstock et al., 1982; Quinlan et al., 2004).

In the subsequent sections, I first review the roles of synaptic glutamatergic receptors in synaptic plasticity, and then review LTP mechanisms with a focus of its role in odor learning.

1.4.1 Synaptic glutamatergic receptors in LTP and odor learning

Glutamate is the primary excitatory neurotransmitter in the PCX. Ionotropic glutamatergic receptors, AMPAR and NMDAR, mediate the majority of excitatory transmission in the central nervous system. The PCX displays laminar differences in glutamate receptor expression. Layer II of the PCX expresses AMPA, NMDA and

kainate receptors, while Ia expresses primarily NMDA receptors (Monaghan and Cotman, 1985; Petralia et al., 1994). In addition, glutamate contributes to excitatory transmission for both AMPA and NMDA receptors in both afferent and associational fibers in the PCX (Jung et al., 1990).

1.4.1.1 NMDARs

NMDA activates a specific type of glutamate receptor ion channel, the NMDA receptor (NMDAR). Such receptors are tetrameric, ionotropic, and are encoded by three gene families NR1, NR2a-d, and NR3a-b. Two NR1 subunits are mandatory for each receptor complex, while combinations of the other subtypes change throughout development (Sheng et al., 1994). The NMDAR contains three membrane-spanning domains, and one cytoplasm-facing membrane loop. Thus, the segments that face the extracellular space and/or the cytoplasm determine which ligands will bind and the protein interactions that can occur (e.g., phosphorylation) (Dingledine et al., 1999). The N terminus, which contains a glycosylation site, is located extracellularly, while the C terminus is intracellular, where binding sites for proteins such as PSD 95 are located (Wood et al., 1995). Alternative splicing that occurs in these domains gives rise to the functional diversity of the receptor (Dingledine et al., 1999).

The activation requirements for NMDARs include co-binding of glutamate, on the NR2 subunit, and glycine, on the NR1 subunit. However, presynaptic release of

glutamate only partially fulfills the activation requirements. A key feature of the NMDAR is the requirement of Hebbian coincidence detection. That is, a presynaptic event, glutamate release from LOT, must occur at the same time as a postsynaptic event, postsynaptic membrane depolarization. This will displace the Mg^{2+} ion that blocks the channel pore, thus allowing the influx of ions (McBain and Mayer, 1994).

NMDARs are permeable to several ions, Na^{+}, K^{+}, and, most importantly, Ca^{2+}. Calcium is a key player in synaptic plasticity, as its influx initiates a series of intracellular signalling cascades that lead to long-term changes within the cell, such as new protein synthesis. Experiments have shown that NMDAR activation is critical for long-term changes in synaptic strength (Tsien, 2000), consistent with their role as mediators of synaptic plasticity (Maren and Baudry, 1995; Debanne, 1996). The functional implications of NMDAR activation have been investigated in other brain regions, such as the hippocampus, during learning. Blocking NMDARs blocks spatial learning in the MWM (Morris et al., 1986; Morris, 1989). However, if animals were trained prior to NMDAR block, learning was retained (Bannerman et al., 1995). Thus, acquisition requires NMDAR activation and synaptic strengthening, but once the rule has been learned, blocking NMDARs does not appear to interfere with already learned memories (Bannerman et al., 1995).

NMDARs in the OB are implicated in early odor preference learning (Lethbridge et al., 2012). *In vitro* recordings show that the ON-MC synapse is capable of LTP in response to high frequency stimulation on the ON, causing glutamate release onto MCs.

In particular, this plasticity is NMDA receptor-dependent (Ennis et al., 1998). Furthermore, MC spike potentiation produced by pairing theta burst ON stimulation with β-adrenoceptor activation *in vitro* was blocked by D-APV, further illustrating the critical role of NMDARs in olfactory synaptic plasticity (Lethbridge et al., 2012).

In the PCX, LTP can be induced at the LOT-pyramidal cell synapse and also at the associational fiber synapses (Stripling et al., 1988). Similar to the OB, both synapses require NMDA receptor activation for LTP induction (Kanter and Haberly, 1990). This NMDAR-mediated plasticity may have essential implications for associative memory models in the olfactory system.

There is evidence for experience-dependent modifications in NMDARs in the PCX. Malinow and Malenka (2002) describe immature synapses in the hippocampus that are dominated by NMDARS, and are referred to as “silent synapses”. With experience, AMPARs are recruited to the synapse, which render the synapse functional with faster kinetic properties. Once this postsynaptic modification occurs, the synapses are much less likely to undergo LTP induction (Isaac et al., 1997). Experiments examining LTP in the adult rat PCX showed little to no LTP induction at the afferent synapses, thus it is likely that synaptic glutamatergic receptor conversion occurs in the PCX (Jung et al., 1990). The same was not true of associational inputs (Kanter and Haberly, 1990), suggesting that these connections may be critical for NMDAR-dependent plasticity in adulthood. Franks and Isaacson (2005) examined the excitatory transmission dynamics that occur in the first postnatal month. As predicted, they found that the contribution of AMPARs

during the first few weeks of life increased relative to the NMDAR contribution, which was regulated by sensory experience. This was only evident at the afferent synapses and not for the associational fiber synapses. In addition, when early sensory experience was delayed using nostril occlusion, the NMDAR down-regulation was interrupted. The authors remarked that this effect is due to an experience-dependent down-regulation of NMDARs at the LOT-PCX synapse. Such a mechanism coincides with a reduced plasticity in the PCX in older rats (Poo and Isaacson, 2007).

Thus, synaptic NMDAR contributions may be modified with experience; however, other developmental changes occur as well, including changes in NMDAR subunit composition. Previous research has demonstrated that at birth, the rat cortical synapses contain NR1 and NR2b subunits. During the first few days of postnatal life, the NR2b subunits are replaced by NR2a subunits (Sheng et al., 1994). This is functionally significant because NMDARs with NR2a subunits have faster kinetics and are less plastic, therefore allowing experience during early life to shape synaptic connections and stabilize synapses. In order to characterize the learning-induced NMDAR subunit modifications, Quinlan and colleagues (2004) employed an odor discrimination paradigm. They hypothesized that the developmental shift from NR2b- to NR2a-containing NMDARs serves to prevent further synaptic modifications, thus maintaining memories. It was found that acquiring the rule of odor discrimination leads to an increase in the ratio of NR2a to NR2b subunits in the PCX, therefore modifying the function of the NMDAR. As a result, the induction threshold for LTP was increased. Together, these results suggest that changes in the NMDAR synaptic contribution and subunit

composition serve to constrain synaptic plasticity, thus protecting previously learned memories and over-strengthening of synaptic connections (Quinlan et al., 2004).

1.4.1.2 AMPARs

AMPARs are glutamate-gated cation channels that mediate the majority of fast excitatory transmission in the brain. Changes in the number of AMPA receptors and/or in their function are a core feature of synaptic plasticity. In fact, AMPAR trafficking has been shown to have a critical role in LTP and LTD learning models (Malinow and Malenka, 2002). Synaptoneurosome preparations in the OB illustrated an increase in membrane AMPARs 24 h following odor conditioning in trained rat pups and not control counterparts. This AMPAR insertion into the membrane in the OB is critical for early odor preference learning, as demonstrated by pharmacologically blocking AMPAR membrane insertion, which prevented learning (Cui et al., 2011). Further electrophysiological experimentation used a nostril occlusion training paradigm and compared *ex vivo* AMPAR responses at the ON-MC synapse in the trained and untrained OB. It was found that 24 h following training, there is a region-specific increase in AMPAR currents in the trained bulbs. This was combined with a decrease in NMDAR currents, suggesting that a decrease in synaptic plasticity mechanisms was observed following the strengthening of the synapses by learning (Yuan and Harley, 2012). Such changes parallel the developmental synaptic changes described earlier. These results tie in nicely with the Franks and Isaacson study (2005), which showed that AMPAR

contribution to the synapse increases, while NMDAR contribution decreases in the PCX in response to early sensory experience (Franks and Isaacson, 2005). This phenomenon leads to a higher LTP induction threshold, thereby preventing modification in synaptic efficacy. It is suggested that AMPARs maintain the change in synaptic strength that occurs with experience (Malinow and Malenka, 2002).

1.4.2 Synaptic plasticity in the piriform cortex

As proposed by Hebb (1949), in order for learning to occur, an event at the presynaptic site must occur simultaneously, or coincide, with a postsynaptic event. This will lead to synaptic strengthening, whereby neurons can communicate more efficiently and will fire with less stimulation.

Studying synaptic properties *ex vivo* provides a useful means to examine experience-dependent changes. Following olfactory learning tasks, Quinlan and colleagues (2004) found a reduction in LTP in the PCX. They postulate that these changes are the result of NMDAR subunit modifications following learning. There is evidence that suggests experience-dependent plasticity in the PCX. The removal of afferent input into the PCX via bulbar lesions in rat pups results in a loss of volume of Layer Ia, while a compensatory increase occurs in Layer Ib (Friedman and Price, 1986; Westrum and Bakay, 1986). The previous evidence for experience-dependent plasticity suggests the PCX may be a site of memory formation in odor learning.

1.4.2.1 LTP at the LOT-PCX synapse

In order to examine the synaptic properties of a particular synapse, electrophysiological recordings are commonly employed. Learning a new task is complemented by a series of profound physiological modifications in certain brain regions. The PCX is ideal for studying extracellular activity, as OB projections to the cortex terminate in a well-defined, cell-sparse dendritic region of the PCX. Moreover, stimulation of the LOT has been shown to model odor-evoked activity in the PCX (Roman et al., 1987).

Stimulation applied to afferent fibers in the PCX evoke field potentials with two main components, an axon, or fiber volley (fast and short) and a postsynaptic potential (slow) occurs in Layer II pyramidal neurons (Saar et al., 1998). LOT stimulation will elicit a monosynaptic EPSP at the postsynaptic PCX that can be recorded in the extracellular space. It is assumed that changes in the recorded slope and amplitude following training, or electrical simulation, reflect changes in synaptic responses (Roman et al., 1987).

Previous work has shown that high frequency theta rhythm stimulation of the afferent LOT fibers can produce a long-lasting, enhanced response in Layer Ia of the aPCX that is stable for hours (Roman et al., 1987; Jung et al., 1990; Kanter and Haberly, 1990). Theta burst stimulation is used typically at the LOT-PCX synapse because theta rhythm occurs in the PCX during sniffing (McCollum et al., 1991). Monosynaptic EPSPs in the PCX can be elicited by stimulating either the afferent LOT fibers or associational

fibers in the PCX. LTP can be divided into temporal phases, two of which are LTP induction and LTP expression. Characterizing these LTP phases at specific synapses can help one further understand the type of information being encoded and how that stored information is organized (Jung and Larson, 1994).

1.4.2.1.1 Induction mechanisms

LTP induction is contingent upon input cooperativity. Low-intensity presynaptic stimulation results in a field potential reflecting the current around the pyramidal cell dendrites, the extracellular EPSP. A more intense stimulation will elicit sufficient postsynaptic depolarization to displace the Mg^{2+} block in the NMDAR channel, inducing LTP (McNaughton et al., 1978). This Hebbian coincidence requirement for simultaneous pre- and postsynaptic activity is more stringent for LOT fibers than association fibers, as the threshold for LTP induction is greater at LOT synapses (Jung et al., 1990). LTP induction at the LOT-PCX or associational fibers-PCX can be blocked by NMDAR antagonists (Kanter and Haberly, 1990). During LTP induction, synaptic function is initially enhanced, but this phase lasts less than one hour, thus there is no *de novo* protein synthesis.

LTP induction requires NMDAR stimulation with coincident postsynaptic depolarization (Collingridge et al., 1983). Once an NMDAR is activated, there is an intracellular influx of calcium ions (MacDermott et al., 1986), a trigger for LTP (Lynch

and Baudry, 1984). Recent work from our lab showed that LTP induction at the LOT-PCX synapse is NMDAR-dependent (Morrison et al., 2013).

1.4.2.1.2 Expression mechanisms

Once LTP induction occurs, LTP expression ensues. Postsynaptic responses can be mediated by NMDARs or AMPARs. LTP expression at both LOT and associational synapses display postsynaptic receptor selectivity, where there is a more robust LTP expression at AMPA receptors compared to NMDA receptors. This was supported by the findings that there was no LTP expression in the presence of DNQX, an AMPA antagonist (Jung and Larson, 1994).

