THE ROLE OF L-TYPE CALCIUM CHANNELS IN EARLY ODOR PREFERENCE LEARNING

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by
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Abstract

These experiments provide evidence that L-Type Calcium Channels (LTCCs) are present in the neonate rat olfactory bulb, and that they are involved in the formation of early olfactory preference memories. Immunohistochemistry staining demonstrated that LTCCs are present in the olfactory bulb, with the highest concentration observed on mitral cell apical dendrites, and on periglomerular cells. Inhibition of LTCCs was sufficient to block early odor learning induced by an intrabulbar infusion of the β-adrenoceptor agonist isoproterenol. Stimulation of LTCCs in the olfactory bulb was not sufficient to induce an early olfactory learning event, but did succeed in rescuing isoproterenol-induced learning from a block of the NMDA receptor. Finally, an NMDA receptor block, but not LTCC block, was necessary to prevent learning induced by an infusion of the GABA receptor antagonist gabazine. These results support the theory that LTCCs contribute to the *conditioned stimulus* of early olfactory learning via the influx of calcium into the cell.

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α-AR	alpha-adrenoceptor
β-AR	beta-adrenoceptor
5-HT	Serotonin / 5-hydroxytryptamine
AC3	Adenylate cyclase type III
ACh	Acetylcholine
AKAP	A-kinase anchor protein
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AMPAR	AMPA receptor
AOB	Accessory olfactory bulb
AON	Anterior olfactory nucleus
ATP	Adenosine triphosphate
C-terminus	cytosolic terminus of the LTCC α1 subunit
cAMP	3',5'-cyclic adenosine monophosphate
CREB	cAMP response element binding protein
CNS	Central nervous system
CS	Conditioned stimulus
DCT	Distal C-terminus of the LTCC α1 subunit
EPL	External plexiform layer
ER	Endoplasmic reticulum
ET	External tuft
fEPSP	Field excitatory postsynaptic potential
GAB	Gabazine
GC	Granule cell
GL	Glomerular layer
GPCR	G protein-coupled receptor
IP3	Inositol triphosphate
IP3R	IP3 receptor
ISO	Isoproterenol
JG	Juxtaglomerular
LOT	Lateral olfactory tract
LTCC	L-Type voltage gated calcium channel
LTP	Long-term potentiation
MAPK	Ras/mitogen-activated protein kinase
MC	Mitral cell
mGluR	Metabotropic glutamate receptor
NE	Norepinephrine / Noradrenaline
NMDA	N-methyl-d-aspartate
NMDAR	NMDA receptor
NIM	Nimodipine
OB	Olfactory bulb
ON	Olfactory nerve
OR	Olfactory receptor
ORN	Olfactory receptor neuron

PCT	Proximal C-terminus of the LTCC α1 subunit
PKA	Protein kinase A
PKA	Protein kinase C
PND	Postnatal day
PG	Periglomerular
PPR	Paired-pulse ratio
RyR	Ryanodine receptor
SA	Short axon
SEM	Standard error of measurement
sTBS	Strong theta burst stimulation
TBS	Theta burst stimulation
TC	Tuft cell
TRPC	Transient receptor protein channel
UCS	Unconditioned stimulus
VGCC	Voltage-gated calcium channel

CHAPTER 1 - INTRODUCTION

1.1 Overview

When rat pups are born, they are deaf and blind. They must use olfactory cues to navigate through the world (Leon & Moltz, 1971). Early odor memories are developed via a simple classical conditioning response by pairing a novel odor (the conditioned stimulus) with one of many potential unconditioned stimuli, including warmth, feeding, tactile stimulation or chemical stimulation within the brain (Galef & Kaner, 1980; McLean et al., 1993; Sullivan & Leon, 1987; Sullivan & Wilson, 1994; Sullivan & Wilson, 2003; Sullivan et al., 2000; Wilson & Sullivan, 1990). Early olfactory learning in neonate rats, therefore, provides us with a simple biological system in which we can study the cellular processes that contribute to learning and memory.

Previous studies have demonstrated that β-adrenoceptor (β–AR) activation (Harley et al., 2006; Sullivan & Wilson, 1989; 1994; Sullivan et al., 2000), α-adrenoceptor (α-AR) activation (Harley et al., 2006) and calcium influx through calcium channels such as NMDA receptors (Cui et al., 2007; Lethbridge et al., 2012; Yuan, 2009) each contribute to the formation of early odor learning. The specific role of each action, however, remains unclear. It has been suggested that the effects of β–AR stimulation and calcium influx combine within the mitral cell of the olfactory bulh and result in the production of plasticity related proteins (Yuan et al., 2003b). In other brain regions, however, β–AR stimulation has been shown to modulate calcium flow into cells by regulating the function of L-type calcium channels (LTCCs; Davare et al., 2001; Marshall et al., 2011). LTCCs have previously been identified in neurons of the olfactory bulb (Schild et al., 1995; Tanaka et al., 1995). This raises the interesting possibility that the

effect of β–AR stimulation during early olfactory learning is to facilitate the flow of calcium into cells.

In this study, we asked if LTCCs are present in the olfactory bulb of neonate rats. We investigated whether LTCC activation is a necessary component of early olfactory learning, and/or if LTCC activation is sufficient to induce early olfactory learning. Using an *in vitro* approach, we attempted to determine if LTCC activation is a necessary component of the generation of mitral cell LTP. Finally, we investigated whether β–AR activation has any direct influence on the function of LTCCs and the flow of calcium into mitral cells of the olfactory bulb.

In this introduction I will present the structure of the olfactory bulb, and I will introduce the most significant neuromodulatory inputs to the olfactory bulb as well as one internally released neuromodulator (vasopressin) that has been the focus of recent work. I will then discuss ways that olfactory information can be processed, ranging from the rate at which an individual inhales through to cortical processing. I will then introduce the potential sources of intracellular calcium for cells in the olfactory bulb. I will review what is known about early odor preference learning, and present two models of early odor preference learning. Finally, I will review the experimental design of this study, and identify the major questions that this study set to answer.

1.2 Olfactory Circuitry

The olfactory bulbs (OB) are a pair of small oblong structures that sit on top of the cribriform plate of the ethmoid bone of the skull. The OBs receive primary sensory

information from olfactory receptor neurons (ORNs) in the olfactory epithelium, and transmit information to the olfactory cortex.

1.2.1 Olfactory Receptor Neurons

Mammals sense smells when odor signals from the environment interact with ORNs in the nasal cavity in an area called the olfactory epithelium. This small area of specialized epithelium (only 5 cm^2 in humans) contains several million ORNs (Morrison & Costanzo, 1990). ORNs are bipolar neurons, and are unique among neurons in that they have a very short lifetime of only 30-60 days, and are continuously being replaced by a basal layer of stem cells in the olfactory epithelium (Morrison & Costanzo, 1990; Graziadei & Monti Graziadei, 1979). ORN dendrites end in a knob-like structure with 10 – 30 cilia projections (Morrison & Costanzo, 1990). These cilia contain olfactory receptors (ORs), a form of G protein-coupled receptor (GPCR) that binds with odor signals (Jones & Reed, 1989). These ORN cilia form a dense mat, which is embedded in a mucus layer. The mucus absorbs odor signals from the air, and captures them so that they can bind effectively with ORs.

Odor signals come in the form of small molecules called odorants (for review see: Shepherd, 1994). Odorants are usually very small (less than 200Da) volatile molecules that are easily carried in the air into individuals' respiratory tracts, and which are easily absorbed into the mucus of the olfactory epithelium. A large multi gene family present both in rats and in humans encodes for over 1000 different forms of ORs (Buck & Axel, 1991). Some ORs (called "generalists") bind a wide variety of odor molecules while others (called "specialists") bind much more selectively. Each ORN only expresses one

form of OR, and *in situ* hybridization tests have shown that ORs are expressed relatively equally so that each of the 1000 forms of ORs is expressed in approximately 0.1% of ORNs (Ressler et al., 1994; Vassar et al., 1994). This massive diversity of ORs allows humans to distinguish between 5000 to 10,000 individual odors (Ressler et al., 1994; Shepherd, 1994).

When an odor molecule binds to an OR, this initiates a conformational change in the GPCR that activates a heterotrimeric G protein (Jones & Reed, 1989). This causes the release of the G protein's α-subunit, which then activates adenylate cyclase type III (AC3). AC3 catalyzes the conversion of adenosine triphosphate (ATP) to 3',5'-cyclic adenosine monophosphate (cAMP). cAMP facilitates the opening of cyclic nucleotide-gated cation channels in the membrane, which causes depolarization of the cell and the generation of an action potential (Bruch & Teeter, 1990; Jones & Reed, 1989).

ORN axons project from the olfactory epithelium through the cribriform plate and terminate in the OB (Morrison & Costanzo, 1990; Pinching & Powell, 1971a). As they travel away from the olfactory epithelium, these myelinated axons form bundles, which together are called the olfactory nerve (ON; Price & Sprich, 1975).

1.2.2 Layers of the Olfactory Bulb

The mammalian brain contains two OBs. Each OB is a highly organized structure with several distinct concentric layers. From the exterior of the OB moving inward, the layers of the OB are the ON layer, the glomerular layer (GL), the external plexiform layer (EPL), the mitral cell (MC) layer, the internal plexiform layer (IPL), the granule cell

(GC) layer and the subependymal zone (Pinching & Powell, 1971a; Pinching & Powell, 1971b; Pinching & Powell, 1971c; Price & Powell, 1970a; Price & Powell, 1970b).

1.2.2.1 Olfactory Nerve Layer

The ON layer is the most superficial layer of the OB (Figure 1). It is made up of the terminal ends of of ORN axons projecting from the olfactory epithelium.

1.2.2.2 Glomerular Layer

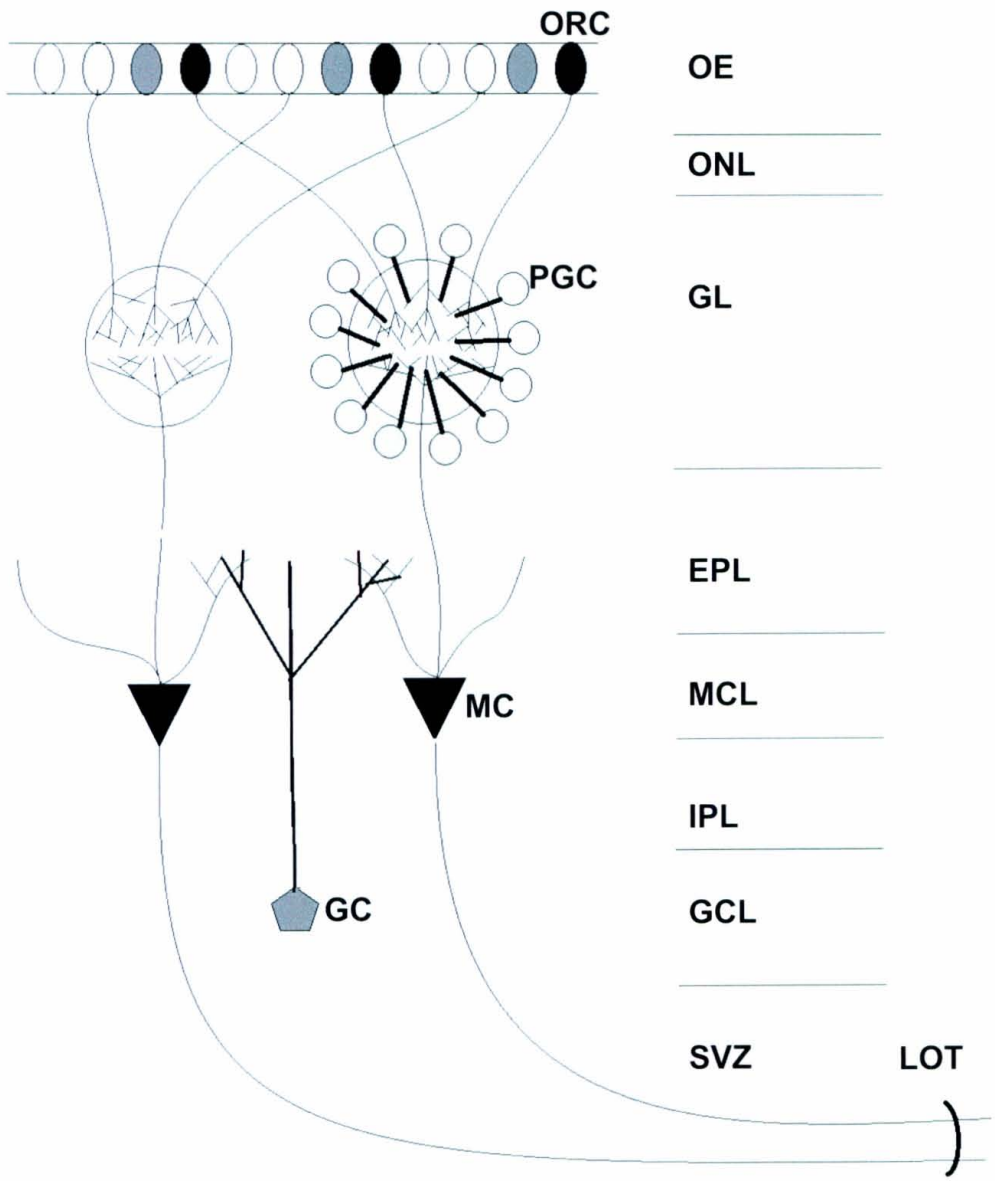
The GL of the OB lies deep to the ON layer (Figure 1). This layer contains round structures called glomeruli. Each bulb contains approximately 3000 glomeruli in rats (Meisami & Safari, 1981), and 1800 glomeruli in mice (Mombaerts et al., 1996). Glomeruli are composed of the highly branched distal dendritic tree of MCs and tuft cells (TCs). Here the MCs and TCs form synapses with the incoming ORN afferents. The outer boundary of each glomerulus is defined by a glial wrapping (Bailey et al., 1999; Kasowski et al., 1999; Pinching & Powell, 1971b).

Three types of small interneurons are present in the GL. These include periglomerular (PG) cells, external tufted (ET) cells and short axon (SA) cells (Pinching & Powell, 1971a; Pinching & Powell, 1971b; Pinching & Powell, 1971c). Together, these interneurons are referred to as juxtaglomerular cells (JG). The cell bodies of all JG cells are located in the PG space, but PG and ET cells project a primary dendrite into the nearest glomerulus. SA cell dendrites are confined to the PG space where they receive input from other JG cells. Together, JG cells form a complex network that mediates the responses within glomeruli to incoming signals via the ORNs.