The locus of changes responsible for LTP may be presynaptic, postsynaptic, or both. In order to examine these learning-induced modifications in the PCX, Saar and colleagues (1998) subjected water-deprived rats to odor discrimination training. Following training, *ex vivo* recordings were performed in the PCX slice. The results showed that presynaptic responses (axon volleys) did not differ between trained and untrained animals, thus ruling out learning-induced changes in axonal excitability at the synapse. In contrast, the ratio of the postsynaptic response to the presynaptic response (fEPSP) was greater in trained animals. This suggests an enhanced synaptic transmission following training. Similar results were found at the OBFC-aPCX synapse, as these connections were also strengthened following discrimination learning. This was

demonstrated by enhanced fEPSP amplitudes in trained animals. There was no change in the paired-pulse facilitation, thus a presynaptic modification was ruled out, suggesting that postsynaptic modifications dominate following learning (Cohen et al., 2008).

1.4.2.1.3 Activity-dependent regulation of NMDARs

Previous work in the PCX has shown that the quantity of NR2a, relative to NR2b, subunits increases following odor discrimination learning (Quinlan et al., 2004). This change in NMDAR subunit composition is also a natural part of development. Franks and Isaacson (2005) demonstrated NMDAR down-regulation following early sensory experience also using electrophysiological recording. Hence, it appears that learning may hasten normal NMDAR down-regulation.

Following learning, the tendency for LTP induction is decreased in the PCX, both in ascending pathways (i.e., LOT-aPCX) and descending pathways (i.e., OBFC-aPCX ; Quinlan et al., 2004). One explanation for this was the change in NMDAR subunit composition. The reduction in LTP induction may make learning at a later stage more difficult, consistent with metaplasticiy in other systems (Abraham and Bear, 1996).

Thus, the next question was whether early learning leads to changes in NMDAR composition that would affect further plasticity as shown by Quinlan et al (2004). In the OB, following early odor preference training, Western blotting revealed a significant down-regulation of the NR1 NMDAR subunit 3 h after odor conditioning, but this did not

persist for 24 h (Lethbridge et al., 2012). This could be due to degradation of the NR1 subunit, or lateral diffusion out of the synaptic membrane. The functional significance of these findings is that a down-regulation of the NR1 subunit may aid in memory consolidation processes by protecting the newly formed memories.

1.4.2.1.3.1 Synaptoneurosome preparation for detecting activity-dependent changes in synaptic receptors

The synaptosome preparation method has been widely used to study presynaptic entities together with associated transmitter release (Hebb and Whittaker, 1958). For postsynaptic events, however, this method is not appropriate. In order to isolate presynaptic and postsynaptic vesicularized components, composite structures called synaptoneurosomes were developed (Titulaer and Ghijsen, 1997). Synaptoneurosomes are useful for small quantities of starting tissue in order to study molecular changes at the synapse. The synaptoneurosome preparation removes internalized receptors from the analysis so that only functional membrane receptors are included in examination. By examining synaptic and extrasynaptic proteins one can further understand how a particular synapse functions before, during, after a given manipulation. For the present study, the synaptoneurosome preparation will be useful for assessing learning-induced changes in NMDAR subunit composition.

1.5 Objectives and hypotheses

The major goals of the present research are to characterize and delineate the role of the PCX in early odor preference learning. As per findings showing an increased *c-Fos* expression in the aPCX (Roth and Sullivan, 2005), we postulated that the aPCX was critically involved in our learning model. In particular, we predicted that if we were to block neural activity in the aPCX prior to training, pups would be unable to learn a preference for a novel odor. We also wanted to explore the role of NMDAR and β-adrenoceptor activation in the aPCX in early odor preference learning.

Objective 1 The first step was to determine whether the aPCX was involved in early odor preference learning. In order to do so, we silenced neuronal activity in the aPCX at different times during training and testing protocols. We bilaterally infused a reversible sodium channel blocker directly into the aPCX prior to training and then tested the following day for odor memory. The same protocol was repeated, but with the infusion occurring prior to testing. This provided insight into the temporal involvement of the aPCX during early odor preference learning.

Objective 2 Next, we examined whether odor preference learning is NMDAR-dependent in the aPCX. We postulated that blocking NMDAR activation in the aPCX prior to odor conditioning would prevent early odor preference learning. In addition, blocking NMDAR activation before testing would not disrupt learning, as this should not disrupt already formed memories.

Objective 3 Next, we sought to determine whether ß-adrenoceptor activation mediates odor conditioning. There has been strong evidence supporting a critical role for ß-adrenoceptor activation in the OB during early odor preference learning *in vivo* and *in vitro*. The PCX is highly innervated by NE fibers. We hypothesized that blocking ß-adrenoceptor activation in the aPCX prior to odor conditioning would also block odor preference learning. In addition, infusions of a ß-adrenoceptor agonist in lieu of stroking may elicit a preference for a novel odor.

Objective 4 Next, we examined LTP induction at the LOT-aPCX synapse. We characterized the synaptic components at the LOT-aPCX synapse. Specifically, we investigated LTP induction using a single theta burst stimulation protocol and compared the LTP magnitude with that induced by additional TBSs. We had hypothesized that early odor preference learning results in LTP-like changes at the LOT synapses in the aPCX. Therefore, these *in vitro* experiments provide a basis for exploring learning-induced LTP changes at the same synapses.

Objective 5 Finally, we examined NMDA receptor subtype composition at 3 h and 24 h following unilateral odor conditioning. Specifically, we investigated the obligate NR1 receptor and the calcium highly permeable NR2b receptor in the aPCX following unilateral odor conditioning. We hypothesized that learning may down-regulate NMDARs or alter NMDAR compositions as seen in other studies.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Animals and ethics statement

All experimental procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland (protocol number: 13–01-QY) with adherence to the guidelines set by the Canadian Council on Animal Care.

Male and female Sprague Dawley rat pups (Charles River) were used in this study. Animals were bred and pups were born on-site at the research facility. The day of birth was designated postnatal day 0 (PD 0) and on PD 1, litters were culled to 12 pups with equal numbers of males and females. Dams were maintained under a 12:12 h reverse light/dark cycle with the dark phase beginning at 12:00 h and had *ad libitum* access to food and water.

2.2 Behavioral studies overview

Behavioral experiments were carried out in a temperature controlled room at approximately 28°C and followed the standard protocol previously established for early odor preference learning (Sullivan and Leon, 1987; McLean et al., 1999), as described below. Three types of behavioral experiments were conducted. First, to test the effects of various drug manipulations on odor preference learning, odor + stroke training (OS^+; peppermint odor exposure paired with stroking the pups) and odor only control

experiments (OS^-; peppermint odor exposure only) were combined with drug infusions into the aPCX either before training or before testing. Infused drugs included lidocaine, muscimol, D-APV, isoproterenol, and propranolol. Next, to test the effect of odor preference training on the activation level of the pyramidal cells in the piriform cortex, pCREB immunohistochemistry was performed in the brains collected shortly following training. Pups were trained with single nostril occluded and sacrificed 10 min following either odor/stroke or odor only training. Student's *t*-tests and one-way ANOVAs were used to determine statistical significance throughout the experiments. No more than ten pups were used in each litter, and no more than two pups in each litter were used for each condition.

2.2.1 Odor conditioning training

Animals were randomly assigned to an OS^+ or an OS^- condition. On PD 7, pups were removed from the nest and placed on unscented bedding for a 10 min habituation period. After habituation, pups receiving conditioning training (OS^+) were placed on peppermint-scented bedding (0.3 mL peppermint in 500 mL bedding) and vigorously stroked with a paintbrush for 30 sec, followed by 30 sec rest, for a total of 10 min. Pups in the non-learning condition (OS^-) were placed in peppermint-scented bedding for 10 min following the habituation period. These pups were not stroked. Once odor conditioning was complete, any excess peppermint bedding was removed to avoid odor contamination and pups were returned to their home cage.

2.2.2 Two choice odor testing

On PD 8, pups were tested for odor preference memory using a two-choice odor preference procedure. The testing apparatus was a stainless steel box (30 x 20 x 18 cm) placed over two testing boxes separated by 2 cm, the neutral zone. One box contained peppermint-scented bedding and the other contained normal, unscented bedding. For testing, pups were removed from the dam and placed in the neutral zone. Pups moved freely for 1 min, while the time spent over each testing box was measured, and were then removed from the test chamber for 1 min. The timer started only if at least one forepaw and one nostril were over the bedding. This was repeated for a total of 5 trials. The average time spent over peppermint bedding was calculated for each pup. Direction preference was eliminated by alternating the orientation of the starting position of the pups in the neutral zone. Behavioral testing was done in a double-blind fashion, which the training and testing were carried out by two individuals, such that the tester had no information of the previous training conditions of the pups.

2.3 Intracranial cannula implantation

On PD 6 pups were anesthetized via hypothermia and placed in a stereotaxic apparatus in skull flat position. A horizontal incision was made just posterior to the eyes so that bregma was visible. Two small burr holes were drilled approximately 2.5 mm anterior and 3.0-3.25 mm bilateral, with respect to bregma. Two guide cannulae (Vita

Needle, MA) with inserted insect pins were lowered from the surface of the brain to a depth of 5.5-6.0 mm and were anchored to the skull with dental acrylic (Lang Dental) and a plastic screw that was firmly attached to the skull surface. Two sutures were made on either side of the cannula and pups were placed on warm bedding to recover before being placed back with the dam.

2.3.1 Cannulae placement verification

Following testing, all pups received bilateral intracranial infusions of 4% methylene blue dye (Fisher Scientific), were sacrificed, and the brains were collected and sliced to confirm cannula placement (Appendix 1). Nissl staining (method see 2.6) was applied on alternate slides to confirm brain structures.

2.4 Intracranial pharmacological infusions

Twenty-four hours following surgical cannula implantation pups underwent odor training. Pups receiving infusions prior to training were removed from the dam and insect pins were removed from the cannulae. Any debris from the home cage or surgery was cleared from the cannulae. Animals were excluded in the event of bilateral cannular infusion blockage. The pups were then placed on plastic hexagonal weighing dishes where they were infused with 1 μl of the desired solution at a rate of 0.25 μl/min, for a total infusion time of 4 min. Infusions were followed by a 6 min resting period to allow

for adequate diffusion of the solution into the brain. Pups were then returned to the dam for 10 min prior to training or testing.

Lidocaine-hydrochloride (4%, Sigma Aldrich), a reversible sodium channel blocker, muscimol (50 mM, Sigma Aldrich), a $GABA_A$ agonist, propranolol-hydrochloride (100 μM, Sigma Aldrich), a β-adrenoceptor antagonist, varying concentrations of the β-adrenoceptor agonist isoproterenol (5 μM, 50 μM, or 500 μM; Sigma Aldrich), and (2R)-amino-5-phosphonovaleric acid (D-APV), an NMDA receptor antagonist (100 μM, Tocris) were infused into the anterior piriform cortex at various time points as described in the results.

2.5 pCREB immunohistochemistry

There were two experimental series in which pCREB immunohistochemistry was employed. The phosphorylation of CREB, specifically at the Ser-133 site, is a transcription factor that presumably reflects learning associational changes (Gonzalez and Montminy, 1989). Additionally, our lab has used pCREB as a marker for neuronal activity. We have found that pCREB expression in MCs in the OB and pyramidal cells in the aPCX increases with high concentrations of odor. Such expression increases dramatically if MCs are disinhibited (e.g., by infusing a $GABA_A$ blocker) suggesting that there is a basal level of pCREB in MCs that can be up-regulated by MC activity (Lethbridge et al., 2012; Fontaine et al., 2013; Morrison et al., 2013). Thus pCREB serves as a great marker for neuronal activity. The first set of experiments examined

pyramidal cell silencing in the aPCX in response to unilateral lidocaine or muscimol local infusions following intense novel odor exposure. The contralateral hemisphere was infused with vehicle (e.g. saline) control, to eliminate the effects due to cannular infusion itself. The second set of experiments compared pCREB expression in animals undergoing odor conditioning and animals in an odor only condition.

2.5.1 Transient silencing using lidocaine

On PD 6 or 7, naïve pups were anesthetised via hypothermia and were placed in a stereotaxic apparatus in skull flat position. A horizontal incision was made so that bregma was visible. Two small burr holes were then drilled approximately 2.5 mm anterior and 3.25-3.5 mm lateral in relation to bregma. A syringe filled with methylene blue dye (4%, Fisher Scientific) was lowered 6 mm into the aPCX and 1 µL of the dye solution was gently infused into the brain. The syringe was held into place for 30 sec to ensure full diffusion of the solution into the tissue. The same protocol was repeated for the opposite hemisphere using lidocaine (4%, dissolved in methylene blue solution, Fisher Scientific) or muscimol (50 mM, dissolved in methylene blue solution, Sigma Aldrich) infusions. Following infusions, the incision was sutured and pups were placed on warm unscented bedding to recover for 20-10 min from the time of the drug infusion. Once the recovery time was over, animals were situated in a covered plastic container that was scented with 30 µL of pure peppermint oil for 10 min. Around the 8 min mark, pups received intraperitoneal (i.p.) injections of chloral hydrate (400 mg/kg, Fisher Scientific) as a

general anesthetic and were kept in the scented container until they were fully unresponsive to tail and foot pinches. Once fully anesthetized, pups underwent transcardial perfusions with ice-cold solutions of saline (0.9%), followed by paraformaldehyde (4%, dissolved in 0.1 M PBS). After 10 min of paraformaldehyde perfusion, the brains were collected and placed in paraformaldehyde overnight at 4ºC. The following day, the tissue was transferred to a sucrose solution (20%) for an additional 24 h.

2.5.2 Pyramidal cell activation following odor preference learning

The next set of experiments was performed to determine whether learning elicits a stronger neural response in the aPCX than mere odor presentation. First, to achieve intra-animal control, unilateral nostril occlusions were performed. Nose plugs were constructed and inserted as described in the section 2.7.1. On PD6 or PD7, pups were removed from the dam and placed on unscented bedding for a 10 min habituation period. Following habituation, the pups underwent either OS^+ or OS^- training in peppermint-scented bedding for 10 min (See section 2.3 Odor conditioning training for detailed protocol). After training, the pups were returned to normal bedding for an additional 10 min before undergoing perfusion and tissue collection, as previously described. This particular timeframe was chosen because pCREB expression is maximal 10 min following odor conditioning (McLean et al. 1999).

2.5.3 Immunohistochemistry

Sucrose-rich brains were mounted for slicing using a Cryomatrix fixative (Thermo Scientific) and immediately placed in a cryostat at -20ºC until fully frozen. Thirty-micrometer coronal sections were cut, collecting two consecutive slices every 200 µm. Slices were mounted on chrome-gelatin coated slides in an alternating fashion, allowing for pCREB and Nissl comparison staining, and kept at -20ºC for the entire slicing period. Following slicing, slides were moved to a 4ºC chamber for 10 min before bringing them to room temperature to dry. A pCREB antibody (1:100, Cell Signalling), dissolved in 0.1 M phosphate buffered solution (PBS) with 0.2% Triton-X-100, 0.02% sodium azide, and 2% normal goat serum, was applied to the appropriate slides to probe for neuronal activity in the aPCX. Control counterpart slides were stored in the fume-hood until Nissl staining ensued. After antibody application, the slides were incubated at 4ºC overnight in a humidified chamber. The following day, slices were washed with PBS (10 min x 3 times) and a biotinylated anti-rabbit secondary antibody solution [normal goat serum & anti-rabbit serum, dissolved phosphate buffer solution with 2% Triton-X-100, 0.002% sodium azide (Vectastain)] was applied to the slices for 1 hr. This was followed by an avidin and biotinylated (A+B) enzyme amplification step (1hr). Finally, sections were stained using a diaminobenzidine tetrahydrochloride (DAB) reaction [50mg DAB (Amresco), dissolved in 50 mL 0.1 M PBS, 50mL dH_2O, & 30 µL 30% H_2O_2] for 2-5 min, which was monitored for completion under an upright light microscope (Olympus). Slides were then dehydrated using concentration-graded ethanol

solutions and cover-slipped. Hemispheres of each slide were compared under the microscope to confirm there was less staining in the LIDO- or muscimol-injected aPCX compared to the dye only hemisphere.

2.5.4 Image analysis for pCREB immunohistochemistry

To quantify pCREB expression in the aPCX, image analysis was performed. This allowed for comparison between occluded and spared hemispheres, as well as between OS^- and OS^+ training conditions. With the use of a Bioquant system (R & M Biometrics), images were obtained using a CCD camera and viewed with a Leitz microscope with a consistent light intensity. Analyzing slices from rostral to caudal (~5-8 slides per brain), an optic density (OD) reading of the background was taken in a non-staining area in the center of the section. A region of interest (ROI) was manually traced in both a lateral and a medial position in pyramidal cell layer (PCL) of the aPCX for both hemispheres. The relative OD (ROD) was calculated using the following formula,

$$ROD = |(OD \text{ of ROI} - OD \text{ of background})| / OD \text{ of background}$$

The medial and lateral ROD measurements were compared in the spared and occluded hemispheres of each animal to ensure that the nostril occlusion was successful. These values were also compared between experimental conditions (i.e., OS^+ versus OS^-). In order to account for individual differences between animals, measurements were normalized and the percent differences were calculated using the following formula,

$$\% \text{ difference} = 100\% - (\text{spared ROD} / \text{occluded ROD})$$

Values displayed indicate the percentage differences between medial and lateral regions of the spared and occluded hemispheres and between OS^+ and OS^- animals.

2.6 Electrophysiological characterization of LOT-PCX synapse

2.6.1 Tissue preparation and extracellular recording

Naïve PD 7-11 animals were used for recording. Animals were anesthetized with halothane inhalation and quickly decapitated. Brain tissue was extracted and placed in a high sucrose artificial cerebral spinal fluid (aCSF; in mM, 83 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 3.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, & 72 sucrose, equilibrated with 95% O_2 and 5% CO_2) for approximately 10 minutes. Sagittal slices (400µm) were cut using a vibrating blade (Vibratome 1000P, Leica Microsystems, ON) and were incubated in an Isotemp 205 chamber (Fisher Scientific) at 34°C in the aforementioned solution for at least 60 minutes before use.

Tissue slices were transferred to an RC-40 open bath recording chamber (Warner Instruments, CT) which was continuously perfused with aCSF (in mM, 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, equilibrated with 95% O_2 and 5% CO_2) at 30-32°C and viewed with an upright microscope (Olympus BX51). A small metal weight with a plastic grid (Warner Instruments, CT) was used to anchor slices and prevent movement and an electrode with a pellet (A-M Systems, WA) acted as a ground and was located within the aCSF solution in the recording chamber.

Extracellular field potentials were recorded with glass pipettes filled with aCSF and placed in layer Ia of the aPCX. A concentric bipolar stimulating pipette (FHC) was lowered into the LOT and delivered one test pulse (200 μsec), ranging from 20-80μA.

Electrophysiological data were recorded with Multiclamp 700B (Molecular Devices), filtered at 2 kHz and digitized at 10 kHz. Data acquisition and analysis were performed with pClamp10 and ClampFit 2.10 (Molecular Devices) and Igor Pro 6.10A (WaveMetrics).

2.6.2 *In vitro* electrophysiology

Prior to baseline recording, the optimal stimulation intensity was determined using a stimulation profile. Single pulse test stimulation was delivered via the stimulating electrode onto the LOT fibers at intensities varying from 20-80 μA, evoking field EPSPs (fEPSPs) in the aPCX apical dendrites in Layer Ia. The optimal stimulation intensity is defined as that at which 50% of the maximum response was evoked. Once the appropriate stimulation intensity was determined, evoked fEPSPs were recorded at 0.05 Hz until a 10 min consistent baseline was established. To induce LTP, theta burst stimulation (TBS; 10 times 5 Hz trains, each train consists of 5 pulses at 100 Hz) was applied to the LOT layer and the resulting response in the aPCX was recorded. Protocols varied from 1, 4, or 8 TBS, each separated by 30 sec. Single test pulse stimulation followed TBS for 30-60 min. The recordings were analyzed to determine the amount of LTP at the LOT-PCX synapse.

In order to determine whether a postsynaptic mechanism was driving the LTP at the LOT-PCX synapse, AMPA input-output measurements were obtained. Stimulation profiles were obtained by stimulating at various intensities (20-80 μA). AMPA input output relationships for field EPSPs were measured both before LTP and 30 min after LTP induction. The size of the presynaptic fiber volley (a measure of input) and the postsynaptic AMPA slope of each field EPSP (a measure of output) were measured and the ratio of the fiber volley (mV) to the AMPA slope (mV/msec) was calculated and plotted as a line of best fit. The input-output curve pre-LTP was compared to that measured 30 min post-LTP.

D-APV (50 mM, Tocris) was used in bath application in subsets of experiments. Drugs were washed into the recording chamber 5 min before the TBS and washed out immediately following the TBS. The sizes of field EPSPs were measured, and paired pulse ratios during isoproterenol wash in were compared before and immediately following the TBS.

2.7 Synaptic expression of NMDAR subunits following odor conditioning

2.7.1 Reversible nostril occlusion and sample collection

Nose plugs were constructed using polyethylene 20 (PE 20) tubing, silk surgical thread, and human hair, as per procedures described by Cummings et al. (1997). Prior to insertion, a small dab of a sterile local anesthetic, 2% Xylocaine (AstraZeneca), a lidocaine hydrochloride jelly, was applied to the left naris. Pups were given one minute to

rest before the plug was gently inserted in the left naris. When the plug was fully inserted, there were approximately 2 mm of hair extending from the nose to allow for easy removal. After nostril plug insertion pups were placed on unscented bedding to habituate to the nose plug, followed by appropriate training protocols.

2.7.2 Tissue preparation and Western blot on PCX synaptoneurosomes

On PD 6 pups were subjected to a reversible nostril occlusion in their left nostril and underwent either odor conditioning or odor exposure, as previously described. At 3 h or 24 h following training, animals were euthanized via decapitation and anterior piriform cortices were collected and flash frozen on dry ice. Samples were stored at -80°C until further processing. Littermate control counterparts underwent nostril occlusions without odor exposure. In addition, one OS^+ and one OS^- pup from each litter underwent two-choice odor testing, as described previously, to verify that pups were learning accordingly with one nostril occluded during training and testing.

2.7.3 Synaptoneurosome isolation

To determine whether learning modifies NMDAR subunit composition, Western blotting was performed on synaptoneurosome tissue preparations of the aPCX. The synaptoneurosome preparation method has been previously shown to provide samples enriched with membrane proteins (Quinlan et al., 1999). All samples were kept on ice or in a 4°C environment during the synaptoneurosome extraction in order to minimize proteolysis. PCX samples were homogenized in oxygenated HEPES buffer [in mM: 50

HEPES, 124 NaCl, 26 $NaHCO_2$, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 3.2 KCl, 1.06 KH_2PO_4, 10 glucose, 1 EDTA, 1 PMSF, complete protease inhibitor cocktail (Roche), complete phosphatase inhibitor cocktail (Roche), saturated with 95%O_2/5% CO_2, pH 7.4], using Teflon-glass tissue homogenizers (Thomas Scientific). Lysed samples were kept on ice for 10 min for incubation in the buffer and were then passed through 3 pre-wetted filters using a 13 mm diameter syringe filter holder (Millipore). The first two filters were 100 µm nylon filters (Small Parts Inc.) and the last filter was a 5 µm pore hydrophilic filter (Millipore). Filtrates were centrifuged at 1000 x g for 20 min at 4°C, following which the pellets were resuspended in HEPES buffer. These pellets represent the synaptoneurosome fraction. Protein concentrations for each sample were calculated using a BCA protein assay kit (Pierce). Small portions of each sample were collected and diluted in water to yield one part sample to ten parts water to use for the protein assay. Samples and standards were added to a 96 well plate and incubated at 37°C for 30 min. A standard curve was determined using the standards which allowed for protein concentration determination for each sample. The volume of lysate required to make 40 µg of protein for each sample was calculated.

2.7.4 Western blot

For the following procedures, all samples collected from the same litter were processed together and transferred to the same nitrocellulose membrane. Sixty µL total volume solutions of lysate (volume required for 40 µg protein), sample buffer (0.3 M TRIS-HCl, 10% SDS, 50% glycerol, 0.25% bromophenol blue, 0.5 M dithiothreitol) and

dH_2O were prepared and boiled for 5 min at 100ºC. Samples were then loaded into lanes of a 7.5% SDS-PAGE gel, along with a protein ladder (Thermo Scientific) and loading buffer for empty lanes. Sample separation occurred through SDS-PAGE, followed by transference to a nitrocellulose membrane (Amersham). Membranes were cut horizontally at the 72 kDa level and the upper portion was probed with a rabbit antibody for NR1 (1:2000, blocked in BSA; Cell Signalling) or NR2b (1:2000, blocked in 0% milk fat, m.f., milk; Millipore) subunits, and the lower portion was probed for β-actin (1:5000, blocked in 0% m.f. milk; Cedarlane). Membranes were incubated in primary antibody and agitated overnight at 4ºC. Secondary antibodies bound to horseradish peroxidase were applied the following day (1:20,000, anti-rabbit; Pierce) for 1 hr and membranes were then washed in Enhanced chemiluminescence Western blotting substrate (Pierce). Finally, blots were developed on X-ray film (AGFA).

Films were scanned onto a computer using an image scanner (CanoScan LiDE 200) and the optic density (OD) of each band was measured using ImageJ software. Each sample was normalized to the corresponding β-actin band that was run on the same gel. In order to view differences in expression following learning, each spared hemisphere was normalized to its naris-occluded counterpart. Experimental values are reported as mean ± SEM of the relative expression of NMDAR subunit in the spared hemisphere normalized to the naris-occluded hemisphere. A one-way ANOVA was used to evaluate differences in the mean OD in OS^+ and OS^- animals at 3 h and at 24 h post odor conditioning.