Figure 1. Olfactory Bulb Circuitry

This cartoon of the olfactory bulb circuitry shows the various layers of the olfactory bulb structure, and highlights the cell types that are the focus of this study. EPL, external plexiform layer; GC, granule cell; GCL, granule cell layer; GL, glomerular layer; IPL, internal plexiform layer; LOT, lateral olfactory tract; MC, mitral cell; MCL, mitral cell layer; OE, olfactory epithelium; ONL, olfactory nerve layer; ORC, olfactory receptor cell; PGC, periglomerular cell, SVZ, subventricular zone

Figure 1



ET cells are excitatory interneurons that link activity of the ON and other JG cells (Hayar et al., 2004). ET cells receive monosynaptic ON input and, when activated, they release glutamate onto PG and SA cells. Inhibitory PG cells release GABA onto the ON terminals and MC distal dendrites within the same glomeruli, regulating intraglomerular inhibition (Murphy et al., 2005). SA cells project to neighbouring glomeruli, and primarily regulate interglomerular inhibition.

When ORNs are activated, they release glutamate onto the distal dendrites of MCs (Berkowicz et al., 1994; Ennis et al., 1996). MCs can pass this odor signal to other MCs within the same glomerulus by either releasing more glutatmate at dendro-dendritic synapses, or by electronic coupling via gap junctions between neighbouring MCs (Schoppa & Westbrook, 2001). The release of glutamate within glomeruli also stimulates the inhibitory PG cells, and, through them, the rest of the JG interneurons.

Up to several thousand ORN axons converge onto a single glomerulus in which they synapse with 15-20 MCs, resulting in a 100-fold decrease in the number of neurons transmitting olfactory information (Ressler et al., 1994). As previously mentioned, each ORN expresses only a single OR, and these receptors are randomly distributed within the olfactory epithelium (Ressler et al., 1994; Vassar et al., 1994). In-situ hybridization and radiolabelled 2-deoxy-D-glucose studies have confirmed that ORNs expressing similar ORs converge onto two, or at most a few, glomeruli in the OB (Falasconi et al 2012; Fletcher et al., 2009; Jourdan et al., 1980; Mombaerts et al., 1996; Ressler et al., 1994; Wachowiak & Cohen, 2001). This convergence means that individual glomeruli, and the MCs that innervate them, are activated downstream of the stimulation of only one type of OR. This demonstrates that the OB contains an odor response map, and opens up the

possibility that an odor response map may be consistent across individuals within the same species. Imaging studies have found evidence that supports this possibility (Vassar et al., 1994; Wachowiak & Cohen, 2001), however they have also demonstrated that the glomeruli-specific response to various odorants is highly variable and subject to factors such as the intensity of the odor stimulus (Fletcher et al., 2009; Wachowiak & Cohen, 2001) and the length of the stimulation (Smear et al., 2011).

Within each glomerulus, there is a subdivision of functional compartments. Immunoflourescence analysis and electron microscopy have both demonstrated that each glomerulus contains interdigitating, but segregated, axonal and dendritic subcompartments (Kasowski et al., 1999; Kosaka & Kosaka, 2005). Axonal subcompartments contain sparse dendritic processes, and are the site of axonal-dendritic synapses between ORNs and MCs and TCs. Dendritic compartments are completely free of axonal processes, and are the location of the majority of dendrodendritic synaptic connections between PG cells and MCs and TCs (Kasowski et al., 1999; Kosaka & Kosaka, 2005). Heterogeneous PG cells project differentially into these two types of glomerular subcompartments. Type 1 PG cells contain high amounts of GABA and dopamine along with its synthesizing key enzyme tyrosine hydroxylase. These cells project their dendrites preferentially towards the axonal subcompartments, and are also activated when ORNs release glutamate at ORN-MC synapses (Kosaka & Kosaka, 2005). Type 2 PG cells contain high amounts of the calcium binding proteins calretinin and calbindin D28K, and project their dendrites preferentially to the dendritic subcompartments of the glomerulus (Kosaka & Kosaka, 2005).

The PG region of the GL consists of the space in between individual glomeruli. This space contains the cell bodies and interglomerular thin dendrites of PG cells, the cell bodies and secondary dendrites of ET cells, the entirety of the SA cells, and the dendrites of MCs and TCs that are projecting towards glomeruli from deeper layers of the bulb (Pinching & Powell, 1971a; Pinching & Powell, 1971c).

1.2.2.3 External Plexiform Layer

The EPL of the OB lies deep to the GL (Figure 1). Secondary dendrites of MCs and TCs project laterally and form dendrodendritic synapses with inhibitory GCs in the deeper portion of the EPL (Price & Powell 1970b). These connections with GCs ensure that only strong odorant stimulants successfully activate MCs, and also serve as an opportunity to further process olfactory information as it is transmitted to the olfactory cortex (Abraham et al., 2010; Assisi et al., 2011). The EPL also contains the cell bodies of TCs (Pinching & Powell, 1971a).

1.2.2.4 Mitral Cell Layer

The MC layer of the OB lies deep to the EPL (Figure 1). This layer is only one or two cells thick, and contains the large cell bodies of MCs in a highly organized lamina (Price & Powell, 1970a; Price & Powell, 1970b). MCs are the largest cell type in the OB (Price & Powell, 1970a). From here, MCs project one primary dendrite to a single glomerulus in the GL, and multiple secondary dendrites obliquely through the EPL. MCs are the primary output neuron of the OB, and they project their axons deep into the OB. MC axons from the OB come together with TC axons from the OB and MC axons from

the Accessory OB (AOB) to form the lateral olfactory tract (LOT) on the olfactory peduncle (Price & Sprich 1975). The axons of the LOT project to the olfactory cortices (Price & Sprich, 1975).

1.2.2.5 Internal Plexiform Layer

The IPL of the OB lies deep to the MC layer (Figure 1). It is a very thin layer, and is sometimes recognized as the superficial component of the GC layer, especially in neonate rats whose bulbs are less developed. This region contains MC axon collaterals and GC dendrites that are projecting superficially (Price & Powell, 1970b) to the EPL.

1.2.2.6 Granule Cell Layer

The GC layer of the OB lies deep to the MC layer and the IPL (Figure 1). This layer contains the cell bodies of GCs (Price & Powell, 1970b). From here, GC primary dendrites project up to the EPL. Smaller GC secondary dendrites project deeper into the GC layer (Price & Powell, 1970b). This layer also contains cell bodies of SA cells (Price & Powell, 1970a; 1970b). These SA cells, which are morphologically and functionally different from the smaller SA cells found in the GL, are morphologically distinct from GCs as they have larger cell bodies, and dendrites that do not project beyond the GC layer (Price & Powell, 1970a). The GC layer also contains MC axons as they project towards the olfactory cortices.

1.2.2.7 Subependymal Zone

The subependymal layer is the deepest layer of the OB, lying deep to the GC layer (Figure 1). This region of the bulb surrounds the remnants of the rostral tip of the lateral ventricle (Price & Powell, 1970b), which is why it is also known as the subventricular layer. This layer only contains ependymal cells, glial cells, and the deepest dendrites of the deepest GCs (Price & Powell, 1970b). The subependymal layer acts as a source of neural progenitor cells that migrate superficially and develop into adult-born GCs and PG cells in the OB (Lois & Alvarez-Buylla, 1993; Luskin, 1993).

1.2.3 Neuromodulatory Inputs to the Olfactory Bulb

The OB receives neuromodulatory input from a wide variety of neurotransmitters (for review, see Fletcher & Chen, 2010).

1.2.3.1 Norepinephrine

The locus coeruleus contains the cell bodies of all noradrenergic cells in the brain, and over 40% of the afferent fibers from this structure project to the OB – far more than to any other structure in the brain (Shipley et al., 1985). The highest density of noradrenergic fibers in the OB is observed in the IPL. From here, the fibers project superficially to the EPL, GC layer and MC layer (McLean et al., 1989). The GL is the least innervated layer, and here most fibers terminate near the periphery of glomeruli without entering the glomerular structure (McLean et al., 1989). It is therefore assumed that these noradrenergic fibers terminate at synapses with PG cells on the periphery of glomeruli. The proportional distribution of noradrenergic fibers through the various

layers of the OB remains consistent throughout development (McLean & Shipley, 1991). The density of NE fiber innervation, however, increases throughout development into adulthood (McLean & Shipley, 1991). In newborn pups, approximately 200 neurons from the locus coeruleus project axons to the OB (McLean & Shipley, 1991), while in adult rats 400-600 noradrenergic fibers have been observed projecting to the bulb (Shipley et al., 1985).

Norepinephrine (NE) plays an important role in maintaining active levels of pCREB and the transcription factor c-Fos in many brain regions of awake animals. NE deprivation in awake individuals results in pCREB and c-Fos dropping to normal sleep-state levels (Cirelli et al., 1996). It has been suggested that the reduced activity of the locus coreulus, and the subsequent reduced activation of transcription factors and the effect on potentiation, may account for why learning does not normally occur during sleep (Cirelli et al., 1996).

NE has also been shown to play a crucial role in early olfactory learning (McLean & Harley, 2004; McLean & Shipley, 1991; McLean et al., 1989; Sullivan & Wilson, 1994; Sullivan et al., 1989; Sullivan et al., 1991; Wilson & Leon, 1988; Yuan et al., 2000). Natural learning involves NE release, and can be blocked with agonists of the NE-binding β-AR receptor. Alternatively, early olfactory learning can be induced in the absence of NE release by activating β-ARs pharmacologically (McLean & Harley, 2004; Sullivan et al., 1989; Sullivan et al., 1991; Lethbridge et al 2012).

1.2.3.2 Serotonin

Retrograde labeling has demonstrated that at least 1300 neurons project from the raphe nuclei to the mature adult rat OB (McLean & Shipley, 1987a). Co-fluorescence labeling demonstrated that the majority of these are serotonergic afferents. Interestingly, serotonin (5-hydroxytryptamine; 5-HT) fibers enter the OB via the ventral and medial portion of the ON layer and project into deeper layers of the bulb. 5-HT fibers terminate in the GL, EPL, IPL and GC layer. The density of 5-HT fibers that terminate in the GL is 2-3 times larger than in other layers of the bulb, and fibers that terminate here are larger than the fibers that terminate elsewhere in the bulb (McLean & Shipley, 1987a).

Neonate rat pups have very sparse 5-HT innervation in the OB (McLean & Shipley, 1987b). The density of 5-HT fibers innervating the bulb rises by PND 4-6, but the higher relative density of fibers in the GL doesn't occur until PND 14-16.

5-HT release in the OB appears to be a necessary component of early olfactory learning. 5-HT depletion blocks odor learning, and this effect is reversed by the introduction of 5-HT receptor agonists (Yuan et al., 2003b) or supra-optimal doses of β-AR agonists (Langdon et al., 1997). It has been proposed that the interaction between 5-HT stimulation and NE stimulation within MCs of the OB enhances the production of cAMP, which is critical for early olfactory learning (Yuan et al., 2000; Yuan et al., 2003b).

1.2.3.3 Acetylcholine

The majority of acetylcholinesterase synthesizing neurons that project to the OB originate in the nucleus of the horizontal limb of the diagonal band (Carson, 1984; Le

Jeune & Jourdan, 1993). All layers of the adult OB contain acetylcholine (ACh) axons, with the highest density expressed in the IPL, EPL and MC layer (Le Jeune & Jourdan, 1991). ACh carrying fibers are already present in the caudal end of the OB a few hours after birth. Innervation of the other areas of the bulb occurs rapidly over the first few days following birth. By postnatal day 17-20 the distribution of the ACh carrying fibers throughout the bulb is largely similar to the distribution observed in the adult bulb (Le Jeune & Jourdan 1991). A small subset of glomeruli on the dorso-medial surface of the OB receive an extremely high density of ACh carrying fibers (Le Jeune & Jourdan 1991, Le Jeune & Jourdan 1993). It has been proposed that these glomeruli are involved in early olfactory learning based on olfactory interactions between mothers and pups (Le Jeune & Jourdan 1991). Within these atypical glomeruli, the main neurons targeted by ACh innervation are PG cells.

It has been suggested that the effect of ACh release in the OB serves to enhance odor discrimination by narrowing the odorant receptive field responses of MC populations (Chaudhury et al., 2009). This would support the development of early odor memories by facilitating a greater discriminability of the learned odor in subsequent exposures (Fletcher & Chen, 2010)

1.2.3.4 Dopamine

There are no known dopaminergic projections to the OB. There is some evidence that a sub-population of PG cells produce dopamine and release it onto MCs via dendrodendritic synaptic connections (Harasz et al., 1977).

1.2.3.5 Vasopressin

Recent work has demonstrated a group of vasopressin producing cells in the adult rat OB (Tobin et al., 2010). These cells are located in the EPL, have a primary dendrite that extends into a single glomerulus, and multiple secondary dendrites that extend laterally to the zone around neighbouring glomeruli. Interestingly, these cells are glutamatergic. They are proposed to be a subpopulation of TCs (Wacker & Ludwig, 2011; Wacker et al., 2012) but unlike traditional TCs, they do not project outside of the OB (Tobin et al., 2010).

Tobin et al., also demonstrated that vasopressin has an important role in forming odor-based social recognition memories. The group interfered with vasopressin function by infusing a V1 receptor antagonist into the OB, or introducing a siRNA targeting V1a receptors. Both techniques successfully blocked the development of odor-based social memories (Tobin et al., 2010), and application of vasopressin or V1 antagonists onto the OB altered the firing pattern of local GCs, suggesting that vasopressin release in the OB may play an important role in filtering olfactory information based on social cues (Tobin et al., 2010; Wacker & Ludwig, 2011; Wacker et al., 2012).

1.2.4 Cortical Projections of the Olfactory Bulb

MC axons comprise the majority of fibers that project from the OB to the olfactory cortex. These axons join a small amount of TC axons from the OB, and axons of MCs from the AOB to form the LOT (Price & Sprich, 1975). The LOT forms on the olfactory peduncle, near the junction of the OB and the anterior olfactory nucleus. At its rostral end the LOT is spread out along the peduncle surface, and it assembles into a

tighter bundle as it progresses along the ventrolateral end of the peduncle. An examination of adult rat LOT cross sections, taken immediately caudal to the olfactory peduncle, revealed that the LOT carries approximately 42,000 axons towards the olfactory cortex (Price & Sprich, 1975). Near the caudal limit of the LOT, the tract carries approximately 38,000 axons.

The LOT projects to subcortical and cortical structures (for review see: Haberly 2001; Kandel et al., 2000). These include the anterior olfactory nucleus (AON), the piriform cortex and the olfactory tubercle, which together form the olfactory cortex. The LOT also projects to the amygdala and the transitional entorhinal cortex. MC axons from the OB project to all of these structures. TC axons from the OB project to the AON and olfactory tubercule, while MC axons from the AOB project only to the amygdala. Recent work (Payton et al., 2012) has suggested that the piriform cortex and the olfactory tubercle process olfactory information in parallel, despite major anatomical differences between their respective afferent innervations from the OB.

Brain regions that receive innervation from the OB project to many subcortical and cortical structures where olfactory information is consciously discriminated (Haberly & Price 1978 a,b). Some areas of the piriform cortex also project back onto the OB (Haberly & Price 1978 a,b). These projections are believed to regulate cellular activity in the OB, and therefore odor discrimination (Cohen et al., 2011; Martin et al., 2004; Martin et al., 2006; Sallaz & Jourdan, 1996). Specifically, it is has been shown that cortical projections terminate on GCs, where they exert control over GC activity patterns (Gao & Strowbridge, 2009) and c-fos expression in GCs (Sallaz & Jourdan, 1996). Cortical

projections have also been shown to regulate the activity patterns of other cells in the OB (Martin et al., 2004; Martin et al., 2006).

1.2.5 Olfactory Processing

Olfactory information is processed at multiple stages as it is transmitted along the olfactory pathway. The incoming information can be processed by changes to the sniffing pattern and the activation pattern of ORNs, as well as by modulation of MC activity via either intraglomerular or interglomerular systems in the OB (for review; Wilson & Mainzen, 2006).

1.2.5.1 Sniffing Rate

Since odorants are carried to the olfactory epithelium on air currents, the rate and volume of inhalations help control the timing and degree of odor sampling by ORs. While early research suggested that modified respiration during testing does not change the OB response to learned odors (Sullivan et al., 1988), more recent work has demonstrated that changes to sniffing patterns can have many effects on the OB response to presented odors (for review: Wachowiak, 2011).

By increasing either the rhythm or flow rate of inhalation, an individual is able to lower the detection threshold of odor signals in the air being sampled (Buonviso et al., 2006) by bringing a larger volume of odorant molecules in contact with ORs at the olfactory epithelium. Recent work has confirmed that respiration-related activation of cells in the CNS is regulated by the activation of ORNs, as opposed to by other brain regions that regulate breathing patterns (Buonviso et al., 2006, Wachowiak, 2011). Just

as ORN activation dynamics are coupled to inhalation frequency (Carey et al., 2009), post synaptic MCs show inhalation driven differences in response latency, rise time and duration (Carey & Wachowiak 2011; Courtiol et al, 2011; Wachowiak 2011). As the inhalation rate increases towards the range of active sniffing (5-10 Hz, theta frequency; Young & Wilson, 1999), MCs demonstrate an increased spike output and temporal precision (Balu et al., 2004). And when ORNs are stimulated in the sniffing range, inhibitory GCs demonstrate an increased synchrony and stronger inhibition of MCs (Young and Wilson, 1999; Schoppa, 2006), and the firing range of ET cells is altered from a spontaneous burst pattern into a pattern that is synchronous with the incoming ORN activation (Hayar et al, 2006). This sniffing-dependent regulation of various cell types within the OB initiates both intraglomerual and interglomerular inhibition and olfactory processing (Murphy et al., 2005; Isaacson and Strowbridge, 1998).

1.2.5.2 Intraglomerular Processing

Many cells types project axons or dendrites into glomeruli of the OB (Pinching & Powell, 1971a; Pinching & Powell, 1971b; Ressler et al., 1994). Most of these cells can modify the olfactory signal being carried through an individual glomerulus. Cellular interactions may increase or decrease the rate of vesicle release by ORNs (McGann et al., 2005; Aroniadou-Anderjaska et al., 2000), inhibitory interneurons may suppress the activation of MCs (Aroniadou-Anderjaska et al., 2000; Berkowick et al., 1994; Kasowski et al., 1999; Murphy et al, 2004; Murphy et al., 2005; McGann et al., 2005), or dendrodendritic connections may amplify and coordinate the timing of MC activation

(Najac et al., 2011; Nicoll & Jahr, 1982; Salin et al., 2001; Schoppa & Westbrook 2001; Yuan & Knopfel, 2006a;b).

Each ORN projects to a single glomerulus in the OB. When activated by an incoming odor signal, an ORN releases glutamate, which activates MC and PG cells inside the glomerulus (Aroniadou-Anderjaska et al., 2000; Berkowick et al., 1994; Kasowski et al., 1999; Murphy et al, 2004; McGann et al., 2005). Inhibitory PG cells can be activated by MCs within the same glomerulus (Murphy et al., 2005), and SA cells from neighbouring glomeruli (Aungst et al., 2003). When activated, PG cells release GABA directly onto MCs. PG dendrites do not form traditional synapses back onto ORNs, but ORN cells do express $GABA_B$ receptors (Bonino et al. 1999). This suggests that GABA is either released from PG cells at non-synaptic dendritic sites, or that dendritically-released GABA can travel to ORN dendrite terminals (Aroniadou-Anderjaska et al., 2000; Bonino et al., 1999). GABA release by PG cells leads to a decrease in glutamate vesicle release by presynaptic ORNs (Aroniadou-Anderjaska et al., 2000; Murphy et al., 2005; McGann et al., 2005; Wachowiak, 2005), and suppresses MCs activated by any remaining ORN glutamate release (Murphy et al., 2005; Schoppa & Westbrook, 2001).

Dendrodendritic connections between TCs and MCs within an individual glomerulus allow for the coordination and amplification of MC depolarization (Isaacson 1999; Najac et al., 2011; Nicoll & Jahr, 1982; Salin et al., 2001; Schoppa & Westbrook, 2001; Yuan & Knopfel, 2005). TCs respond to weak ORN stimulation, even ORN stimulation that is not strong enough to generate direct MC activation (Najac et al., 2011). TCs are then capable of activating MCs via dendrodendritic synapses. This

feedforward activation by TCs can summate with direct ORN stimulation of MCs to reach the activation threshold for MCs in response to weak ORN activation (Najac et al., 2011; Yuan & Knopfel, 2005). When MCs are activated directly by a strong ORN stimulation, feedforward excitation through dendrodendritic connections with other MCs and TCs generates a biphasic EPSC in MCs, in which the fast component is generated by direct ORN input and the slow component is the result of feedforward excitation (Najac et al., 2011). This serves to amplify the incoming odor signal (Najac et al., 2011). In these experiments by Najac et al., (2011), NMDARs were blocked with D-APV, so the recorded responses were all AMPA-mediated EPSPs.

It has been proposed that MC self-excitation may serve to increase the signal-to-noise ratio by amplifying active inputs from ORNs, which may improve the response selectivity of cortical neurons responding to olfactory input (Salin et al. 2001). Dendrodendritic connections and gap junctions also serve to increase sensitivity to the odor information encoded in incoming olfactory signals, and couples the response of all MCs projecting from a glomerulus so that the MCs act as a functional unit in processing olfactory information (Christie et al., 2005; Schoppa & Westbrook, 2001; Yuan & Knopfel, 2005).

1.2.5.3 Interglomerular Processing

Olfactory processing occurs laterally within the OB between cells associated with separate glomeruli via connections in both the GL and GC layer of the bulb. At the GL, SA cells project their axon between glomeruli, where they inhibit presynaptic release by ORNs and postsynaptic depolarization of MCs (Aungst et al., 2003; Pinching & Powell,

1971c; Price & Powell, 1970a). At the GC layer, MC secondary dendrites extend laterally to form dendrodendritic synapses with each other or with GCs (Isaacson & Strowbridge, 1998; Parrish-Aungst et al., 2010; Price & Powell, 1970b; Rall & Shepherd, 1968).

SA cells, despite their name, actually have quite long axons that can project up to 850 μm through the GL (Aungst et al., 2003). For a point of reference, glomeruli have a diameter of 50 – 100 μm (Aungst et al., 2003). These cells have 3-5 short dendrites that connect to 2-4 separate glomeruli. Like other JG cells, SA cells are activated by glutamate release from ORNs. SAs express both GAD-67 derived GABA and dopamine, unlike PG cells which express only GAD-65 derived GABA. (Aungst et al., 2003; Kosaka et Kosaka, 2007; Parrish-Aungst et al., 2011). SA cells therefore exert an inhibitory influence on neighbouring glomeruli that surround their home glomerulus. This leads to an on-centre, off-surround processing of olfactory information (Aungst et al., 2003). It has been demonstrated that sensory deprivation leads to a reduction in GABA and dopamine synthesizing enzymes in SA cells (Cho et al., 1996; Parrish-Aungst et al., 2011), but not PG cells (Parrish-Aungst et al., 2011). This likely leads to decreased levels of neurotransmitter release by SA cells and therefore less SA-mediated inhibition in neighbouring glomeruli, which would increase the sensitivity and decrease the selectivity of those glomeruli.

GCs form dendrodendritic synapses with laterally projecting MC secondary dendrites (Price & Powell, 1970b; Rall & Shepherd, 1968). GCs are activated when backspreading action potentials stimulate glutamate release from the MC lateral dendrite, which binds to GC-bound NMDA receptors (Abraham et al., 2010; Chen et al., 2000).

The GABAergic GCs induce both feedback inhibition onto the activated MC, as well as inhibition of neighbouring MCs (Rall & Shepherd, 1968; Shepherd et al., 2007; Yokoi et al., 1995). The importance of GC inhibition is believed to be in refining the specificity of each glomerular unit. First proposed by Rall & Shepherd (1968), this model assumes that adjacent glomeruli in the OB respond to the presence of similar but slightly different odorant molecules. While an odorant will generate the strongest signal in its proper target glomerulus, it could evoke a small response in neighbouring glomeruli that target similarly shaped odorants. The effect of GC inhibition is to suppress the weak stimulation generated in neighbouring glomeruli ensuring that only MCs from the correctly targeted glomerulus sends information onto the cortex (Rall & Shepherd, 1968; Shepherd et al., 2007; Yokoi et al., 1995). This helps an individual discriminate quickly and accurately between similar odors (Abraham et al., 2010).

Recent work has demonstrated that canonical transient receptor potential channels (TRPCs) are activated on GCs following NMDAR activation (Stroh et al., 2012). TRPC activation plays a critical role in generating a long lasting depolarization response in GCs, which is believed to be responsible for the asynchronous component of GC inhibition (Chen et al., 2000; Isaacson & Strowbridge, 1998; Strohl et al., 2012).

1.2.5.4 Odor Processing by the Olfactory Cortex

Once odor information is conveyed by MCs and TCs to the olfactory cortex, the information is transformed in a variety of ways that alters the conscious perception of the odor. Odor processing within the olfactory cortex involves interactions among many subregions of the cortex (for review; Wilson & Sullivan, 2011).

Due to the extensive innervation of the olfactory cortex back onto the OB (Haberly & Price 1978a,b), which terminates primarily on inhibitory GCs (Gao & Strowbridge, 2009), odor learning often involves top-down regulation of cellular activity in the OB by the olfactory cortex (Cohen et al., 2011; Martin et al., 2004; Martin et al., 2006; Sallaz & Jourdan, 1996). Cortical projections have been shown to regulate the firing patterns of cells in the OB in response to odor presentation (Martin et al., 2004; Martin et al., 2006), as well as the expression of the proto-oncogene *c-fos* in GCs, which is an indirect measure of increased cellular activity (Sallaz & Jourdan, 1996).

1.3 The LTCC

Calcium entry into cells of the OB plays a crucial role in both the transmission of olfactory information and activation of intracellular plasticity-related processes involved in forming an olfactory memory. Extracellular calcium enters the cell in a controlled fashion by moving through calcium channels. We hypothesize that the L-type calcium channel (LTCC) is involved in this process.

1.3.1 LTCC Structure and Function

Voltage-gated calcium channels (VGCCs) are membrane bound proteins responsible for calcium currents found in all excitable cells (Tuckwell, 2011). VGCCs, and the currents that result from their activation, are subdivided into 5 subgroups; T (for "transient"), L (for "long-lasting)", P/Q (for "Purkinje"), R (for "resistant") and N (for either "neuronal", or "neither T nor L") (Dolphin, 2006; Dolphin, 2009; Tuckwell, 2011). Of these, the T-subtype is considered low-threshold/low voltage activated, while L, P/Q,

R, and N are considered high-threshold/high voltage activated channels (Catterall, 2000; Tuckwell, 2011).

All VGCCs are comprised of up to four subunits. The principal conducting pore of the channel is the $\alpha 1$ subunit, of 190 kDa. This protein contains about 2000 amino acids. The protein has four repeated domains (I through IV), each of which contains six transmembrane segments (S1 through S6) and a membrane-associated loop between S5-S6 (Catterall, 2000). The other subunits are the extracellular $\alpha 2\delta$ dimer of 170 kDa, the intracellular β subunit of 44 kDa, and the transmembrane γ subunit of 33 kDa (Catterall, 2000; Takahashi et al., 1987).

There are 10 different forms of the $\alpha 1$ subunit, and these are used to define and identify 10 distinct subgroups of VGCCs (Catterall, 2000; Dolphin, 2006; Dolphin, 2009; Tuckwell, 2011). The category of LTCCs contains four subtypes, known as $Ca_v1.1$ - 1.4. $Ca_v1.1$ is found mainly in skeletal muscle, $Ca_v1.4$ is found primarily in retinal cells, and $Ca_v1.2$ and 1.3 are found in cardiac cells and neurons (Catterall, 2000; Hell et al, 1993; Tuckwell, 2011) including neurons of the OB (Schild et al., 1995; Tanaka et al., 1995).