CHAPTER 3 – RESULTS

3.1 Piriform cortex silencing impairs early odor preference learning

In order to establish the critical involvement of the aPCX in early odor preference learning, lidocaine or muscimol was infused either before training or prior to testing to transiently silence cells in that area. On PD 7 lidocaine, muscimol, or saline was bilaterally infused directly into the piriform cortex 10 min before OS^+ (or OS^-) training and then pups were tested 24 h later for odor preference using a two choice preference test. Figure 2A shows that lidocaine- and muscimol-infused OS^+ animals spent less time over peppermint-scented bedding (lidocaine, 40.73 ± 4.23% on the peppermint side, n = 17; muscimol, 20.34 ± 4.79% on the peppermint side, n = 7) compared to their saline-infused counterparts (saline OS^+, 54.81 ± 3.26%, n = 19). Saline-infused OS^- animals were comparable to lidocaine pups (39.02 ± 2.70%, n =18). A one-way ANOVA reveals a significant difference among groups ($F_{(3,57)} = 10.77$, $p < 0.001$). Post hoc Fisher least significant difference (LSD) testing demonstrates significant differences between saline-infused OS^+ and saline-infused OS^- groups ($p < 0.01$), saline-infused OS^+ and lidocaine-infused animals ($p < 0.01$), and saline-infused OS^+ and muscimol-infused groups ($p < 0.001$). Animals that underwent OS^+ training on PD 7, followed by lidocaine or muscimol infusions prior to testing on PD 8, showed a reduced preference for peppermint (lidocaine, 36.86 ± 7.92%, n = 6; muscimol, 38.57 ± 6.04, n =11) compared to their saline-infused counterparts (58.05 ± 5.31%, n = 7). Additionally, animals who received

odor exposure only combined with saline infusions showed a lower preference for peppermint compared to OS^+ saline-infused animals (27.97 ± 5.23%, n =8). A one-way ANOVA shows significant between-groups differences ($F_{(3, 28)} = 3.81$, $p < 0.05$). Post hoc testing demonstrates significant differences between saline-infused OS^- and saline-infused OS^+ animals ($p < 0.01$), saline-infused OS^+ and lidocaine-infused animals ($p < 0.05$), and saline-infused OS^+ and muscimol-infused animals ($p < 0.05$). Results are summarized in Figure 2B. These results support a critical role for the aPCX in memory encoding and recall processes during odor preference learning.

To verify that pyramidal cells were silenced in response to lidocaine or muscimol infusion, pCREB immunohistochemistry was performed following a unilateral lidocaine infusion and a high concentration novel odor exposure. CREB phosphorylation is a transcription factor that may indicate neuronal activity. Figures 2C and 2D show pCREB expression in the pyramidal cell layer of the aPCX in the same animal (Fig. 2C is rostral to Fig. 2D) at low (middle panel) and high (lower panel) magnifications. Quantitative results showed that hemispheres injected with lidocaine show much less staining than saline-injected hemispheres (ROD: 69.8 ± 12.3% of the saline injected side, $t = 6.50$, $p = 6.333E^{-4}$; n = 7). Nissl staining (upper panel) confirms that pyramidal cell bodies are still present in the piriform cortex in both hemispheres. No spread to the olfactory bulb or the posterior PC was observed using both methylene blue infusion spread assessment and, more critically, pCREB IHC reactivity in these two structures. This suggests that there was no silencing of adjacent areas. We cannot exclude the possibility that the infusion of the drugs may spread to the very adjacent anterior olfactory nucleus. However, from our

impression, the most rostral portion of the aPCX was not affected; suggesting drug spread was more limited to middle portion of the aPCX. There were also no long-lasting effects of lidocaine and muscimol at 24 h – at the time of testing, judged by pCREB IHC in aPCX itself (n = 4, visual observations).

Visual inspection of the OB and pPCX (not pictured) show equal pCREB expression between bulbs and hemispheres, respectively, therefore indicating that cell silencing was localized in the aPCX. This silencing effect, however, subsided 24 h following lidocaine or muscimol infusions (visual observations), indicating that the drug effects were transient.

3.2 Learning is dependent on NMDARs in the piriform cortex

To further characterize the molecular machinery required for early odor preference learning in the piriform cortex, the role of NMDARs was tested. NMDARs were blocked using D-APV 10 min prior to OS^+ training. When animals were tested the following day, it was found that blocking NMDARs in the aPCX prevents early odor preference learning (Figure 3). Such animals spent significantly less time over peppermint-scented bedding compared to their saline-infused counterparts (D-APV, 41.43 ± 5.02%, n = 17; saline odor +stroke, 54.81 ± 3.22, n = 19). In addition, saline-infused OS^- animals (39.02 ± 2.70%, n = 18) were comparable to D-APV-infused animals. A one-way ANOVA shows that there were significant differences between all groups ($F_{(2,51)} = 5.41$, $p < 0.01$). Post hoc testing further shows that saline-infused OS^- animals spent much less time over peppermint compared to saline-infused OS^+ animals (p

< 0.01) and D-APV infusion before OS^+ training significantly reduced the time spent over peppermint compared to saline-infused OS^+ animals ($p < 0.05$). These results suggest that NMDAR activation in the aPCX is critical for early odor preference learning. However, once the memory is formed, NMDAR activation in the aPCX is not required and pups can recall such memories in the absence of NMDAR activation.

3.3 β-adrenoceptors in the piriform cortex are critical for learning

β-adrenoceptor activation is required for early odor preference learning in the OB (Lethbridge et al., 2012; Sullivan et al., 1989), and since the aPCX is highly innervated by NE fibers, we tested whether β-adrenoceptor activation in the aPCX plays a causal role in our learning model. To test this, β-adrenoceptors were blocked by infusing propranolol, a β-adrenoceptor antagonist, directly into the piriform cortex 10 min before OS^+ training. Propranolol-infused animals (39.59 ± 4.82%, n = 8) showed a preference for peppermint similar to that of non-learning saline-infused animals (39.02 ± 2.70%, n = 18) and a lower preference for peppermint compared to saline-infused OS^+ animals (54.81 ± 3.23%, n = 19; Figure 4). Thus, the natural release of NE from the LC during stroking leads to activation of β-adrenoceptors in the aPCX, which is critical for early odor preference learning.

To further explore the role of β-adrenoceptors in early odor preference learning, stroking was replaced with bilateral intracranial infusions of varying concentrations isoproterenol, a β-adrenoceptor agonist, during odor exposure. Animals infused with low-to-moderate concentrations, 5 μM isoproterenol (38.86 ± 4.29%, n = 6) or 50 μM

isoproterenol (38.06 ± 8.68%, n = 6), did not learn to prefer peppermint, however, animals infused with 500 μM isoproterenol (58.41 ± 10.98%, n = 6) did show a preference for peppermint, which was blocked by co-infusion of propranolol (100 μM). A one-way ANOVA shows significant differences between all groups ($F_{(6,60)} = 3.66$, $p < 0.01$; Fig. 4). Post hoc testing reveals significant differences between saline-infused OS^- and saline-infused OS^+ animals ($p < 0.01$) and between saline-infused OS^+ and propranolol-infused animals ($p < 0.05$). In addition, there was a significant difference between saline-infused OS^- animals and 500 μM isoproterenol-infused animals ($p < 0.01$). The 500 μM isoproterenol group is significantly different from the propranolol co-infusion group ($p < 0.01$). These results suggest that β-adrenoceptor activation is necessary for early odor preference learning and that increasing levels of norepinephrine in the piriform cortex is sufficient to induce learning that is independent of olfactory bulb input.

Note: Groups in Figure 3A and 4 were constructed at the same time over many litters, but data were separated into different figures to represent different information. As mentioned in the section 2.2, no more than two pups from the same litter were assigned to the same condition. Those saline control animals have been accumulated over many litters and were analyzed together to serve as a comparison for all other experimental groups.

3.4 Odor + stroke training enhances pCREB expression in the lateral

region of the anterior piriform cortex

To compare differences in pCREB expression in the piriform cortex that occur as a result of learning with those in response to odor perception in that area, nostril occlusion studies were conducted. This allowed for intra-animal comparisons, where the occluded hemispheres were compared to the spared hemispheres. Pups underwent OS^+ or OS^- training and the differences in pCREB expression in both the OB and aPCX were observed (Fig. 5A & 5B, respectively). Relative optic densities (ROD) were measured in the region of interest (ROI) in the piriform cortex in both hemispheres, measuring a lateral and a medial region in order to investigate whether regional differences exist. Data were normalized to account for individual differences and are expressed as a percentage difference [100% - (spared/occluded); Figure 5C]. Using a paired sample t-test, OS^- animals showed no significant differences in normalized pCREB expression between medial (1.81 ± 0.70%) and lateral (2.10 ± 0.42%) regions of the aPCX ($n = 7$, $t = 0.36$, $p > 0.05$), while OS^+ animals showed much higher staining in the lateral region (3.60 ± 0.76%) of the aPCX than in the medial region (1.74 ± 0.43%, $n = 5$; $t = 5.15$, $p < 0.01$). Comparing medial and lateral regions between training conditions, a two sample t-test found no significant differences in the medial regions of the aPCX between OS^- and OS^+ animals ($t = 0.080$, $p > 0.05$). However, there were significant differences in the lateral regions of the aPCX between OS^- and OS^+ animals, with stronger staining in the OS^+ animals ($t = 1.87$, $p < 0.05$). These results show that learning (i.e., odor plus stroking) produces higher CREB phosphorylation than odor exposure only, specifically in the lateral regions of the piriform cortex. This suggests that pCREB-induced post-

transcriptional modifications in the aPCX are associated with early odor preference learning.

3.5 LTP at the LOT-pyramidal cell synapses *in vitro*

For characterizing synaptic plasticity at the lateral olfactory tract (LOT)-piriform cortex synapse, *in vitro* electrophysiology was used to induce LTP. A stimulating electrode was lowered into the LOT, while extracellular evoked field potentials were recorded from Layer Ia of the piriform cortex. Once a baseline was established, TBS was applied and subsequent recording 30 min post-LTP induction was compared to baseline levels. Figure 6A shows LTP following 1 TBS. A paired sample t-test comparing the normalized fEPSP slope (mV/ms) of the baseline to the post-TBS fEPSP recording reveals that they are significantly different (post-LTP: 108.8 ± 3.8% of baseline, $n = 15$, $t_{(14)} = 2.31$, $p < 0.05$) and that LTP was induced at the LOT-piriform cortex synapse. Furthermore, an 8 TBS protocol induced a moderately larger LTP (post-LTP: 116.5 ± 3.3% of baseline; $n = 14$, $t_{(13)} = 4.93$, $p < 0.01$) compared to 1 TBS, however a two sample t-test revealed that this difference is not statistically significant ($t_{(29)} = 1.50$, $p > 0.05$; Figure 6B). Therefore, the LOT-piriform cortex synapse is plastic and is capable of strengthening synaptic communication, which is commonly found in learning.

Previous behavioral results revealed that NMDARs were critical for early odor preference learning. To complement these behavioral results, D-APV was used in bath application during recording. When D-APV was present, LTP was not inducible.

However, once it was washed out and aCSF was perfused back into the recording chamber, LTP was inducible following 1 TBS (n = 5; Fig. 6C). These results suggest that LTP induction at the LOT-piriform cortex synapse is NMDAR-dependent.

3.6 Post-synaptic expression following *in vitro* LTP

Figure 7A shows a typical (upper) pyramidal evoked field potential in response to different stimulation intensities and the changes following LTP (lower). Both display a similar fiber volley (FV), however, the EPSP slope is larger following LTP. To characterize the postsynaptic changes that occur following LTP, AMPA components were isolated and compared before and after LTP. AMPA input-output (I/O) relationships were determined by comparing the presynaptic FV with the EPSP slope. A one-tailed paired t-test revealed that the AMPA I/O was greater post-LTP compared to pre-LTP ($t_{(14)} = -1.87$, $p < 0.05$; Fig. 7B), implying that a postsynaptic mechanism is contributing to the plasticity at this synapse.