The LTCC unit functions by undergoing a conformational change in response to depolarization of the membrane in which it is bound (Catterall 2000; Dolphine 2006; Dolphine 2009; Tuckwell, 2011). A transmembrane segment of the $\alpha 1$ subunit serves as the voltage sensor for the unit. This segment moves outwards and rotates when the membrane is depolarized, which initiates a conformational change in the rest of the $\alpha 1$ subunit, effectively opening up the channel so that calcium ions are free to flow along their electrochemical gradient from the extracellular space into the intracellular space (Catterall 2000; Dolphine, 2006). Each of the VGCC subgroups has unique activation

thresholds and properties, which themselves are also variable given the specific chemical environment of the cell (Dolphine, 2009; Tuckwell, 2011).

In the CNS, LTCCs are expressed in a wide variety of neurons (Tuckwell, 2011; West, 2001). LTCC function has been implicated in the amplification of synaptic input (Bui et al., 2006; Dixon et al., 2012; Grande et al., 2007), including in ORNs in response to odor signal stimulation (Gautam et al., 2006; Trombley & Westbrook, 1991). Calcium influx via presynaptic VGCCs, including LTCCs, has been implicated in the regulation of vesicle release (Mercer et al, 2011; Neher & Sakaba, 2008; Silva et al., 2012). LTCC function has also been linked to the activation of transcription factors in neurons throughout the CNS (Catterall, 2000; Dolmetsch et al., 2001; Impey et al 1996; Marshall et al 2011; Murphy et al 1991; Satin et al., 2011; Tuckwell 2011; West et al 2001), but especially in the hippocampus where LTCC-regulated gene transcription is believed to be related to synaptic plasticity and memory formation (Fisher & Johnston, 1990; Holmgaard et al., 2008; Impey et al., 1996; Lacinova et al., 2007).

Calcium influx via LTCCs activates intracellular signaling cascades, which are responsible for initiating other activities within the cell. The cytosolic region of the LTCC contains a binding site for the calcium-binding messenger protein calmodulin, and successful calmodulin binding is necessary to initiate intracellular signaling. This demonstrates that the LTCC is directly involved in activating signal pathways to the nucleus, rather than just providing a source of calcium for separate processes (Dolmetsch et al., 2001). Once calmodulin senses the presence of calcium, it activates the Ras/mitogen-activated protein kinase (MAPK) pathway, which leads to the activation of cAMP and the phosphorylation of cAMP response element binding protein (CREB)

(Dolmetsch et al., 2001; Impey et al., 1996; Marshall et al., 2011; Murphy et al., 1991; West et al., 2001). CREB is a transcription factor that modulates the transcription of genes that include the cAMP response element in their promoters (Silva et al., 1998). Increases in cAMP and CREB phosphorylation have been implicated in the transcription of intermediate early genes involved in synaptic plasticity and long-term learning (McLean & Harley, 2004; McLean et al. 1999; Cui et al., 2007; Silva et al., 1998; Yuan et al., 2003a).

1.3.2 LTCC Distribution

LTCCs are membrane bound proteins, anchored in the phospholipid bilayer with both intracellular and extracellular components. LTCCs have been found to be located primarily on the soma and proximal dendrites of neurons (Westenbroek et al., 1990; Hell et al., 2003), specifically cultured neurons from the OB (Schild et al., 1995). This location close to the cell body supports the theory that calcium entry via LTCCs is involved in intracellular signaling to the nucleus (Dolmetsch et al., 2001; Impey et al., 1996; Marshall et al., 2011; Murphy et al., 1991).

In some neurons, LTCCs have also been implicated in regulating intracellular calcium levels at presynaptic terminals, which partly controls transmitter release at the synapse (Neher & Sakaba, 2008; Mercer et al., 2011). Some evidence suggests that LTCCs play this role in ORNs (Trombley & Westbrook, 1991) where they are present at the presynaptic cleft (Mercer et al., 2011).

1.3.3 LTCC Regulation

The $Ca_v1.2$ and 1.3 forms of LTCCs play an important role in the regulation of pacemaker activity in heart muscle cells (Hell, 2010; Zhang et al., 2011). Up-regulation of LTCCs in heart cells has been implicated in the "fight-or-flight" response in vertebrates (Fuller et at, 2010; Hell, 2010; Hulme et al., 2006). In cardiac muscle cells, LTCCs exist in a receptor-channel complex along with β-ARs, a type of metabotropic GPCR, and adenylyl cyclase (Fuller et al., 2010; Hell, 2010). In the fight-or-flight response, NE stimulates β-ARs, which in turn stimulate LTCCs to allow more calcium into the cell. This leads to a stronger compression of the heart (Bean et al., 1984; Dolphin 2009; Fuller at al., 2010; Hell 2010; Hulme et al., 2006). Recent work has suggested that LTCC-β-AR complexes are present in some neurons, and that a similar regulation of LTCCs by β-ARs may be at play in these cells (Davare et al., 2001; Gray & Johnston, 1987; Marshall et al., 2011). To date, it is unknown if such complexes occur in the OB.

When excess calcium is present in the intracellular space, the calcium-activated protease calpain cleaves the cytosolic-terminus (C-terminus) of the LTCC α1 subunit at residue 1800 (Hulme et al., 2005). The free distal end of the C-terminus (DCT) interacts with the proximal C-terminus (PCT), causing a conformational change in the α1 subunit that inhibits the flow of calcium through the LTCC during subsequent voltage-driven activations of the channel (Hell, 2010; Hulme et al., 2005). Calcium influx from NMDARs is sufficient to induce calpain cleavage of the α1 subunit C-terminus in hippocampal neurons (Hell et al., 1996). Activation of the β-AR portion of the LTCC-β-

AR complex relieves this inhibition (Bean et al., 1984; Davare et al., 2001; Fuller at al., 2010; Gray & Johnston, 1987; Hell 2010; Hulme et al., 2006; Marshall et al., 2011).

The LTCC-β-AR complex is activated *in vivo* when NE binds to the β-AR unit. This binding activates the β-AR-bound adenylyl cyclase, which converts ATP into cAMP (Hell, 2010). As the local concentration of cAMP increases, cAMP molecules bind to the regulatory subunits of endogenous PKA (protein kinase A), which leads to the release of the catalytic PKA subunits. The active PKA binds to A-kinase anchor protein (AKAP), which anchors the PKA onto the C-terminus of the LTCC's α1 subunit (Gao et al., 1997; Hell et al., 2010; Marshall et al., 2011). This binding stimulates phosphorylation of the Ser1928 site on the DCT, which undergoes a conformational change (Gao et al., 1997; Hulme et al., 2006). This relaxes the interaction between the DCT and PCT, which relieves the inhibitory effect of the DCT on the PCT and the channel activity. Finally, the release of this inhibition allows an increased level of calcium to enter the cell the next time the LTCC is activated, providing a stronger calcium signal to initiate intracellular cascades via MAPK and other potential calcium-activated pathways (Davare et al., 2010; Fuller et al., 2010; Gao et al., 1997; Hell, 2010; Marshall et al., 2011). The resulting increase in LTCC efficiency from the relief of this inhibition is in the range of 100-300% in cardiac heart cells and neurons (Davare et al. 2010; Gao et al., 1997).

1.4 Other Calcium Sources

1.4.1 NMDARs

1.4.1.1 NMDAR Structure and Function

The NMDAR is an ionotropic glutamate receptor. There are three possible subunits for the receptor, which include the GluN1 subunit, a family of four distinct GluN2 subunits (A through D), and two GluN3 subunits (A and B; see Cull-Candy et al., 2001; McBain & Meyer, 1994; Collingridge et al., 2009). Each subunit type has multiple isoforms, owing to alternative splicing and post-translational modification. Functional NMDARs are heteromeric complexes containing GluN1 and some form of GluN2 subunits. The GluN3 subunits cannot combine to form a functional receptor on their own, but they do combine with GluN1/GluN2 subunit complexes (Cull-Candy et al., 2001).

The subunit composition of the NMDAR has a significant impact on the functional properties of the receptor. All NMADR units are permeable to potassium, sodium and calcium. The NR2A and NR2B subunits are the most predominant NR2 subunits found in functional NMDARs in the forebrain (Erreger et al., 2005). NR2A containing NMDARs have a high open probability and fast deactivation, decay and rise times (Erreger et al., 2005). NR2B containing NMDARs, on the other hand, have a low open probability and slow deactivation, decay and rise times (Erreger et al., 2005). NR2B containing NMDARs, therefore, respond to glutamate stimulation by generating larger EPSCs and by allowing a greater calcium influx into the cell. This means that

NR2B containing NMDARs have a stronger effect on the cell with each individual activation (Lethbridge et al., 2012).

1.4.1.2 NMADR Activation Requirements

In its resting state, the NMDAR has a voltage-dependent magnesium block (McBain & Meyer, 1994; Nowak et al., 1984). Glutamate stimulation of the receptor is therefore insufficient to reliably activate the channel. To activate the NMDAR, it is necessary that the cell be depolarized to remove the magnesium block, in conjunction with glutamate binding to the receptor to open the channel (Nowak et al., 1984).

1.4.2 GluR2-Lacking AMPAR

AMPARs, like NMDARs, are a subfamily of ionotropic glutamate receptors. Due to their fast transmission, they are usually involved in mediating primary glutamate signal transmission (Greger et al., 20007). AMPARs are tetramers comprised of two pairs of closely related subunits – in other words, they are dimers of dimers. Each subunit contains three transmembrane domains (M1, M3 and M4), as well as a region that loops into the membrane and lines the channel pore (M2). The properties of the M2 region determine the functional characteristics of the AMPAR (Greger et al., 2007; Seeburg et al., 2001).

There are four types of AMPAR subunits, identified as GluR1-4. The GluR2 subunit differs from the other three types in that it is modified post-transcriptionally to contain the amino acid arginine at the critical channel site of the M2 region, in place of glutamine (Seeburg et al., 2001). The substitution of a positively charged arginine

molecule for the neutral glutamine at this narrow position in the channel pore makes the pore impermeable to the divalent calcium ion. The presence of any GluR2 subunits is therefore sufficient to make an AMPAR calcium impermeable.

Most excitable cells express calcium-impermeable AMPARs that contain the GluR2 subunit, while most inhibitory interneurons express GluR1 homomers, which are calcium permeable (Seeburg et al., 2001; Tanaka et al., 2000). However, a recent study (Abraham et al., 2010) demonstrated that GCs in the OB express GluR2-AMPARs, and their removal increases the inhibitory effect of the GCs on MCs.

GluR2-lacking AMPARs have been implicated in early olfactory learning. Insertion of GluR1 AMPARs in glomeruli were observed 3 hours after odor training, and the increase disappeared within 48 hours, which is consistent with the time-course of the short-term odor memory (Cui et al., 2011).

1.4.3 mGluRs

Metabotropic glutamate receptors (mGluRs) are activated by glutamate, the same as the ionotropic glutamate receptors NMDAR and AMAPR. Unlike the ionotropic receptors, however, mGluRs are GPCRs and do not contain a channel that allows ions to flow into the cell. mGluRs, like all GPCRs, have seven transmembrane helices, an extracellular N terminus that includes the ligand binding site, and an intracellular C terminus (Niswender & Conn, 2010; Pin et al., 2003). When activated, the mGluR undergoes a conformational change, which activates its associated G-protein inside the cell. The G-protein, depending on its type, goes on to affect various effector molecules such as enzymes, ion channels and transcription factors (Niswender & Conn, 2010).

Thus, while mGluRs do not act as a direct source of calcium, their activation can have an indirect effect on calcium influx and cell excitability.

Recent work had demonstrated that group 1 mGluRs in neurons of the cerebellum activate LTCCs (Zheng & Raman, 2011). And in the nucleus tractus solitarius, mGluR stimulation was observed to facilitate LTCCs and inhibit N and P/Q type VGCCs (Endoh, 2004). The LTCC facilitation was mediated by both protein kinase C (PKC), and inositol-1,4,5-trisphosphate (IP3).

mGluRs are expressed widely in the OB, with particularly heavy expression of group 1 mGluRs observed on MCs (Sahar et al., 2004; Shigemoto et al., 1992). Activation of group 1 mGluRs via ON stimulation leads to a slow potentiation of MCs and calcium influx in MC dendritic tufts, even in the presence of NMDAR antagonists (Yuan & Knopfel, 2006b). It is possible that this effect is the result of mGluR facilitation of LTCCs, as had been demonstrated in other regions of the brain, but this has yet to be tested.

1.4.4 Intracellular Calcium Release

Calcium can enter a neuron's cytosol not only from extracellular sources, but also when it is released from intracellular stores. Within neurons, calcium is stored in high concentrations inside the endoplasmic reticulum (ER). The ER extends throughout the neuron, reaching the distal ends of both dendrites and axons. Due to its presence throughout the cell, the ER is sometimes referred to as the "neuron within a neuron." (Berridge, 2002; Stutzmann & Mattson, 2011). The controlled release of calcium from the ER modulates many activities within the neuron, including neurotransmitter release,

synaptic plasticity, and the activation of signaling pathways and gene transcription (Berridge, 2002; Bouchard et al., 2003; Carlson et al., 1997; Li et al., 1998; Stutzmann & Mattson, 2011).

Multiple intracellular signals may lead to the release of calcium from the ER. One signal is IP3, which binds to the IP3 receptor (IP3R). IP3 is a second messenger generated by GPCRs or tyrosine kinase-like receptors bound to the cell's plasma membrane (Stutzmann & Mattson, 2011). When IP3 binds to ER-bound IP3Rs, calcium is released into the cytosol. Calcium itself can bind to IP3Rs, and modulate the function of the channel in a concentration-dependent manner. Low concentrations (<300 nM) will increase the open probability of the channel, while high concentrations will inhibit the opening of the channel (Stutzmann & Mattson, 2011). This means that calcium release from one channel will facilitate the release of more calcium from neighbouring channels on the ER membrane until the local concentration of calcium in the cytosol becomes too high.

The other ER-membrane bound receptor that facilitates calcium release is the ryanodine receptor (RyR; Fill & Copello, 2002). This nonspecific cation channel is activated by the binding of calcium itself. RyRs are responsible for the phenomena of calcium-induced calcium release. As is the case for IP3Rs, however, the effect of calcium binding on RyRs is concentration-dependent and RyRs are inhibited by high concentrations of cytosolic calcium levels (Fill & Copello, 2002; Stutzmann & Mattson, 2011).

The release of intracellular calcium plays an important role in cells of the OB. A study of OB neurons in cell culture demonstrated that both projection (MC/TC) and

intrinsic (PG/GC) cells express both IP3Rs and RyRs throughout their soma and dendrites (Carlson et al., 1997). Activation of mGluRs has been observed to mediate calcium release from internal stores in GCs of the OB (Heinbockel et al., 2007). It's believed that intracellular calcium release plays an important role in driving GC-mediated inhibition (Heinbockel et al., 2007). Intracellular calcium release has also been observed in astrocytes of the OB, where it is believed to play an important role in the astrocytes' response to GABA signaling (Doengi et al., 2009), as well as their response to glutamate stimulation via mGluR5 receptors (Biber et al., 1999; Glaum et al., 1990).

1.5 Early Odor Preference Learning

Neonate rat pups are born blind, and unable to thermoregulate. They must stay near their mother in order to receive warmth, food and protection. Pups learn and use odor cues to locate their mother (Leon & Moltz, 1971). Pups learn to recognize smells associated with their mother via a simple association between her odor and the tactile stimulation they receive from her and littermates (Galef & Kaner, 1980; Sullivan & Leon, 1987). Taking advantage of this biological system, it is possible to train rat pups via a simple classical conditioning protocol to respond positively towards an odor that they would normally find mildly aversive (CS) when it is paired with one of many unconditioned stimuli (UCS) including warmth, feeding, tactile stimulation or chemical stimulation of defined brain regions (McLean et al., 1993; Sullivan & Leon, 1987; Sullivan & Wilson, 1994; Sullivan & Wilson, 2003; Sullivan et al., 2000; Wilson & Sullivan, 1990). Peppermint is traditionally used as the CS in early odor learning experiments because pups have a natural aversion to the smell and because the glomeruli

that respond to peppermint are located on the dorsolateral quadrant of the OB, which is a convenient location to access for chemical stimulation and electrical recording (McLean et al., 1999).

1.5.1 Advantages of Studying Early Odor Preference Learning

Early odor preference learning provides an excellent model for studying learning and memory as all necessary and sufficient inputs for learning can be localized to a simple cortical structure, the OB. Learning can be induced via a simple and reliable one-time training event. Since the neonate pups' eyes are not yet fully developed, inadvertent associations or distraction by visual cues is not a concern. And, finally, the learning effect is easily quantified, as mobile pups clearly move towards or away from odor sources during testing.

1.5.2 Potential Mechanisms of Early Odor Preference Learning

To date, two models have been proposed to explain how early olfactory learning occurs; the GC-MC disinhibition model, and the MC potentiation model.

1.5.2.1 GC-MC Disinhibition Model

Early work on neonate olfactory learning uncovered a crucial role for NE release and subsequent β-AR activation in the OB during learning. It was demonstrated that NE release is required for memory acquisition (Sullivan & Wilson, 1989; 1994; Sullivan et al., 2000), but not for memory expression (Sullivan & Wilson, 1991). It was also demonstrated that early odor learning led to changes in the activity of MCs, which carry

the odor signal out from the OB. Odor learning reduced MC habituation during training (Wilson & Sullivan, 1992). After a memory was formed, training resulted in an enhanced probability of MC inhibition by GC, even in neonate OBs (Wilson & Leon, 1988).

These findings led Wilson and Sullivan to propose a GC-MC disinhibition model of early olfactory learning (1994). According to this theory, learning occurs due to changes at the reciprocal GC-MC dendrodendritic synapse (Wilson & Sullivan, 1994; Sullivan & Wilson, 2003). These were considered to be odor-specific changes in the lateral and feedback inhibition of MCs by GCs. Within this model, NE stimulation of β-ARs acts as a UCS that, when paired with the CS of odor input, inhibits the GCs and leads to MC disinhibition.

1.5.2.2 MC Potentiation Model

Further research, however, demonstrated that the β-AR agonist isoproterenol has a relatively weak effect on GCs. Rather, GCs demonstrated a much stronger response when stimulated with α–adrenoceptor (α-AR) agonists (Mouly et al., 1995; Trombley, 1994; Trombley & Shepherd, 1992). These studies also demonstrated that NE stimulation of α-ARs affects MC excitation via a presynaptic modulation of calcium influx (Trombley, 1994). Furthermore, NE release during the generation of an early olfactory memory was shown to phosphorylate CREB in MCs (McLean et al., 1999). Similarly, systemic injection of the β-AR agonist isoproterenol, which is a sufficient UCS to generate an early olfactory memory (Sullivan & Wilson, 1989; 1994), also led to the phosphorylation of CREB in MCs (Yuan et al., 2000). Finally, it was observed that

application of isoproterenol could compensate for 5-HT depletion, and rescue early olfactory learning from a 5-HT depletion block (Langdon et al., 1997; Yuan et al., 2000). This is in line with evidence from the rat neocortex that suggests that 5-HT stimulation potentiates β-ARs and promotes the generation of cAMP (Morin et al., 1992).

This led McLean and his colleagues (Yuan et al., 2003b) to propose a MC potentiation model for early olfactory learning. According to this theory, the UCS in early olfactory learning was the production of cAMP in MCs. When this UCS converges with the CS of odor evoked glutamate release at the ON-MC synapse, the result is the phosphorylation of CREB (Yuan et al., 2003b). CREB phosphorylation, in turn, is responsible for facilitating the production of plasticity related proteins (McLean & Harley, 2004; Cui et al., 2007; Silva et al., 1998; Yuan et al., 2003a), which serves to potentiate the ON-MC synapse in the glomeruli (Yuan et al., 2003b).

An important strength of the MC plasticity model is that the mechanisms it suggests are involved in early olfactory learning parallel the model of serotonin mediated sensory learning proposed in *Aplysia* by Kandel et al., (2001). As recognized by Sullivan & Wilson (2003) this supports the intriguing possibility that the intracellular mechanisms involved in the generation and storage of memories may be conserved across species.

Since the MC potentiation model was first proposed, further evidence has been presented to support it. Yuan (2009) observed that stimulating the ON with theta burst stimulation (TBS), which mimics the rat's natural sniffing pattern, produced long term potentiation (LTP) of the glomerular field excitatory post synaptic potentials (fEPSPs). TBS stimulation in the presence of isoproterenol generated a significant calcium response in MC cell bodies (Yuan, 2009). These results confirm that these conditions, which

closely represent the conditions during *in vivo* learning, generate a response in the MC nucleus.

It has also been demonstrated that early olfactory learning leads to a change in the AMPA/NMDA ratio expressed in MC dendrites (Yuan & Harley, 2012). Early odor memory acquisition involves an increased expression of AMPARs in the postsynaptic membrane, and memory stability involves a decreased NMDAR-mediated response (Yuan & Harley, 2012). Finally, Lethbridge et al (2012) provided further evidence that NMDARs located in the MC distal dendrites are involved in *in vivo* odor memory formation. The NMDAR GluN1 subunits in MC distal dendrites are phosphorylated 5 minutes following early olfactory training, and they are down-regulated 3 hours following training, which suggests a role in memory formation. The GluN2B subunits in the same location are down-regulated 24 hours after training, which suggests a role in memory stability (Lethbridge et al., 2012).

1.5.3 Gap in the Model – β-Adrenoceptor Modulation of Calcium Dynamics in the MC?

β-AR activation (Sullivan & Wilson, 1989; 1994; Sullivan et al., 2000) and calcium influx (Cui et al., 2007; Lethbridge et al., 2012; Yuan, 2009) have both been established as necessary components of early odor memory formation. Previously, it was suggested that coincidence detection of β-AR activation and calcium influx facilitates calmodulin phosphorylation of CREB in MCs (Yuan et al., 2003b). However, it is possible that β-AR activation directly facilitates the flow of calcium into the cell. A previous study (Yuan et al., 2004) investigated whether LTCCs are present in cells of the

OB and if they are under the influence of β-AR activation, but failed to find any evidence of LTCC expression or β-AR modulation on calcium dynamics within the OB.

Other groups, however, have demonstrated that LTCCs are present in cells of the OB (Schild et al., 1995; Tanaka et al., 1995). β-AR modulation of calcium signaling plays an important role in learning and memory in the hippocampus and amygdala (Gray and Johnston 1987; Huang et al. 1993, 1996), and β-AR activation modulates calcium flow through LTCCs in cardiac myocytes (Hell, 2010), as well as in neurons (Davare et al., 2001; Marshall et al., 2011). We wanted, therefore, to further examine the possibility that LTCCs may be present in cells of the neonate rat OB, and that they may serve an important function during early odor preference learning.

1.6 Experimental Design

1.6.1 Are LTCCs Present in the Neonate Olfactory Bulb?

Previous studies have demonstrated that LTCC are present in cells of the OB (Murphy et al., 2005; Schild et al., 1995; Tanaka et al., 1995), and ORNs (Trombley & Westbrook, 1991). To confirm these previous findings as a first step in understanding LTCC functioning in the OB, we performed immunohistochemistry testing with the anti-LTCC $\alpha 1_C$ subunit antibody to check the cell-specific expression of LTCCs in the neonate rat pup OB.

1.6.2 Is LTCC Activation Necessary for Odor Preference Learning?

MC-bound NMDARs play an important role in early olfactory learning (Lethbridge et al., 2012; Yuan & Harley, 2012; Yuan et al., 2003b). *In vitro* experiments have shown that stimulation of the ON with TBS paired with β-AR activation leads to NMDAR-dependent potentiation of ON-evoked MC firing (Lethbridge et al., 2012). Application of TBS and β-AR activation has also been shown to evoke an increased calcium response in the MC bodies (Yuan, 2009). Because LTCCs are generally located near the soma (Schild et al., 1995; Westenbroek et al., 1990; Hell et al., 2003), and LTCCs have been implicated in the activation of intracellular signaling cascades related to synaptic plasticity (Fisher & Johnston, 1990; Holmgaard et al., 2008; Impey et al., 1996; Lacinova et al., 2007), we suspect that LTCCs may play an important role in the calcium influx observed in MCs.

In order to determine if LTCC function is necessary for the development of early odor preferences, we performed intrabulbar infusions of the LTCC antagonist nimodopine during odor preference training.

1.6.3 Is LTCC Activation Necessary for the Generation of MC-LTP?

LTP is believed to be the cellular equivalent of a memory trace in associative learning (Bliss and Lomo 1973; Brown et al. 1988; Barnes 1995; Malenka 1994). In the OB, TBS stimulation of the ON afferent has been shown to generate LTP of field EPSPs at glomerular ON synapses (Yuan, 2009).

In order to determine if LTCC function is necessary for the development of LTP at the ON synapse, we performed *in vitro* electrophysiology studies in which we attempted to induce LTP at the glomerular ON synapse in the presence of nimodipine.

1.6.4 Is LTCC Activation Sufficient to Induce Odor Preference Learning?

Previous studies have indicated that LTCC function is sometimes regulated by β-AR activation (Davare et al., 2001; Hell, 2010; Marshall et al., 2011). Other studies have demonstrated a functional relationship between NMDAR and LTCC activation (Silva et al., 2012; Turner et al., 2009), and have suggested that LTCCs may function downstream from NMDARs.

In order to determine if LTCC activation is sufficient to induce an early odor preference, we attempted to induce an early odor preference using an intrabulbar infusion of the LTCC agonist BayK-8644 as the UCS. We also performed early olfactory training paired with an intrabulbar infusion of the NMDAR antagonist D-APV and the LTCC agonist BayK-8644 to determine if activation of LTCCs could rescue early olfactory learning from a D-APV block.

1.6.5 Are LTCCs in the Olfactory Bulb Dependent on the Co-Activation of β-Adrenoceptors?

β-AR activation via NE release from the locus coeruleus, or the application of the β-AR agonist isoproterenol, is known to be a critical component of early olfactory learning. It has been shown that β-AR activation can promote MC cAMP production (McLean et al., 1999; Yuan et al., 2000; McLean & Harley, 2004), and relieve MC

inhibition from PG cells (Murphy et al., 2005), both effects which do or could contribute to the promotion of early odor preference learning. However, it remains to be tested whether β-AR activation is directly implicated in generating a calcium influx in the MC layer. Having determined that LTCC activation is necessary for early olfactory learning, we were interested in examining if β-ARs are involved in regulating LTCC action in neurons of the OB, as has been demonstrated in other neurons (Davare et al., 2001; Marshall et al., 2011).

In order to determine if LTCC activity is dependent on the co-activation of β-ARs we induced an early olfactory preference by relieving intraglomeular inhibition with an intrabulbar infusion of the GABA-A receptor antagonist gabazine. This training method does not involve the activation of β-ARs. To determine if LTCCs were involved in this β-AR-free learning, we attempted to block this learning with an intrabulbar infusion of the LTCC antagonist nimodipine.

CHAPTER 2 – MATERIALS & METHODS

2.1 Animals

Sprague Dawley rat pups (Charles River Laboratories) were used in this study. Pups were born on-site at the research facility, and were housed with the dam in polycarbonate cages. Dams were kept under a reverse 12 h light/dark cycle with lights off at 12:00 pm (noon). Food and water were accessible *ad libitum*. Animals were housed, and behavioural training and testing were performed, in temperature-controlled rooms maintained at approximately 28°C. The pups' day of birth was considered postnatal day (PND) 0. Litters were culled to 12 pups on PND 1, and an equal number of male and female pups were maintained in the litter, and subsequently used in testing, whenever possible. Immunohistochemistry testing with the anti-$Ca_v1.2$ antibody was also performed on juvenile rats (PND 20) and adult wild type C57BL/6J mice (>1 month old) as comparisons.

All experimental procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland, and follow the guidelines set by the Canadian Council of Animal Care.

2.2 Immunohistochemistry

Samples were collected from naïve PND 6 pups for $Ca_v1.2$ staining, ($n = 6$). Animals were deeply anesthetized with an intraperitoneal injection of chloral hydrate (1.5 g/kg, Sigma-Aldrich) and then perfused transcardially with ice-cold saline solution for one minute, followed by perfusion of ice-cold fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) for 30 minutes. Brains were removed and post-fixed for

one hour in the same fixative solution. Brains were then immersed in buffered 20% sucrose solution overnight at 4°C, and stored under these conditions until used for immunohistochemistry.

For sample sectioning, brains were removed from the storage sucrose solution and quick-frozen on dry ice. The OB was cut into 30 μm coronal sections in a cryostat at –20° C. Sections were mounted directly onto chrome-gelatin coated slides, air-dried at room temperature, and then incubated in phosphate buffer solution containing the primary antibodies.