3.7 Learning-induced NMDAR subunit expression changes in the piriform cortex

NMDAR composition in the aPCX is modified with development, specifically NR2b subunits are replaced with NR2a as synapses mature (Quinlan et al., 2004). To test

whether odor conditioning also leads to subunit composition modifications, Western blot analyses were performed and subunit expression in the learning (i.e., spared) hemisphere was compared to the non-learning (i.e., occluded) hemisphere. At 3 h following OS^+ learning, there was a significant down-regulation in NR1 (0.668 ± 0.361) that was not found 3 h following OS^- training (1.399 ± 0.556). At 24 h, there was a significant up-regulation in NR1 in OS^+ (1.589 ± 0.405) animals, and not odor only animals (1.019 ± 0.513). Animals that did not receive any training and were subjected to nostril occlusions only showed NR1 levels around baseline at 3 h (1.253 ± 0.773) and 24 h (1.243 ± 0.297). A one-way ANOVA revealed significant differences between all groups ($F_{(5,41)} = 3.165$, $p < 0.05$; Fig. 8). Post hoc Fisher LSD tests showed that OS^+ 3 h pups expressed significantly less NR1 compared to both no odor 3 h animals ($p < 0.05$) and OS^- 3 h animals ($p < 0.01$). In addition, no odor 24 h pups showed significantly higher NR1 expression than OS^+ 3 h animals ($p < 0.05$), while OS^+ 24 h animals yielded significantly higher NR1 expression compared to OS^+ 3 h ($p < 0.05$) and OS^- 24 h animals ($p < 0.05$). Together, these results suggest that learning leads to NMDAR NR1 subunit expression down-regulation 3 h following odor conditioning and a compensatory up-regulation 24 h following OS^+ training. These results tie in with work done in the OB, which also found a transient down-regulation of bulbar NR1 subunits 3 h following training.

With respect to NR2b, there was a significant variation within the data. One-way ANOVA did not show significant differences between groups ($F_{(5,22)} = 2.314$, $p > 0.05$; Fig. 9). No odor 3 h animals (0.880 ± 0.241), no odor 24 h animals (1.251 ± 0.704), OS^- 3hr (1.926 ± 0.728), OS^- 24 h (1.829 ± 0.798) did not differ from baseline levels of NR2b

subunit expression. Animals that underwent OS^+ 3 hr training (0.594 ± 0.418) showed lower than baseline level of NR2b, while OS^+ 24 h (2.865 ± 0.721) yielded higher than baseline level of NR2b expression (compared to no odor and OS^- groups), although these did not reach statistical significance. It has been shown that NR2b can be degraded by an endogenous enzyme calpain in an activity dependent manner (Simpkins et al., 2003) and this leads to two bands detected at 170 kDa and 115 kDa. Our analysis was based on the 170 kDa as suggested by the manufacturer manual. However, the cleavage of NR2b by the endogenous calpain complicates the comparisons between groups underwent different activities (there were different degrees of activities therefore different degradations of NR2B among groups).

CHAPTER 4 – DISCUSSION

4.1 Summary of major findings

The present study sought to characterize the role of the anterior piriform cortex in early odor preference learning. First, we visualized cellular inactivity in the aPCX following local infusions of lidocaine or muscimol, both used for cell silencing. Once we established that there was less pCREB expression following such infusions, we examined the effects of transiently silencing cells using these agents in the aPCX prior to odor conditioning or prior to testing. Animals were unable to learn a preference for peppermint during pyramidal cell silencing during training, and were unable to recall these memories if the cells were inactive during testing. These results concur with previous studies that support a role for the aPCX in odor learning where odor-shock protocols elicited an increased *c-fos* expression in the aPCX, and not the pPCX, following conditioning (Roth and Sullivan, 2005).

To further characterize early odor preference learning in the aPCX, we examined the role of NMDAR activation during odor conditioning. NMDAR antagonist application in the aPCX prior to training prevented learning, however, blocking NMDARs prior to testing did not prevent the recall of the existing memory, indicating that NMDAR blockage did not interfere with odor perception. Next, we examined the role of β-adrenoceptor activation during odor conditioning. Animals were unable to learn if β-

adrenoceptor activation was blocked during odor conditioning, however, β-adrenoceptor activation in lieu of stroking was sufficient to induce learning.

Next, using CREB phosphorylation as a marker for neural activation, we confined learning to one hemisphere using a reversible nostril occlusion and compared pCREB expression in the learned hemispheres of pups that underwent odor training to those that were merely exposed to a novel odor. This confirmed that learning produces a more robust pCREB expression than simply odor perception. It has been suggested that sustained increases in pCREB expression trigger the intracellular signalling cascades that support long-term memory processes (McLean et al., 1999), suggesting that odor conditioning leads to a robust expression of early transcription markers implicated in learning and memory in the aPCX.

To complement our behavior data, we examined the plasticity mechanisms governing the LOT-aPCX synapse using a long-term potentiation model. We were able to induce LTP at the LOT-aPCX synapse that was dependent upon NMDAR activation. In addition, examining changes in AMPA input-output relationships following LTP, we found that a post-synaptic mechanism is strongly involved in the synaptic strengthening that is found following TBS. Together our behavior and electrophysiology results show that the aPCX is plastic and is critical for early odor preference learning and that this type of learning requires NMDAR activation.

Finally, we examined the NMDAR subunit expression modifications that occur as a result of learning. Western blotting of aPCX synaptoneurosomes revealed that 3 h following learning, NR1 subunit expression shows a marked down-regulation, which is

compensated at 24 h, when there is a significant NR1 subunit up-regulation. This may reflect a homeostatic process that NMDAR is up-regulated to "match up" with increased AMPA receptors at synapses. The data suggest that the NMDAR undergoes significant regulation during the different learning and memory phases, where down-regulation coincides with the initial phase of odor memory formation, the change is transient and reversible at the 24 h.

4.2 Evidence for aPCX as a critical locus in early odor preference learning

Odor-shock experiments have demonstrated that metabolic changes occur in the aPCX as a result of odor conditioning. Roth and Sullivan (2005) measured *c-fos* expression following learning, while Rainiki and colleagues (2009) measured 2-DG uptake following mild shock-odor conditioning. Both groups showed increases in neuronal activity specifically in the aPCX following learning, suggesting that this area may be implicated in odor learning in young rat pups.

As previously shown, lidocaine and muscimol infusions elicit transient cell silencing. In order to examine the effects of temporarily inactivating cellular activity in the aPCX during odor conditioning, pups received bilateral infusions of lidocaine or muscimol prior to training or testing. Such infusions prevented learning preferences for the novel odor. Thus, when the aPCX is not involved in the memory encoding or recall processes, early odor preference learning and/or memory expression is prevented. OB

and aPCX have extensive reciprocal projections, as there are heavy projections from the aPCX to the inhibitory granule cells in the OB. Both aPCX itself and feedback mechanisms to the OB were interrupted in the silencing experiments. Either way, the present experiments show that the aPCX plays a critical role in early odor preference learning and subsequent experiments served to delineate this role.

4.3 NMDAR activation in the aPCX is critical for early odor preference learning

The aforementioned experiments support a critical role for the aPCX in early odor preference learning. The prevailing view is that synaptic plasticity supports learning. A key player in synaptic plasticity is the NMDAR. The afferent synapses, but not associational synapses, exhibit an age- and activity-dependent decline in NMDAR-mediated LTP after the first postnatal month of life (Poo and Isaacson, 2009), when such synapses show higher LTP induction thresholds (Franks and Isaacson, 2005). Many forms of associative learning require NMDAR activation which is especially intriguing as it fulfills the Hebbian (1949) requirement of coinciding pre- and postsynaptic events that lead to synaptic strengthening. Thus, early odor preference learning may exhibit NMDAR activation requirements. Our results show that animals who receive bilateral infusions of an NMDAR antagonist into the aPCX prior to odor conditioning are unable to learn an odor preference. However, if animals received the infusion prior to testing, learning was intact. This ruled out the possibility that NMDAR blocking in the aPCX

interferes with odor perception. Thus, NMDAR activation in the aPCX as well as in the OB (Lethbridge et al., 2012) is required for early odor preference memory encoding.

Further electrophysiological experiments were performed to correlate with the *in vivo* behavioural study. Previous work has demonstrated an NMDAR-dependent LTP at the afferent synapses in the aPCX *in vitro* (Kanter and Haberly, 1990) and we were able to replicate such results by showing that LTP induction was not possible in the presence of D-APV.

4.4 β-adrenoceptor activation in the aPCX is necessary for early odor preference learning

During stroking, the LC releases NE, which has widespread projections to olfactory structures in the forebrain. This natural increase in β-adrenoceptor activation, when paired with presynaptic glutamate release, is the basis for associative learning in the OB model and here we test the same associative hypothesis in the aPCX model. In the OB, β-adrenoceptor activation has been proposed to have a critical role in initiating the learning-related cAMP/PKA/CREB cascade (McLean et al., 1999; Yuan et al., 2003). Direct infusion of a β-adrenoceptor agonist into the OB is sufficient to induce early odor preference learning in the rat pup (Sullivan et al., 2000b; Lethbridge et al., 2012). Both the OB and the aPCX are heavily innervated by NE fibers and are highly interconnected. Our results showed that blocking β-adrenoceptor activation with propranolol in the aPCX prior to odor conditioning prevented early odor preference learning, as these pups did not

learn a preference for peppermint when paired with stroking. This supports a critical role for β-adrenoceptor activation in both the OB and the aPCX in early odor preference learning.

4.4.1 β-adrenoceptor activation in the aPCX is sufficient to induce learning

As previously stated, β-adrenoceptor activation in the aPCX is necessary for early odor preference learning. That is, endogenous NE that is released during tactile stimulation plays a critical role in our learning model. This leaves us with the question of whether β-adrenoceptor activation is sufficient to induce learning. In other words, if we pharmacologically increase β-adrenoceptor activation during odor presentation, without tactile stimulation, will this induce learning? According our findings, β-adrenoceptor activation in the aPCX is also sufficient for early odor preference learning. We found that direct infusions of high concentration isoproterenol led to preference learning, however, this effect was blocked in the presence of propranolol, showing that these results were specific to β-adrenoceptors. These results are interesting because in the OB, isoproterenol works optimally at more moderate (e.g., 50 μM) concentrations (Lethbridge et al., 2012). This may relate to the more diffuse representation of odors in the aPCX in contrast to the OB where an effective moderate concentration would reach all or most of the odor encoding region. Importantly, this odor preference was induced independent of β-adrenoceptor activation in the OB. The present data suggest the isoproterenol-induced OB odor preference would not occur if β-adrenoceptors were blocked in aPCX.

In the OB, it is suggested that direct β-adrenoceptor activation may activate NMDARs on the postsynaptic membrane (Yuan et al., 2003). This may also enhance calcium influx through postsynaptic NMDARs that occurs naturally from novel odor presentation.

4.5 Cell silencing in pyramidal cell layer of aPCX

CREB phosphorylation has been shown as a reliable marker for cellular activation in the olfactory system (Lethbridge et al. 2012) and is causal in our early odor preference model (McLean et al., 1999; Yuan et al., 2000). In order to test the efficacy and longevity of agents known to transiently silence cells, lidocaine, a sodium channel blocker, was unilaterally infused into the aPCX. Either thirty minutes, or 24 h following infusions, pups were transcardially perfused, tissue was collected and immunohistochemistry (IHC) was performed to probe for pCREB expression. We found that there was significantly less pCREB expression in the aPCX pyramidal cell layer in the drug-infused hemispheres compared to saline-infused hemispheres 30 min following infusions. This was consistent with the report that the action of lidocaine lasts 30-60 min. One concern, however, is that the effects of silencing from lidocaine may be too far-reaching, and that it is a "messy" drug. Lidocaine is a weak sodium channel blocker and would also silence surrounding fibers (Bean et al., 1983). For that reason, muscimol, a robust $GABA_A$ antagonist, was used to create a cell specific temporary lesion. Comparing both drugs, Martin (1991) measured glucose uptake following drug infusions and found that lidocaine elicited a

greater radius of hypometabolism compared to muscimol, showing that lidocaine may spread further from the point of origin compared to muscimol. In our study, both drugs yielded similar results with respect to pCREB expression at 3 h and 24 h post-conditioning, thus lidocaine and muscimol appear suitable as reversible lesion inducers for future aPCX studies.

4.5.1 Learning-induced changes in pCREB expression

Learning induced pCREB increases were also found in the aPCX, consistent with a role of aPCX in early odor preference learning encoding. In the OB, tactile stimulation in the presence of a novel odor will elicit increased pCREB expression in neonate rats. This is thought to occur through β-adrenoceptor activation (elicited by stroking) along with increased levels of cAMP. This leads to a synergy of the calcium-enhanced adenyl cyclase activated by β-adrenoceptors and likely activation of NMDA receptors occurring in response to novel odor (Yovell and Abrams, 1992), which together lead to enhanced CREB phosphorylation (McLean et al., 1999). In order to view any differences in pCREB expression following odor conditioning with those in response to mere odor exposure, pups underwent a unilateral nostril occlusion that was paired with either OS^+ training or OS^- training. The nostril occlusion technique is useful for avoiding background odor confounds that may result between different animals and for which it can be difficult to control. IHC revealed that pups that underwent odor conditioning showed enhanced pCREB expression in the lateral aPCX region compared to odor only pups. These results

were specific to the lateral portions of the aPCX, where the medial portions did not differ among OS^+ and OS^- animals. It is unknown whether this learning-induced pCREB effect will occur if the experiment was performed after the critical period, after which synaptic maturation modifies such physiological properties as reduced synaptic plasticity at the LOT-aPCX synapses (Poo and Isaacson, 2007).