To test for the presence of LTCCs, a solution of the anti-$Ca_v1.2$ antibody (rabbit polyclonal, 1:200, Millipore: Billerica, MA, USA, catalog number AB5156, purified peptide from 8480865 of $\alpha 1_C$ subunit of rat brain voltage-gated calcium channel: accession number P22002), dissolved in phosphate buffer saline with 0.2% Triton-X-100, 0.02% sodium azide and 2% normal goat serum was applied to the slices. Some slices were incubated with a solution that also contained a control peptide (Millipore) to ensure that staining observed on test slices was not non-specific staining. Samples were incubated with the primary antibody overnight in a 4° C humidified chamber. The following day, sections were washed in PBS, and then incubated in a biotinylated anti-rabbit secondary antibody (Vectastain Elite, Vector Laboratories: Burlingame, CA, USA), followed by an Avidin and biotinylated enzyme (A+B) amplification step. Finally, slices underwent a diaminobenzidine tetrahydrochloride reaction (30 μl of 30% H_2O_2 added to 100 ml of 2.3 mM diaminobenzidine tetrahydrochloride). The progress of the staining was monitored under an upright light microscope (Olympus), and took approximately 2 to 5 minutes. Slices were then dehydrated and coverslipped with

Permount (Fisher Scientific). Staining patterns were determined via a visual inspection of the treated slices. Staining intensity was not quantified.

Photomicrographs of the slices were taken through a Leica DMR SE upright microscope equipped with a DVC 1310 camera (MBF Bioscience: Williston, VT, USA). Objectives used include HCX PL FLUOTAR 5x/0.15-PH1, HC PL FLUOTAR 10x/0.30-PH1; HC PL FLUOTAR 20x/0.50-PH2.

2.3 Intrabulbar Infusion and Behaviour Experiments

2.3.1 Guide Cannulae Unit and Infusion Cannulae Production

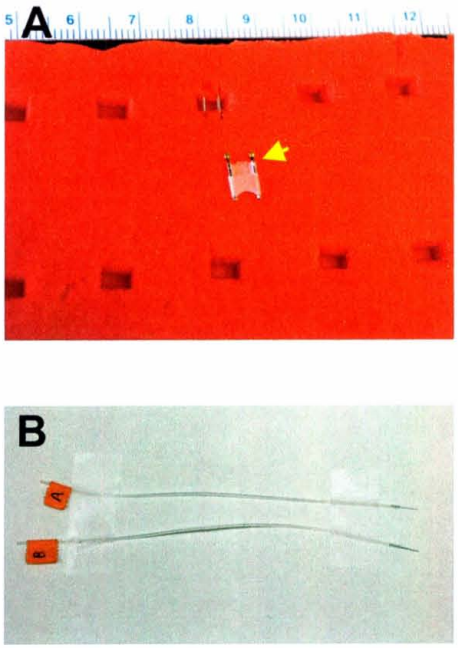
Guide cannula units were constructed by anchoring stainless steel guide cannulae (Vita Needle Company: Needham, MA, USA; 23 gauge – outer diameter 0.025", inner diameter 0.013"; cut to 6 mm length) in dental acrylic (Lang Dental: Wheeling, IL, USA). Each unit was comprised of two parallel guide cannulae, separated laterally by 4 mm, each extending 0.5 mm below the bottom of the acrylic block. Insect pins were inserted into the guide cannulae to prevent pre-test blocking (Figure 2A).

Infusion cannulae units consisted of a stainless steel cannula (Small Parts Inc: Seattle, WA, USA; 30 gauge – outer diameter 0.012", inner diameter 0.006"; cut to 13 mm length) inserted into PE20 polypropylene tubing (Clay Adams: Sparks, MD, USA; cut to 20 cm). The steel cannulae were inserted into the tubing so that 7 mm of cannula extended beyond the end of the tubing (Figure 2B).

Figure 2. Custom Made Cannula.

(A) Photograph a guide cannulae unit assembly. Two guide cannulae are held in parallel inside a block of dental acrylic with 0.5 mm extending below the bottom of the acrylic block. (B) Photograph of infusion cannula unit. A stainless steel cannula is inserted into PE20 polypropylene tubing so that 7 mm of cannula extends beyond the end of the tubing.

Figure 2



2.3.2 Surgery and Guide Cannula Implementation

Prior to surgery, PND 5 pups were anesthetized via hypothermia by placing them under ice for 15-20 minutes. Pups were placed in a stereotaxic apparatus with bregma and lambda in the same horizontal plane. Anesthesia was maintained by covering the pups' bodies in ice during the surgical procedure.

The skull was exposed and two small holes were drilled through the skull over the dorsolateral surface of each OB using a micro drill (Fine Science Tools: North Vancouver, BC, Canada). The guide cannula units were lowered so that the bottom of each guide cannula sat in one of the holes. A plastic screw head (Small Parts Inc.) was glued upside down onto the skull just posterior to the drilled holes. Using the screw as an anchor, the guide cannula unit was fixed to the skull with dental acrylic (Figure 3A). The skin was sutured together and pups recovered from their anesthesia on warmed bedding for at least 30 minutes before being returned to the dam (Figure 3B).

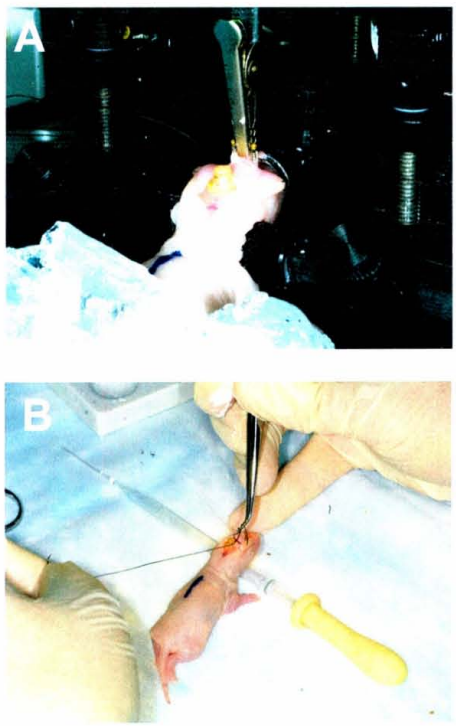
2.3.3 Training – Intrabulbar Infusion

On training day, pups received an intrabulbar infusion of drugs or vehicle while sitting on peppermint-scented bedding. The infusion solutions were administered via the infusion cannulae. The free-end of each infusion cannula was attached to the needle of a microsyringe (Hamilton Company: Reno, NV, USA; 10 μl). The microsyringes were held in a multi-syringe pump (Chemyx: Stafford, TX, USA), which controlled the rate and volume of the infusion.

Figure 3. Cannula Implantation Surgery

(A) Photograph of guide cannula insertion during surgery. Guide cannulae unit is being held in place with the alligator clip. A screw head has been glued on the skull above the cortex to be used as an anchor for the guide cannulae unit. The pup is being kept hypothermic by the application of ice on its body. (B) Photograph of post-surgery suturing. Following this, the pup will be placed on warmed bedding while it recovers from the hypothermia.

Figure 3



Peppermint-scented bedding was prepared 30 minutes prior to the beginning of training by mixing 500 ml of control bedding with 30 μl of peppermint extract. During training, the insect pins were removed from the guide cannulae, and the infusion cannulae were gently inserted into the guide cannulae attached to the pup's skull (Figure 4). Pups were given three minutes to adjust to the presence of the infusion cannulae, and then they were placed on the peppermint-scented bedding.

Thirty seconds after being placed on the bedding, a 1 μl infusion was administered over 4 minutes. Pups remained on the peppermint-scented bedding for an additional 6 minutes (bringing the total time on peppermint to 10.5 minutes), at which point the infusion cannulae were removed and the pups were returned to the dam.

To test if LTCC function is required for learning, we asked if an LTCC antagonist would block isoproterenol-induced learning. During odor training, pups received a bilateral infusion of a vehicle control (sterile saline, with 0.1% ethanol), a positive learning control (isoproterenol, 50 μM), or isoproterenol together with nimodipine (10 μM), dissolved in the vehicle solution.

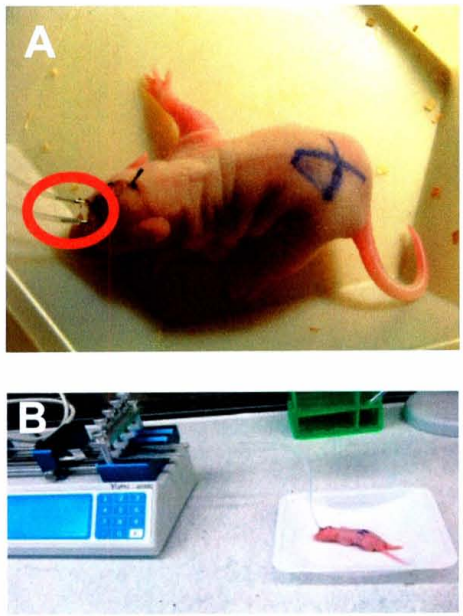
To test if LTCC activation could rescue isoproterenol-induced odor learning from a D-APV-block, pups received an infusion of a vehicle control (sterile saline with 2% DMSO and 0.8% Tween 20), a positive learning control (isoproterenol, 50 μM), a learning-block control (50 μM isoproterenol, 500 μM D-APV), isoproterenol with D-APV and BayK-8644 (20 μM, 200 μM or 2 mM), or BayK-8644 (2 mM), dissolved in the vehicle solution.

Finally, to test if β-AR activation is required for LTCC function in odor learning, pups received an infusion of a vehicle control (sterile saline, 0.1% ethanol), a positive

Figure 4. Drug Infusion During Odor Preference Training

(A) Photograph of pup during infusion. Red oval highlights the infusion cannulae, which are inserted into the pup's implanted guide cannulae. (B) Photograph of pup, infusion cannulae and infusion pump. The pump regulates the volume and rate of infusion.

Figure 4



learning control (100 μM gabazine), gabazine with nimodipine (10 μM), or gabazine with nimodipine and D-APV (500 μM), dissolved in the vehicle solution.

2.3.4 Two-odor Choice Testing

Testing was performed on PND 7, 22-25 hours post-training. Testing was performed in between 12:00 pm and 2:00pm in a climate controlled room maintained at 28 degrees Celsius. The stainless steel testing box (30 cm x 20 cm x 18 cm), was placed on top of two training boxes, which were separated by a 2 cm neutral zone. One training box contained peppermint-scented bedding, and the other non-scented control bedding (Figure 5). Peppermint-scented bedding was prepared as described above. Pups were placed individually into the testing box for 5 separate 1-minute trials. In each trial, the pup was placed in the testing box over the neutral zone, and allowed to move freely. When a pup's nose moved from the neutral zone to over either the peppermint or control bedding, the experimenter began recording time. The amount of time the pup spent over each of the two types of bedding was recorded for each trial. After each trial, the pup was removed from the test box and placed in a cage with unscented bedding during the 1-minute inter-trial interval. To account for a potential inherent preference to turn either left or right, the pup was placed in the testing box in alternating orientations (with the peppermint scent on either their right or left side) at the beginning of each trial.

The total amount of time each pup spent over peppermint or control bedding was calculated. Values reported are the mean ± SEM of the percentages of time pups spent over the peppermint scented bedding divided by the total time spent over either bedding type. A one-way ANOVA was carried out and post-hoc Fisher tests were used to

Figure 5. Two-Odor Choice Preference Test.

Top-down view of pup during two-odor choice preference test. Pup was placed in NZ, and is in the processing of moving towards the peppermint-scented bedding. NZ, neutral zone.

Figure 5

determine statistical significance. Data from pups that demonstrated little mobility (not moving from the neutral zone in ≥3 trials of 5) were excluded from the analysis.

After testing, animals were sacrificed in order to confirm correct placement of the guide cannula and infusion. This was performed via a visual inspection on the skull, and then of the OBs themselves (Figure 6). Only pups that had received an infusion in the lateral OB were included in the analysis.

2.4 Electrophysiology Experiments

2.4.1 Slice Preparation

On PND 5-11, naïve rat pups were anaesthetized via halothane inhalation and decapitated. The pup's brain was quickly dissected and placed into ice-cold aCSF containing the following (in mM): 83 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 3.3$MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, and 72 sucrose, equilibrated with 95% O_2/5% CO_2. Slices of the OB were cut along the horizontal plane at 400 μm using a vibrating slicer (Leica VT 1000P). Slices were hemisected and incubated at 34°C for 30 min in the same high-glucose aCSF solution. Slices were then incubated in solution at room temperature until use.

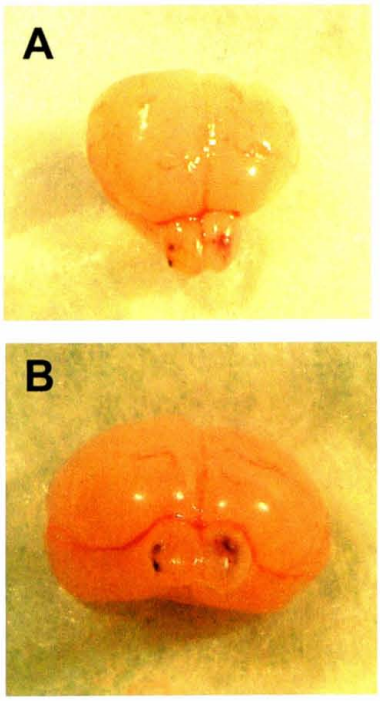
2.4.2 Electrophysiology Recordings

During recording, slices were superfused with magnesium-free aCSF containing the following (in mM): 122 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 22 glucose, equilibrated with 95% O_2/5% CO_2.

Figure 6. Site of Targeted Infusions into the Lateral Olfactory Bulb

Photographs of a rat brain, harvested following odor preference testing to confirm the site of targeted infusion. Following testing, methyl blue was infused through the guide cannulae to stain the infusion site. (A) Photograph taken from the top, with the brain lying in the horizontal plane. Olfactory bulbs, with methyl blue stain are pointing towards the bottom of the page. (B) Photograph taken from the front, with the brain in the sagittal plane. Olfactory bulbs are pointing up from the page.

Figure 6



Slices were viewed with an upright microscope (Olympus BX51) using differential interference contrast optics. Field recordings were obtained using glass pipettes filled with the magnesium-free aCSF. Recording pipettes were positioned in the centre of glomeruli within the mid-lateral region of the OB whose ON innervation was clearly visible. A concentric bipolar stimulating pipette (FHC) was positioned in the ON layer, contacting ON fibers that were innervating the recorded glomeruli. All recordings were acquired at 30°-32°C.

A stimulation profile was measured for each slice used to determine the maximum and median stimulation thresholds. Baseline recordings of the fEPSP were obtained by stimulating a single test stimulus (mean stimulation threshold for the slice, within 20-100 mA) every 20 sec for 5 minutes. LTP induction was performed using an established protocol (Yuan, 2009). To induce LTP at the ON-MC synapse, a single strong theta burst stimulation (sTBS; 10 bursts of high frequency stimulation at 5 Hz, each burst containing five pulses at 100 Hz, same stimulation as test stimuli) was applied to the ON. To test for the induction of LTP, post-stimulation recordings of fEPSP were obtained by stimulating the ON with a single test stimulus (same the baseline stimuli) every 20 sec for 60 minutes.

To test if LTCC activation was required for the induction of LTP at the ON-MC synapse, we applied a sTBS in the presence of nimodipine. The drug was applied via bath wash at a concentration of 10 μM for 5 minutes before the application of the sTBS, after which the drug was washed out with magnesium-free aCSF.

Electrophysiology data were recorded with Multiclamp 700B (Molecular Devices), filtered at 2 kHz and digitized at 10 kHz. Data acquisition and analysis were

performed with pClamp10 (Molecular Devices) and Igor Pro 6.10A (WaveMetrics). Results were interpreted by dividing the post-stimulation fEPSP responses by the average baseline response of that same slice. LTP was indicated by a significant increase in the post-stimulation fEPSP response relative to baseline responses.

CHAPTER 3 - RESULTS

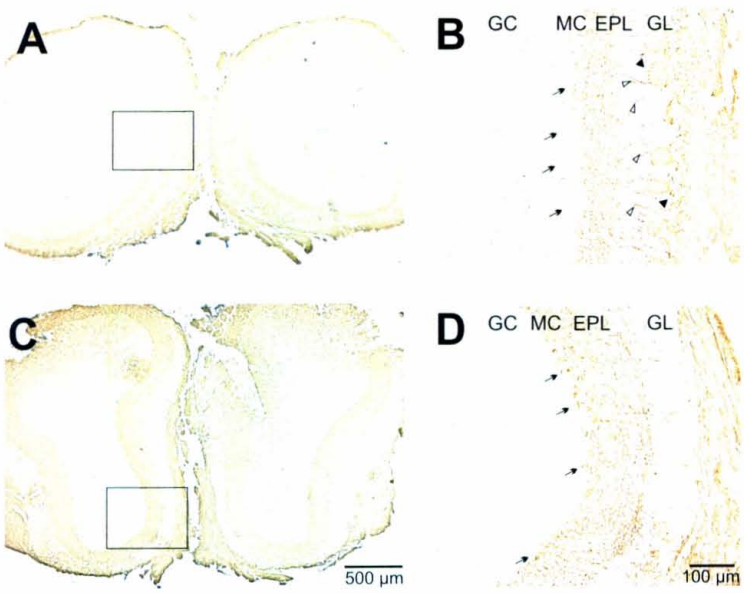
3.1 LTCCs are Present in the Neonate Rat Pup Olfactory Bulb

To test if LTCCs are expressed in the neonate pup OB, we performed immunohistochemistry on OB slices harvested from naïve PND 6 pups (Figure 7 A&B). The primary antibody bound to the $Ca_v1.2$ subtype of LTCCs. Figure 7 shows that significant staining was observed in the OB. Specifically, staining is consistently strong in the ON layer, glomeruli, and the internal portion of the EPL of the OB. MC bodies (arrows, Figure 7B) are weakly stained. However, the shaft of the MC apical dendrites in the EPL are heavily stained (hollow arrowheads, Figure 7B). In addition, the heavy staining in the internal portion of the EPL is assumed to be on MC primary and secondary dendrites, as it is known that this is where MC secondary dendrites extend laterally (Price & Powell, 1970b). $Ca_v1.2$ staining is also observed in PG cells (solid arrowheads) surrounding glomeruli. $Ca_v1.2$ staining in juvenile adult rats (PND 20-23) showed an overall weaker pattern of staining (data not shown). We also compared $Ca_v1.2$ expression patterns between rats and C57BL/6J mice (Figure 7C&D). Interestingly, $Ca_v1.2$ expression is observed strongly in MC bodies (arrows, Figure 7D) in mice and is weaker in the GL. This suggests there is a difference in $Ca_v1.2$ expression patterns between the two species. Nevertheless, these results confirm that LTCCs are present in the neonate OB, and that they are expressed in MCs, which are the substrate for early odor preference learning according to the MC-potentiation model (Yuan et al. 2003; Yuan 2009; Lethbridge et al., 2012).

Figure 7. Distribution of LTCCs in the Olfactory Bulb of a Neonate Rat and an Adult Wild-Type Mouse.

(A) A coronal section of the olfactory bulb from a PND 6 rat pup. (B) Increased magnification of the medial area of the olfactory bulb, as indicated in A. (C) A coronal section of the olfactory bulb from a wild type mouse. (D) Increased magnification of the medial area of the olfactory bulb, as indicated in C. Arrows indicate individual mitral cell somas with significant staining. Solid arrowheads indicate periglomerular cells surrounding glomeruli. Hollow arrowheads indicate mitral cell primary dendrites extending through the EPL to GL. EPL, external plexiform layer; GC, granule cell layer; GL glomerular layer; MC, mitral cell layer.

Figure 7



3.2 LTCC Function is Required for Isoproterenol-Induced Odor Preference Learning

To test if LTCC function is required for odor preference learning, we infused the LTCC antagonist nimodipine into the dorsolateral OB during odor training. Figure 8 shows that an infusion of isoproterenol successfully induced an odor preference in the animals (60.11 ± 4.33%; one-way ANOVA, $F_{(2,31)} = 13.18$, $p < 0.01$). Infusion of the vehicle control (30.85 ± 3.14%) or nimodipine with isoproterenol (39.00 ± 4.98%) did not induce an odor preference. A post-hoc Fisher test of the mean demonstrated a significant difference between the isoproterenol-only and isoproterenol with nimodipine groups ($t = 3.46$, $p < 0.01$), and between the isoproterenol-only and vehicle control groups ($t = 5.08$, $p < 0.01$). These results suggest that LTCC function is required for isoproterenol-induced odor preference development.

3.3 fEPSP Experiments

Because slice preparation and electrophysiology recordings are technically challenging skills, we chose not to begin testing the effect of nimodipine on LTP induction until we could reliable produce LTP in at least 60% of control slices. Despite significant effort, this level of efficiency was never achieved. We attempted to induce LTP in 16 slices, over a period of 2 weeks. LTP was successfully induced in 3 of these experiments. Example fEPSP recordings from a slice that did produce LTP during this procedure (Figure 9A) and from a slice that did not produce LTP (Figure 9B) are provided. Figure 9C presents the averaged fEPSP data from all 16 slices.

Figure 8. Nimodipine Blocks Isoproterenol-Induced Learning.

Bars indicate the percentages of time spent in the peppermint side in a two-choice test box in different experimental groups. $^{**}p < 0.01$. Error bars, mean ± SEM. Animals that received a 1 μm infusion of ISO + odor at training spent significantly more time over the peppermint-scented bedding than the vehicle only group and the ISO+Nim group when tested 24 h following training. There was no significant difference between the ISO+Nim and vehicle only groups. VC, vehicle control; ISO, isoproterenol; Nim, nimodipine.

Figure 8

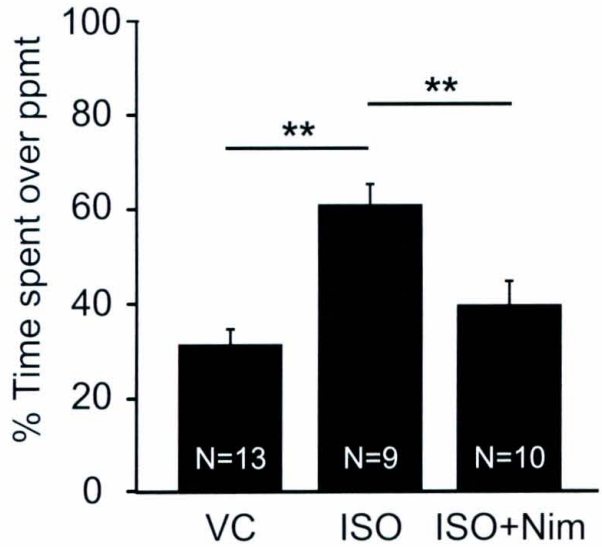
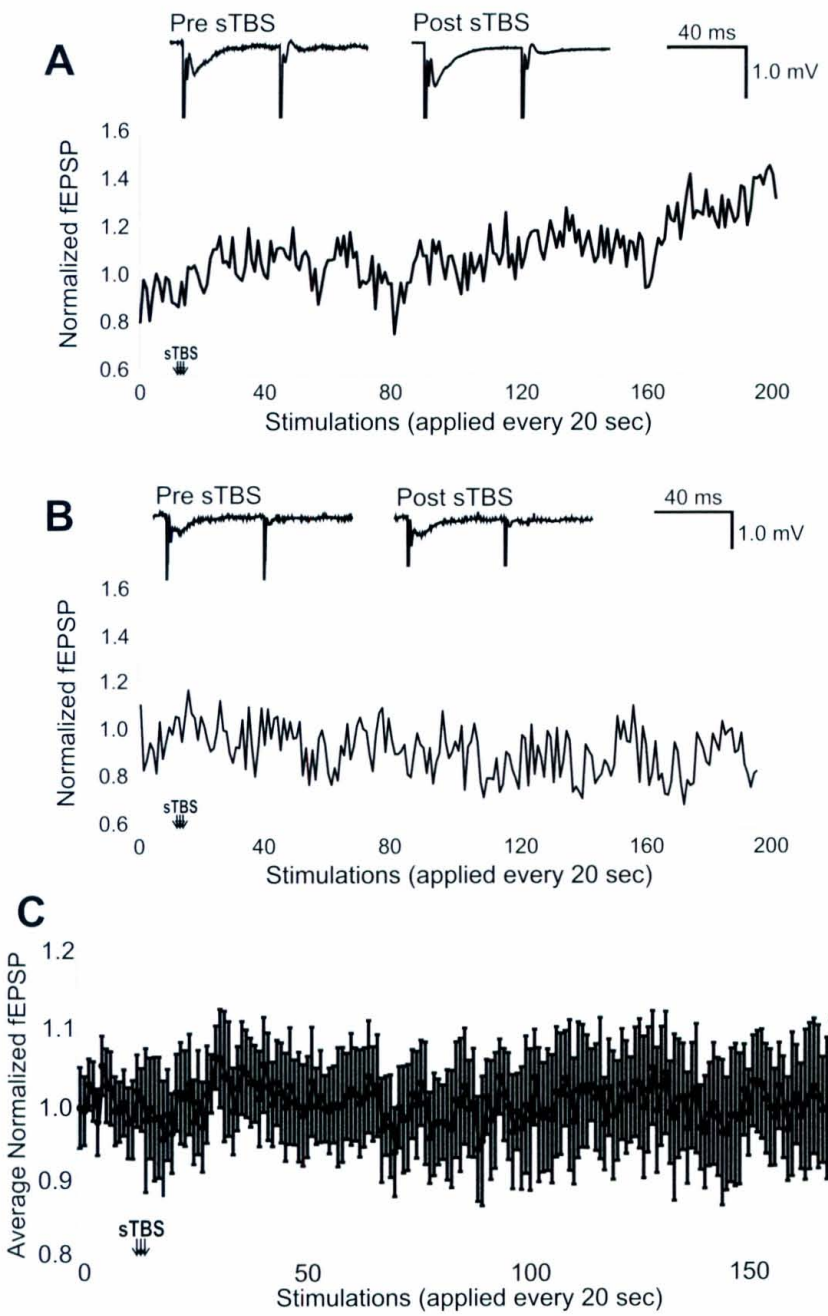


Figure 9. Sample fEPSP Measurements from sTBS Experiments

(A) Sample recordings from slice b010912, which did experience LTP. The tracings are representative samples of fEPSP recordings before (Pre) and after (Post) the sTBS was applied. The graph shows the Normalized fEPSP recording from slice b010912 throughout the trial. sTBS was applied after 15 stimulations, as noted in the graph. Normalized fEPSP recordings were calculated by dividing each fEPSP measurement by the average of the 15 baseline stimulations. (B) Sample recordings from slice a010412, which did not experience LTP. The tracings are representative samples of fEPSP recordings before (Pre) and after (Post) the sTBS was applied. The graph shows the Normalized fEPSP recording from slice a010412 throughout the trial. sTBS was applied after 15 stimulations, as noted in the graph. Normalized fEPSP recordings were calculated by dividing each fEPSP measurement by the average of the 15 baseline stimulations. (C) Averaged Normalized fEPSP recordings from 16 slices used during sTBS experiments. sTBS was applied after 15 baseline stimulations, indicated by arrows. Normalized fEPSP recordings were calculated by dividing each fEPSP response by average baseline responses. Note how the average fEPSP response does not change significantly over time, indicating that LTP has not been achieved in the majority of the slices. fEPSP, field excitatory postsynaptic potential; sTBS, strong theta burst stimulation.

Figure 9


A
Pre sTBS
Post sTBS
40 ms
1.0 mV
Normalized fEPSP
1.6
1.4
1.2
1.0
0.8
0.6
sTBS
0
40
80
120
160
200
Stimulations (applied every 20 sec)
B
Pre sTBS
Post sTBS
40 ms
1.0 mV
Normalized fEPSP
1.6
1.4
1.2
1.0
0.8
0.6
sTBS
0
40
80
120
160
200
Stimulations (applied every 20 sec)
C
Average Normalized fEPSP
1.2
1.1
1.0
0.9
0.8
sTBS
0
50
100
150
Stimulations (applied every 20 sec)

To confirm the presence or absence of LTP, we compared the average pre- and post-sTBS peak amplitude of the fEPSP responses. The pre-sTBS value was the average of the peak amplitude from the initial 15 baseline stimulations (minutes 0-5). The post-sTBS value was the average peak amplitude from stimulations 175-190 (minutes 55-60). A paired t-test was performed to compare the two groups. Figure 10 shows that there is no significant difference between the pre- and post-sTBS groups (the post-sTBS is normalized to pre-sTBS; $t = 0.85$, $p > 0.41$, $n = 16$), which indicates that the samples on average did not experience LTP following the application of a sTBS. This is despite the fact that in 3 out of 16 experiments there was measureable LTP of the fEPSP glomerular response (as demonstrated in Figure 9A).