4.6 Evidence for aPCX plasticity in early odor preference learning

Previous work characterizing the synaptic properties at the afferent synapses in the aPCX has shown that LTP induction is possible with theta burst stimulation applied to the LOT fibers (Kanter and Haberly, 1990). Theta burst stimulation frequencies are employed here because they are thought to mimic sniffing patterns in the OB-aPCX circuitry (Kepecs et al., 2006). We investigated this at the LOT-aPCX synapse and, as expected, were able to induce LTP in the pyramidal cell apical dendrites. Moreover, we tried a stronger stimulation and found a more robust response following an 8 TBS protocol compared to the 1 TBS protocol. *In vitro* work done in our lab examined the effects of bath-applied isoproterenol on LTP expression at the LOT-aPCX synapse. Such application enhanced the LTP magnitude in response to the 8 TBS protocol. The decrease in PPR following isoproterenol suggests that this augmentation was due to a presynaptic event, such as increased excitatory neurotransmitter release (Morrison et al., 2013). These results suggest that the afferent synapses in the aPCX are capable of transient synaptic

modification and that neuromodulation can enhance this plasticity in pups in the sensitive period.

4.6.1 LTP-like changes governed by post-synaptic mechanism

LTP induction and expression may occur as the result of a presynaptic mechanism (i.e., more excitatory neurotransmitter release from presynaptic terminals), a postsynaptic mechanism (i.e., an increased efficacy for ligand binding, or simply more postsynaptic receptors), or both. In order to determine whether a postsynaptic mechanism is governing LTP induction at the LOT-aPCX synapse, one can compare the input-output profiles before LTP with those after LTP. An input-output compares the presynaptic response (fiber volley) with the postsynaptic response (EPSP slope) and in doing so, it was found that there is a postsynaptic change that occurs as a result of TBS. Work from our lab investigated the presynaptic component by comparing paired-pulse ratios prior to and 30 min following LTP induction. It was found that there was no change in PPR following LTP, thus ruling out a presynaptic expression mechanism governing LTP at the afferent synapse in the aPCX (Morrison et al., 2013). Taken together, these results suggest that the LTP expression mechanism is a post-synaptic phenomenon at the LOT-aPCX synapse. One possibility is AMPAR insertion into the postsynaptic membrane, therefore creating more binding sites for the presynaptic glutamate release. In the OB, if AMPAR insertion into the membrane is blocked prior to odor conditioning training, these animals do not display odor preference memory (Cui et al., 2011). Another possibility is increases

in binding efficacy at the postsynaptic membrane, where the same amount of presynaptic excitatory neurotransmitter release and postsynaptic binding will lead to a greater postsynaptic depolarization and ion influx.

4.7 NMDAR subunit modifications in the aPCX following early odor preference learning

The NMDAR itself is regulated age- and activity-dependently, and undergoes composition changes throughout development. For example, the NR2b, which is prominent at birth, is replaced by NR2a subunits as pups develop during the first few weeks of life (Quinlan et al., 2004). The absolute number of NMDAR or its composition can be altered by olfactory learning (Quinlan et al., 2004; Lethbridge et al., 2012). The increase in NR2a subunits over NR2b may occur in response to neuronal activity, which has been shown to increase NR2a transcription and increase synaptic delivery of NR2a-containing receptors (Hoffmann et al., 2000). Also, NR2b subunits are more susceptible to endocytosis compared to NR2a subunits, which may contribute to the increase in NR2a to NR2b over development (Lavezzari et al., 2004). Furthermore, as NR2a levels increase with respect to NR2b, there is less Ca^{2+} influx into the postsynaptic cell and less CaMKII availability (Erreger et al., 2005). This interferes with synaptic plasticity, as such an occurrence would raise the threshold for LTP. In this project, we tested whether learning would lead to NMDAR subunit modification in the aPCX. Specifically, we probed for NR1 subunits as this is the obligate NMDAR subunit. We found that when learning was

confined to one hemisphere there was a significant learning-specific down-regulation 3 h following odor conditioning. At 24 h post-training, however, there was a compensatory up-regulation. Animals that were presented with a novel odor without tactile stimulation, as well as those that only received nostril occlusions without odor presentation, did not show significant NR1 subunit modifications.

Experiments probing for NR2b subunit expression in the same animals were unsuccessful as the bands displayed substantial component separation. This is a possible limitation with the study, as Simpkins and colleagues (2003) describe an activity-dependent degradation of NR2b by calpain, an NMDAR-activated enzyme. This enzyme does not appear to cleave NR1 or NR2a, and thus seems specific to NR2b.The subunit remains on the cell surface following cleavage, and therefore would be incorporated into the synaptoneurosome preparation. NR2b is particularly interesting with respect to learning and synaptic plasticity because the proximal promoter region of the NR2b gene has a CREB binding site (Sasner and Buonanno, 1996), which, as mentioned, is implicated as a mediator of early odor preference learning and of long-term memory formation. Modifications in NMDAR subunit expression may lead to functional changes at the synapse, thus by studying changes that occur through development and learning we can further understand how neuronal functions are modified in the presence or absence of different subtypes. For example, NR2a-containing receptors typically open and close faster in response to glutamate compared to NR2b-containing receptors (Chen et al., 1999), which may modify NMDAR-mediated EPSPs. In addition, NR2b-containing receptors carry almost double the charge (Erreger et al., 2005) and carry more Ca^{2+} per

current unit (Sobczyk et al., 2005) compared to NR2a-continaing receptors. Thus, receptor composition is important in synaptic plasticity.

Our results suggest that at least the absolute number of NMDAR (indexed by NR1) show a down-regulation at 3 h however, at 24 h following odor conditioning, there is an up-regulation. The purpose of this up-regulation may be for balancing the AMPA to NMDAR ratio, thus re-establishing homeostatic balance in excitatory transmission.

4.7.1 NMDAR down-regulation may stabilize active synapses

NR1 is the mandatory subunit in a functional NMDAR, thus an NR1 down-regulation may reflect a down-regulation of functional NMDARs. This would coincide with an increased threshold for LTP induction and reduced synaptic plasticity, which occurs naturally as glutamatergic synapses in the PCX mature with sensory experience (Franks and Isaacson, 2005). NMDAR down-regulation stabilizes active synapses and reduces their plasticity for new memory encoding. Synaptic plasticity requires NMDAR activation, therefore NMDAR down-regulation in the PCX during early life may render important odors, such as those associated with maternal care, more salient by preventing synaptic interference from new odors experienced later. Taken together with work done in the OB (Lethbridge et al., 2012), it would appear that memories that do not require new protein synthesis (i.e., less than 3 h) (Grimes et al., 2012) produce a transient NMDAR down-regulation, which may occur as the result of lateral diffusion of the receptors out of the synaptic membrane or increases in NMDAR degradation

(Montgomery et al., 2005; Morishita and Malenka, 2008). Furthermore, since LTP induction thresholds are increased following experience-dependent loss of NMDARs, we might predict less LTP in slices taken from trained aPCX at 3 h. Similarly it might be more difficult for trained pups to acquire new information about odors at this time point, although the system should be 'normalized' with respect to plasticity at 24 h (Franks and Isaacson, 2005).

4.7.2 NMDAR up-regulation may balance the AMPAR to NMDAR ratio

It is thought that during early life, NMDARs predominate in synaptic transmission, however as circuits mature, AMPARs slowly contribute to much of the excitatory transmission until their eventual domination. AMPARs play a key role in learning and memory. Specifically, it is thought that AMPAR phosphorylation ultimately leads to AMPAR membrane insertion into the postsynaptic membrane, leading to structural scaffolding protein expansion at the synapse (Cui et al., 2011; Lynch and Baudry, 1984). Cui and colleagues (2011) showed an up-regulation in membrane AMPARs 24 h post odor conditioning in the OB glomerular layer. The up-regulation of NR1 subunits, and possibly NR2b subunits, 24 h following learning may reflect receptor recruitment to the synapse in order to "catch up" to the increase in membrane AMPARs, thereby maintaining an even AMPAR to NMDAR ratio, reflecting a maintaining of homeostasis in glutamatergic transmission in spite of an increased receptor density at the synapse.

4.8 Synergistic interactions between aPCX and OB support early odor preference learning

Feedback loops are ubiquitous throughout the cortex, and are prominent in the OB and PCX. As previously mentioned, the OB plays a critical role in early odor preference learning. Specifically, bulbar NMDARs mediate early odor preference learning (Lethbridge et al., 2012) and bulbar β-adrenoceptor activation is necessary and sufficient for this learning model (Sullivan et al., 2000b; Lethbridge et al., 2012). However, due to the highly interconnected circuitry between the aPCX and the OB, we have shown that although the OB plays a crucial role, if there is no activity in the aPCX, learning will not occur. Furthermore, cellular modifications confined to the aPCX are sufficient to induce learning, without an OB potentiation mechanism (Lethbridge et al 2012). This implies that OB potentiation mechanisms further strengthens PCX potentiation that is the critical site for odor perception and learning; but without it, a strong PCX potentiation mechanism is sufficient for learning to occur.

However, PCX feedback to the OB is still intact and may play a critical role in odor learning. Boyd and colleagues (Boyd et al., 2012) employed optogenetic techniques to create selective feedback projections from the aPCX to the OB in transgenic mice. Light-activated channelrhodopsin (ChR2) and fluorescent reporter protein, mCherry, were expressed specifically in the aPCX using adeno-associated viral vectors and produced brightly fluorescent labeled cells in the glomerular and granule cell layer in the ipsilateral OB, with weaker expression in the mitral cell layer and external plexiform

layers. This suggests that pyramidal cells in the aPCX input into two distinct stages of OB processing: 1) aPCX to periglomerular layer to external plexiform layer, and 2) aPCX to granule cell layer to mitral cell layer. When granule cells are activated they produce inhibition and will suppress MC and TC firing. This inhibition is thought to contribute to MC and TC recurrent and lateral inhibition (Halabisky and Strowbridge, 2003). Additionally, the weak direct MC activation from the aPCX elicited small excitatory currents that were not strong enough to elicit action potentials. Boyd and colleagues (2012) postulated that these were created from nonsynaptic sources and can be overcome by the strong inhibition from the granule and periglomerular layers. Neville and Haberly (2003) proposed that feedback from the aPCX is necessary for beta oscillations in the OB, which complements these findings. Furthermore, such feedback loops can promote cell firing synchrony between structures, which is critical for odor coding (Laurent, 2002) and possibly plasticity. MC and TC inhibition is important for controlling excitation integration timing, thereby fine-tuning MC and TC firing patterns during odor processing. This OB-aPCX feedback circuit is dynamic, as cortical feedback to the OB will change activity in the OB, which will modify activity in the aPCX, which will modify OB activity. Thus, in a way, the aPCX can modulate its own input and odor coding in the OB is regulated by the aPCX.

4.9 Conclusions and future directions

The goal of the current study was to first, determine whether the aPCX had a critical role in early odor preference learning, and then to characterize what that role is. Based upon the aforementioned experiments and results, we can conclude that the aPCX is involved in early odor preference learning. Until recently, the OB was considered the locus for this form of learning, however in light of recent research, it can now be assumed that both the OB and the aPCX play an integrative role in these processes.

Studying the fundamental processes that underlie learning and memory are key to solving some of the pathologies associated with interruptions in such processes. If one can determine what exactly a memory is, how it is stored, and it is recalled, then we can determine an intervention strategy for diseases such as Alzheimer's Disease, or memory problems associated with traumatic brain injuries or stroke. Thus, knowing how simpler systems process and encode information will provide us with a deeper understanding of how more complex brain processes work.

Future studies that expand on this work should continue to delineate the role of the aPCX in our learning model. For example, one may test the hypothesis that transient down-regulation of NMDAR subunits stabilizes synapses, thereby preventing further learning from occurring at the same synapse. This will allow us to further characterize the role of the NMDAR and its subunits at various synapses in the aPCX, such as associational synapses. In addition, one can study how AMPAR and NMDAR currents change following learning at the LOT to pyramidal neuron synapses in the aPCX, and also look at NMDAR and AMPAR phosphorylation in the aPCX following learning. Finally, it would be interesting to characterize the different types of learning (e.g.,

aversive learning) and explore the role of the aPCX and pPCX during different developmental time points.

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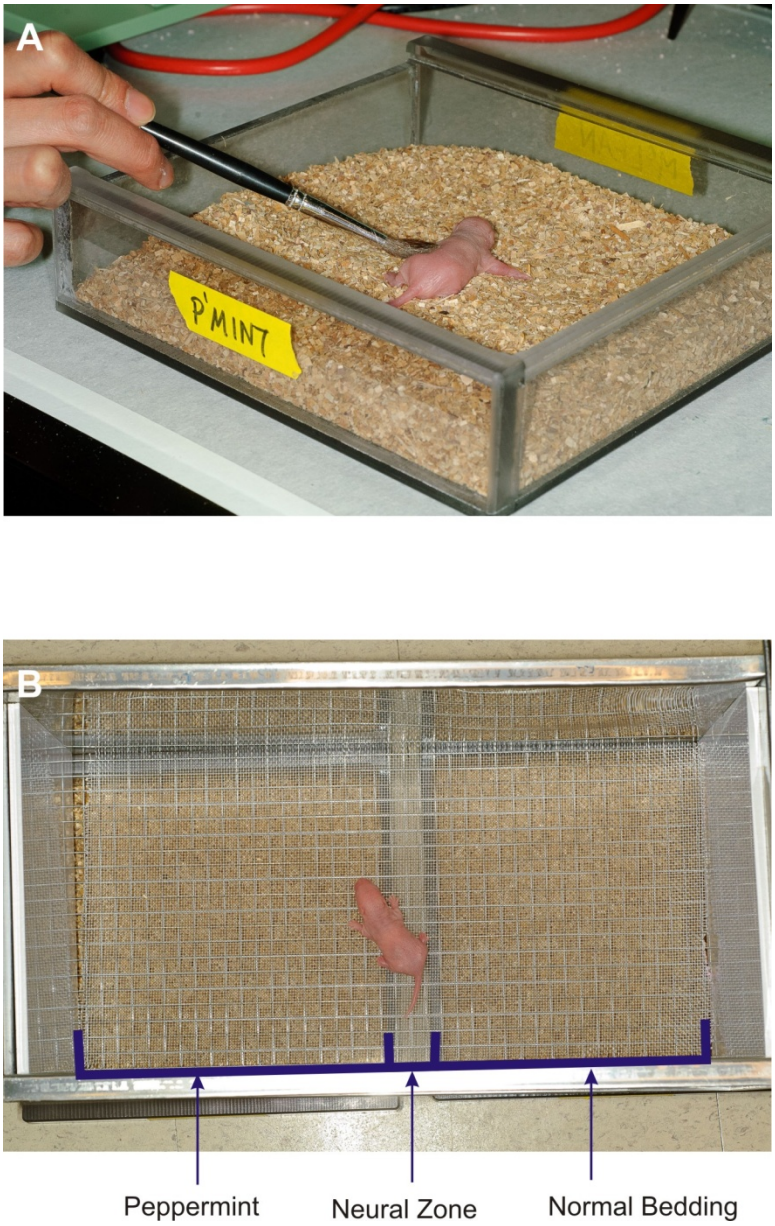


Figure 1. Early odor preference training and testing protocol.
A. For odor plus stroking training, on PD 7 pups are removed from the nest and placed on peppermint bedding. They are stroked with a paintbrush for 30 sec followed by 30 sec rest for 10 min total. ***B.*** On PD 8 pups undergo two choice odor testing. Pups are placed in the neutral zone between peppermint bedding and neutral bedding. They can roam freely for 1 min and are then removed for 1 min. This is repeated five times and the total amount of time over each bedding is recorded.

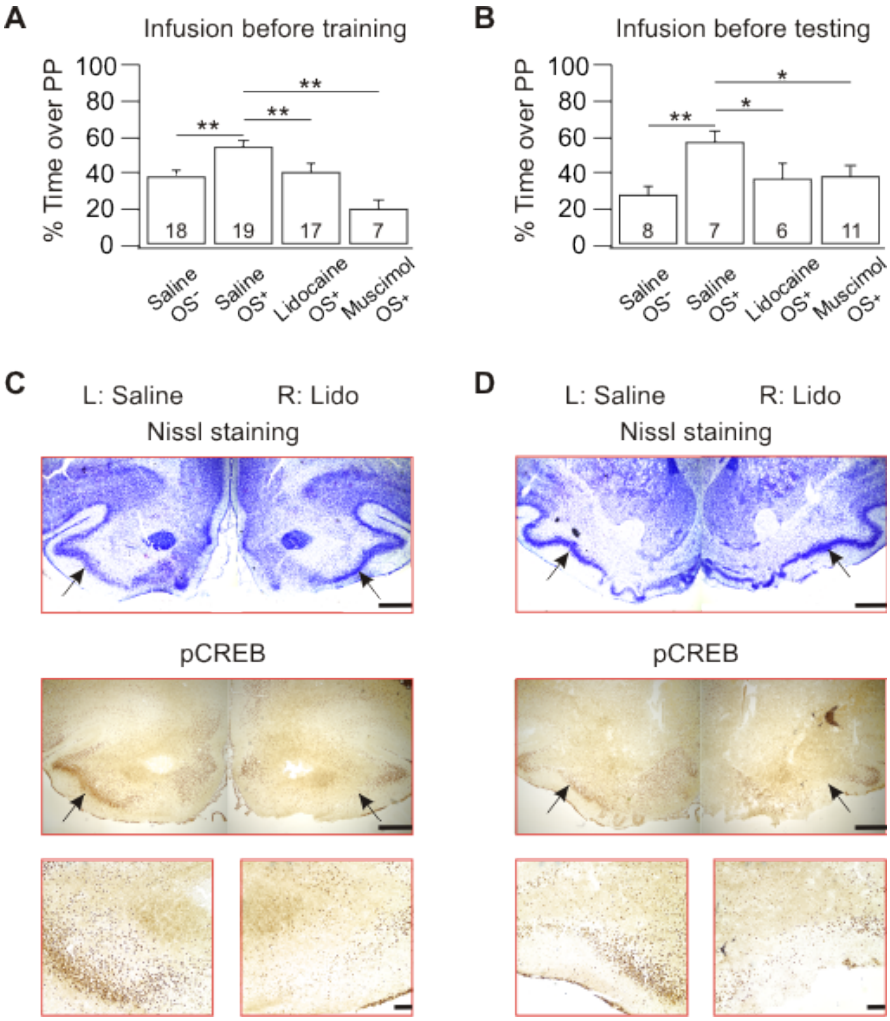


Figure 2. Piriform cortex silencing impairs early odor preference learning.

A. Lidocaine (4%) or muscimol (50 mM) infusions into the anterior piriform cortex 10 min prior to odor + stroke training produced less time spent over peppermint bedding compared to saline-infused counterparts. ***B.*** Lidocaine or muscimol infusion 10 min before testing prevented early odor preference learning. Bars represent percentage of time spent over peppermint-scented bedding during a two-choice test. ***C, D.*** Nissl staining and immunohistochemistry of pCREB expression in the piriform cortex 10 min following unilateral lidocaine injection. Note both *C* and D are from the same animal, *C* is rostral and *D* is caudal. Black arrow in low magnification indicate the pyramidal cell layer where less pCREB staining is evident following lidocaine injection compared to saline injection. High magnification is shown of a portion of the pyramidal cell layer. Scale bars, 500 µm for low magnification and 100 µm for high magnification. **p,0.01. *p,0.05. Error bars, mean ± SEM.

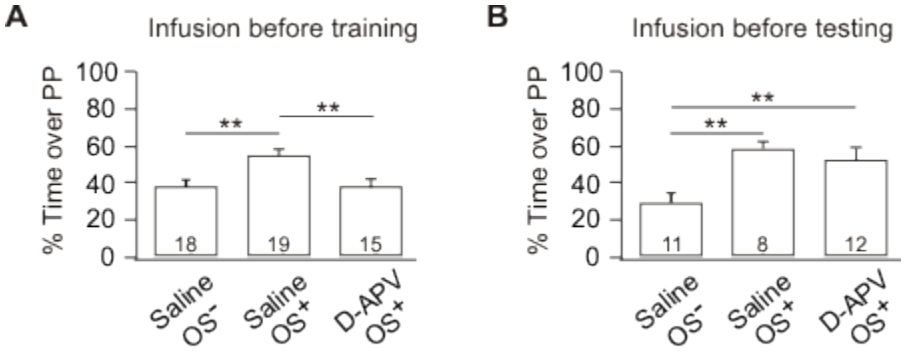


Figure 3. Learning is dependent on NMDARs in the piriform cortex.

A. NMDA receptor antagonist D-APV (100 μM) blocked early odor preference learning when infused 10 min prior to odor + stroke training. ***B.*** D-APV did not prevent learning when infused prior to two-choice odor testing, thus did not interfere with odor perception. Bars, percentage of time spent over peppermint-scented bedding in a two choice test. **p,0.01. Error bars, mean ± SEM.

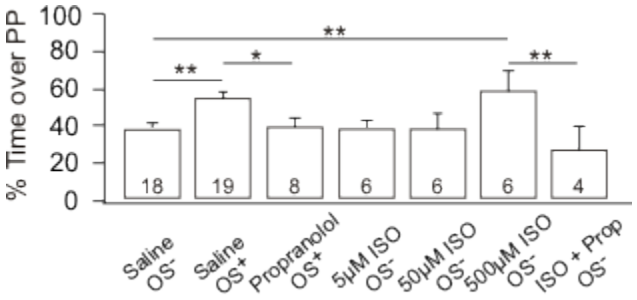


Figure 4. β-adrenoceptors in the piriform cortex are critical for learning. Blocking β-adrenoceptors with propranolol (100 μM) prior to odor + stroke training prevented early odor learning. Animals infused with 500 μM isoproterenol (ISO) during odor only training showed an increased preference for peppermint-scented bedding compared to lower ISO concentrations (5 μM & 50 μM) and saline-infused animals. Co-infusion of propranolol (100 μM) with ISO (500 μM) blocked the ISO-induced learning effect. Bars, percentage of time spent over peppermint-scented bedding in a two choice test. **p,0.01. *p,0.05. Error bars, mean ± SEM.

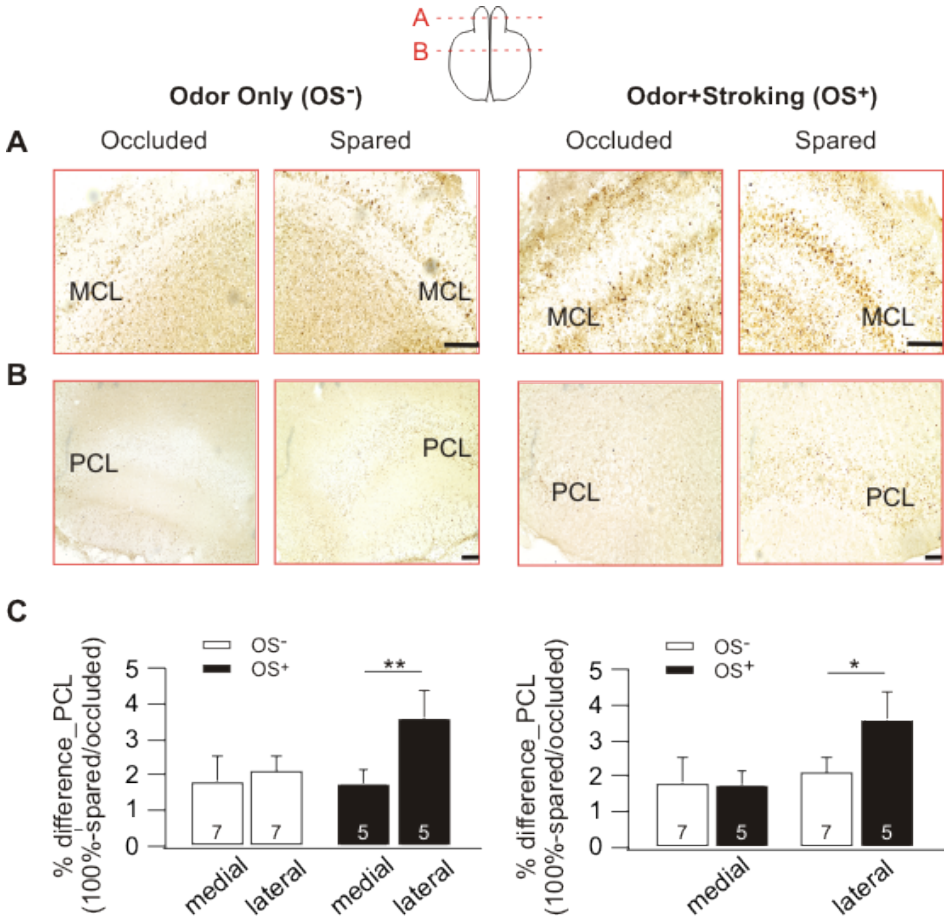


Figure 5. OS$^+$ enhances pCREB in the lateral region of the anterior piriform cortex. ***A.*** Immunohistochemistry (IHC) of pCREB expression in the mitral cell layer (MCL) in the olfactory bulb shows more staining in the spared bulb compared to occluded bulb in both odor only and odor + stroke animals. Overall, odor + stroke animals display more pCREB expression compared to odor only animals. ***B.*** pCREB IHC in the pyramidal cell layer (PCL) in the piriform cortex shows more staining in the spared hemisphere compared to the occluded hemisphere. Higher staining is shown in odor + stroke animals compared to odor only animals. Scale bars, 100 µm. ***C.*** Analysis of relative optic density of pCREB staining in PCL in spared and occluded hemispheres 10 min following either odor only or odor + stroke training shows more pCREB staining in odor + stroke animals, particularly in the lateral anterior PC. Bars, percentage differences in the PCL [100% - (spared/occluded)]. **p,0.01. *p,0.05. Error bars, mean ± SEM.

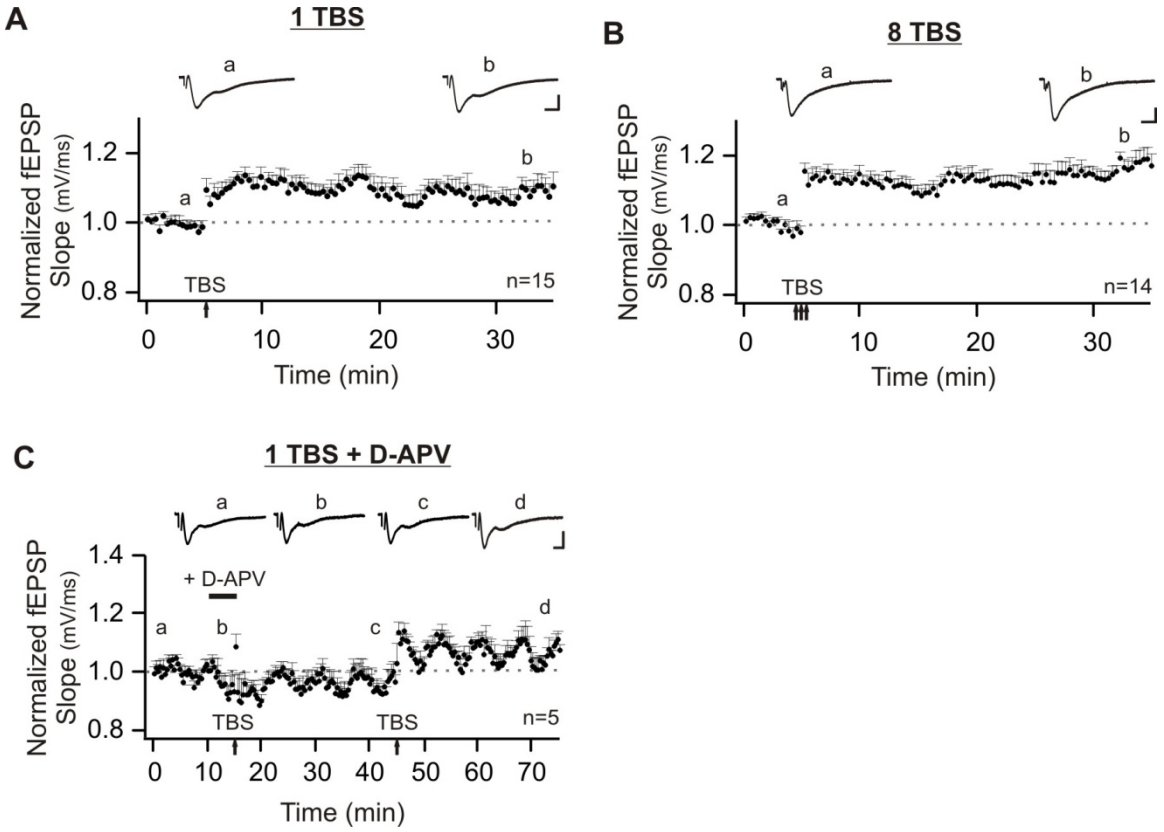


Figure 6. LTP at the LOT-pyramidal cell synapse *in vitro*.

A. Long term potentiation (LTP) was induced at the lateral olfactory tract (LOT)-piriform cortex pyramidal cell synapse following a 1 theta burst stimulation (TBS) protocol. ***B.*** 8 TBS produced a larger LTP at the LOT-piriform cortex synapse compared to 1 TBS protocols. ***C.*** NMDAR blockage using D-APV during TBS blocked LTP of the LOT-piriform cortex field EPSP. LTP was inducible with 1 TBS once D-APV was washed out.

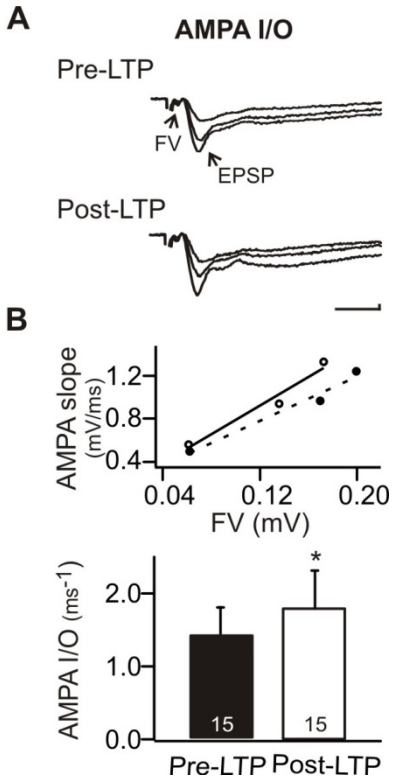


Figure 7. Post synaptic expression following *in vitro* LTP.

A. Traces from stimulation profile before LTP and after LTP. ***B.*** Isolation of AMPA components following LTP induction. The ratio of the change in presynaptic volley to the AMPA slope was greater post-LTP compared to pre-LTP. Broken line, pre-LTP. Solid line, post-LTP. 1 TBS is equal to 10 times 5HZ trains, each train is 5 pulses at 100Hz. *$p<0.05$ Error bars, mean ± SEM.

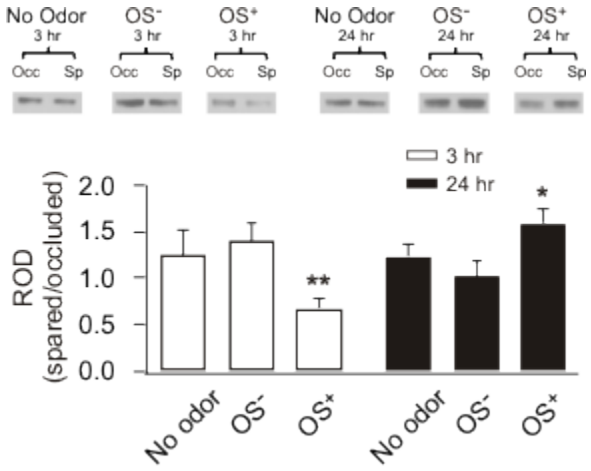


Figure 8. Learning-induced NR1 NMDAR subunit modifications in the aPCX. NR1 subunit expression of the NMDAR shows a significant down-regulation 3 hrs following OS^+ training. Twenty-four hours following OS^+ training there is a compensatory up-regulation in the NR1 NMDAR subunit.**p,0.01. *p,0.05. Error bars, mean ± SEM.

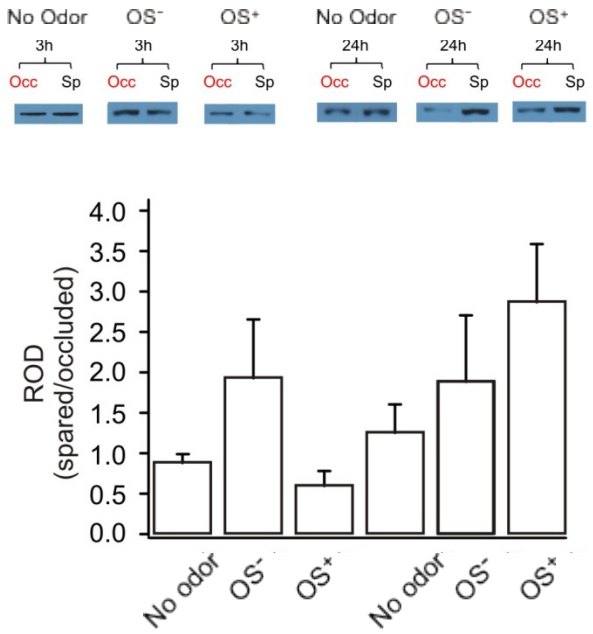
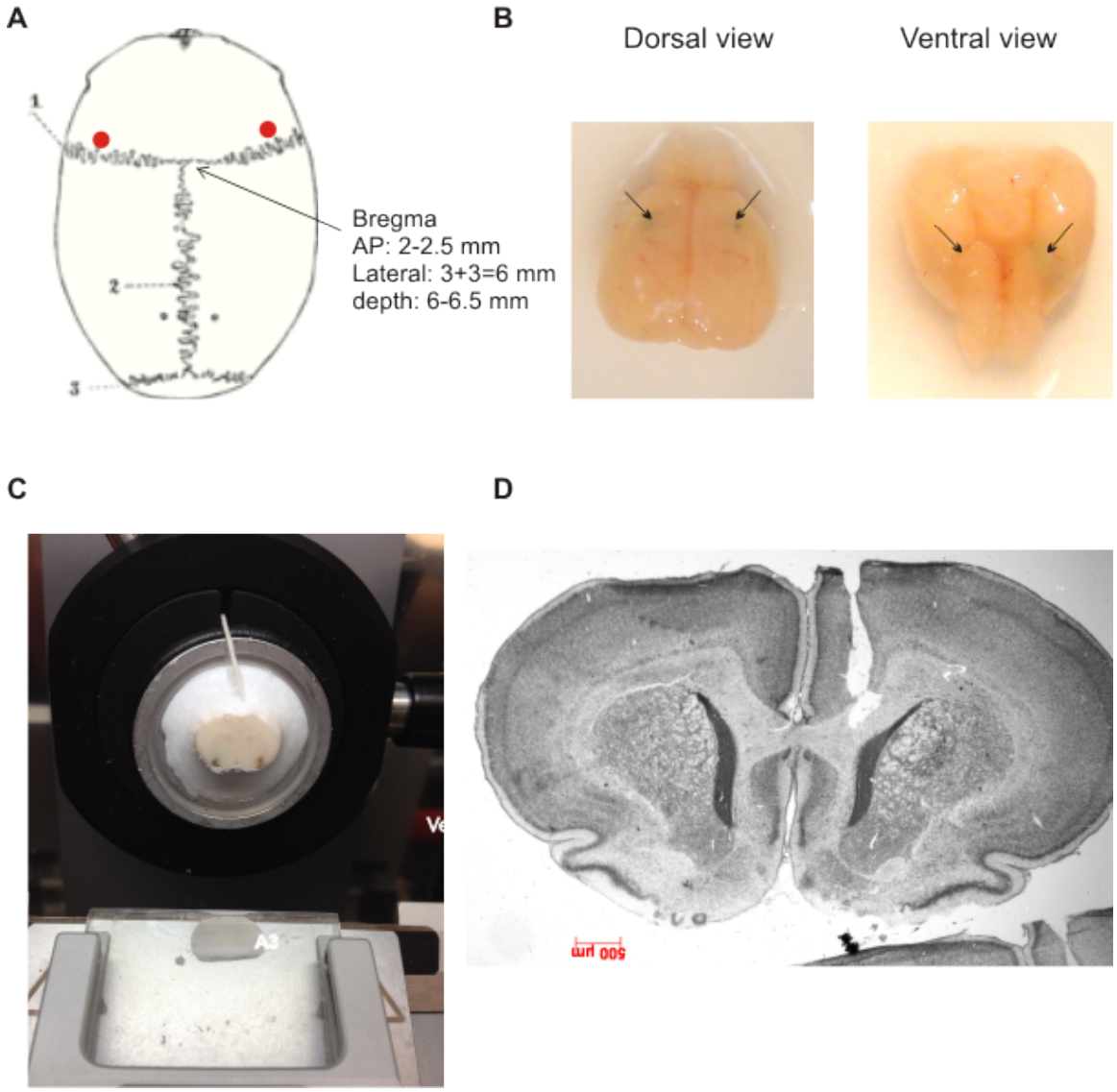


Figure 9. Learning-induced NR2b NMDAR subunit modifications in the aPCX. NR2b subunit expression of the NMDAR shows a possible trend of down-regulation 3 hr following OS^+ training and up-regulation 24 hr following OS^+ training. Error bars, mean ± SEM.

Appendix 1: Cannulae implantation configuration and confirmation



A. Schematic showing the positioning of implantation. ***B.*** An example brain with infused dye (arrows). ***C.*** Brain sectioning showing the dye locations. ***D.*** Corresponding level of ***C*** by Nissl staining.