3.4 LTCC Activation Rescues Isoproterenol-Induced Learning From a D-APV Block, but is Not Sufficient to Induce Learning By Itself

Recent work from our lab (Lethbridge et al., 2012) demonstrated a critical role of NMDARs in odor preference learning induction. Blocking NMDARs at the ON-MC synapse with D-APV prevented early odor preference learning. To test if LTCC activation could rescue isoproterenol-induced learning from a D-APV-block, we infused an LTCC agonist (BayK-8644) during odor training. Figure 11 shows that while infusion of isoproterenol with D-APV did not induce an odor preference (38.25 ± 4.37%; one-way ANOVA, $F_{(6, 46)} = 3.12$, $p < 0.05$), an infusion of isoproterenol-only (58.29 ± 4.49%) or isoproterenol with D-APV and 20 μM BayK-8644 (63.50 ± 9.61%), 200 μM BayK-8644 (61.83 ± 8.12%) or 2 mM BayK-8644 (66.17 ± 10.25%) all led to the development of an odor preference. Infusion of the vehicle control (33.25 ± 2.93%) did not induce an odor

Figure 10. sTBS of the Olfactory Nerve is Not Sufficient to Induce LTP in the fEPSP Response of Olfactory Bulb Glomeruli

Bars indicate the average normalized fEPSP peak amplitude measured pre- and post-sTBS. Error bars, mean ± SEM.

Figure 10

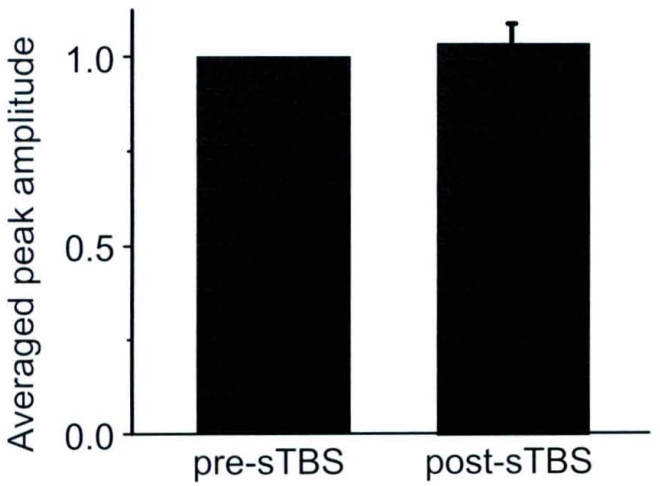
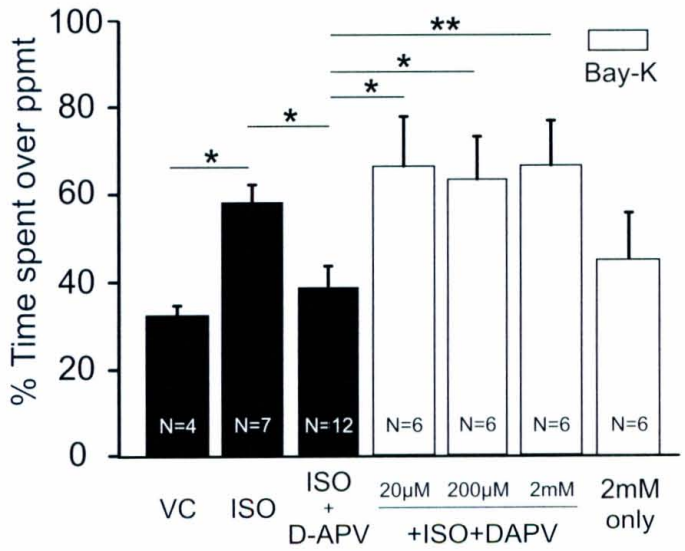


Figure 11. BayK-8644 Rescues Learning from a NMDAR Block, but It Is Not Sufficient to Induce Learning on Its Own.

Bars indicate the percentages of time spent in the peppermint side in a two-choice test box in different experimental groups. *p<0.05. **$p < 0.01$. Error bars, mean ± SEM. Animals who received an infusion of ISO-only, or ISO, D-APV and either 20μM, 200μM or 2mM BayK-8644 spent significantly more time over peppermint-scented bedding than the vehicle control or ISO+D-APV groups. Animals who received a 1 μm infusion of just BayK-8644 did not spend significantly more time over peppermint-scented bedding, as compared to the vehicle control or ISO+D-APV groups. VC, vehicle control; ISO, isoproterenol; BK, BayK-8644.VC, vehicle control; ISO, isoproterenol; BK, BayK-8644.

Figure 11


% Time spent over ppmt
100
80
60
40
20
0
**
*
*
*
*
Bay-K
N=4
N=7
N=12
N=6
N=6
N=6
N=6
VC
ISO
ISO + D-APV
20μM
200μM
2mM
+ISO+DAPV
2mM only

preference. Interestingly, an infusion of 2 mM BayK-8644 only (45.33 ± 10.16%) did not induce odor preference. A post hoc Fisher Test of the mean demonstrated a significant difference between the vehicle control and isoproterenol with D-APV and 20 μM BayK-8644 ($t = 2.46$, $p < 0.05$), 200 μM BayK-8644 ($t = 2.32$, $p < 0.05$) and 2 mM BayK-8644 ($t = 2.68$, $p < 0.05$) groups, as well as between the vehicle control and isoproterenol-only groups ($t = 2.10$, $p < 0,05$). Significant differences were also demonstrated between the isoproterenol with D-APV group and the isoproterenol with D-APV and 20 μM BayK-8644 ($t = 2.65$, $p < 0.05$), 200 μM BayK-8644 ($t = 2.48$, $p < 0.05$) and 2 mM BayK-8644 ($t = 2.93$, $p < 0.01$) groups, as well as between the isoproterenol with D-APV and the isoproterenol-only group ($t = 2.21$, $p < 0.05$). These results suggest that LTCC activation with a wide-range of BayK-8644 concentrations successfully rescues isoproterenol-induced odor preference from a D-APV block, but that LTCC activation by itself is not sufficient to induce an odor preference.

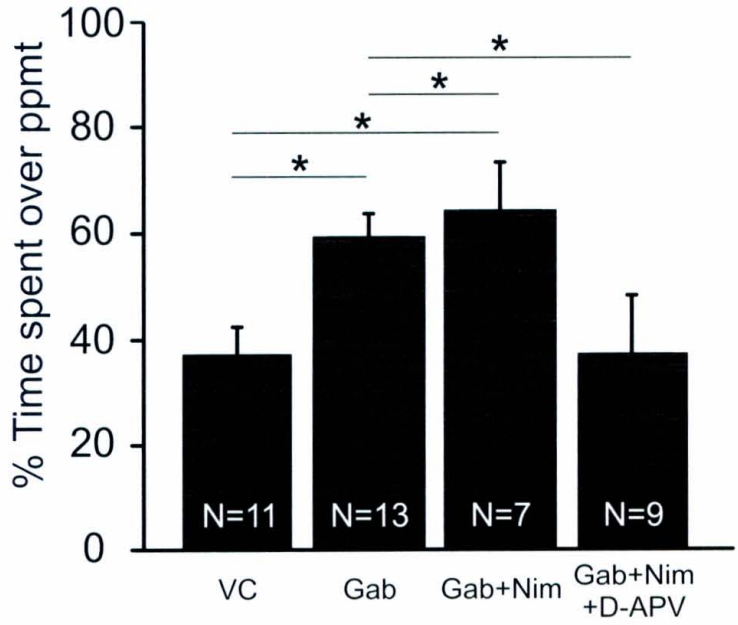
3.5 NMDAR Blockage is Necessary to Prevent Gabazine-Induced Odor Preferences

To further test if β-AR activation is required for LTCC function on MCs, we induced an odor preference independent of β-AR activation, and tested if LTCC inhibition with nimodipine inhibits this learning. We infused gabazine, a GABA-A antagonist, to induce odor preference learning. Figure 12 shows that an infusion of gabazine-only induced an odor preference (57.85 ± 4.25%; one-way ANOVA, $F_{(3, 36)} = 3.84$, $p < 0.05$), compared to the vehicle infusion (36.18 ± 5.16%). Interestingly, addition of nimodipine to the infusion cocktail did not prevent the gabazine-induced odor

Figure 12. NMDA Receptor Blockage is Necessary to Prevent Gabazine-Induced Odor Preferences.

Bars indicate the percentages of time spent in the peppermint side in a two-choice test box in different experimental groups. *p<0.05. **$p < 0.01$. Error bars, mean ± SEM. Animals who received a 1 μm infusion of gabazine spent significantly more time over the peppermint-scented bedding. Animals who received an infusion of both gabazine and nimodipine did not spend significantly more time over the peppermint-scented bedding. VC, vehicle control; Gab, gabazine; Nim, nimodipine.

Figure 12


% Time spent over ppmt
100
80
60
40
20
0
*
*
*
*
N=11
N=13
N=7
N=9
VC
Gab
Gab+Nim
Gab+Nim
+D-APV

preference (62.71 ± 8.85%). This result suggests that gabazine-induced learning is not dependent on LTCCs in the MC layer. Further addition of D-APV to the gabazine and nimodipine cocktail blocked odor preference development (36.66 ± 10.72%). A post hoc Fisher Test of the mean demonstrated a significant difference between the vehicle control and both the gabazine-only ($t = 2.41$, $p < 0.05$) and the gabazine with nimodipine ($t = 2.50$, $p < 0.05$) groups. A significant difference was also observed between the gabazine with nimodipine and D-APV group and both the gabazine-only ($t = 2.26$, $p < 0.05$) and the gabazine with nimodipine ($t = 2.39$, $p < 0.05$) groups. While these results did not shed light on the relationship of LTCCs and β-ARs on MCs, they suggest that activation of the NMDA receptor is sufficient for gabazine-induced learning.

CHAPTER 4 – DISCUSSION

4.1 Summary

Our results show that LTCCs are present in the naïve neonate OB, and, demonstrate cell-specific localization of LTCCs throughout the bulb. Our work also shows that OB LTCCs are involved in early odor learning and short-term memory formation. LTCCs play a critical role in natural learning. LTCC activation is not, however, sufficient to induce learning on its own, and LTCCs do not appear to play an important role in gabazine-induced learning that suppresses PG inhibition. These results provide further evidence in support of the MC potentiation model of early olfactory learning (Yuan et al., 2003b), which proposes that the UCS and CS of odor learning converge within the MC, and result in CREB phosphorylation and the production of plasticity related proteins within the MC (McLean & Harley, 2004; Cui et al., 2007; Silva et al., 1998; Yuan et al., 2003a). We suggest that, within this model, the function of LTCCs is to contribute to the CS via the influx of calcium in response to an odor-evoked response. It remains unclear from our results if the role of β-ARs within the MC potentiation model is tied exclusively to contributing to the UCS of cAMP production, or if β-AR activation also contributes to the CS by facilitating calcium influx via LTCCs.

4.2 LTCC Activation is Necessary to Induce Natural Early Odor Preference Learning

In nature, pups develop an odor preference when novel odors are paired with tactile stimulation from the dam, which stimulates NE release from the locus coeruleus (Galef & Kaner, 1980; McLean et al., 1989; Sullivan & Leon, 1987). Isoproterenol

infusion facilitates the development of odor preference learning because it mimics natural NE release into the OB by stimulating β–ARs (Langdon et al., 1997; Sullivan & Wilson, 1989; 1994). To test if LTCCs are involved in early odor preference learning, we co-infused isoproterenol with the LTCC antagonist nimodipine. Pups that received an intrabulbar infusion of isoproterenol developed a preference for peppermint-scented bedding, while pups that received an infusion of isoproterenol along with nimodipine did not develop an odor preference (Figure 9). This suggests that LTCC activation is a necessary component of the natural odor preference learning process.

Previous data in our lab (Lethbridge et al., 2012) have shown that activation of MC-bound NMDARs is required for early odor preference learning. They demonstrated that a co-infusion of the NMDAR antagonist D-APV was sufficient to block isoproterenol-induced learning. To further confirm if LTCCs are involved in early olfactory learning, we co-infused the LTCC agonist BayK-8644 along with an infusion of isoproterenol and D-APV to see if LTCC activation could rescue the isoproterenol-induced learning from the D-APV block. In our experiments, pups who received a BayK-8644 infusion successfully developed a preference for peppermint-scented bedding, overcoming the D-APV block (Figure 11). This provides further evidence that natural odor preference learning is a LTCC-dependent process.

4.3 β-adrenoceptor Regulation of Glomerular LTCCs Remains Unclear

According to the MC potentiation model of early olfactory learning proposed by McLean and colleagues (Yuan et al., 2003b), learning arises when the CS of odor evoked glutamate release at the ON-MC synapse is paired with the UCS of cAMP production in

MCs. When these two events occur together, this leads to the calcium/calmodulin-regulated phosphorylation of CREB, and the subsequent production of plasticity-related proteins (McLean & Harley, 2004; Cui et al., 2007; Silva et al., 1998; Yuan et al., 2003a). Previous work has demonstrated that pairing odor exposure to β-AR stimulation (either natural or pharmacologically induced) is sufficient to induce an early odor preference learning (Sullivan et al., 2000; Yuan et al., 2003b). From these findings, it has been assumed that β-AR activation contributes to the UCS of cAMP production in MCs (Sullivan & Wilson, 1989; 1994; Yuan et al., 2003b). It has also been shown, however, that β-AR activation is involved in calcium modulation during learning events in the hippocampus and amygdala (Gray and Johnston 1987; Huang et al. 1993, 1996), and that β-ARs regulate LTCC functionality in cardiac myocytes (Hell 2010), as well as in neurons (Davare et al., 2001; Marshall et al., 2011). We were therefore interested in examining if β-AR activation contributed to the CS by facilitating calcium influx via LTCCs, in addition to the β-AR's accepted role in contribution to the UCS of cAMP production, during early odor preference learning.

In order to examine this question, we induced an early odor preference without stimulating β-ARs in the OB. This was accomplished by infusing the GABA-A receptor antagonist gabazine into the lateral OB. This relieved the baseline inhibitory influence exerted by PG cells on both ONs and MCs, so that the incoming odor signal was strong enough to activate intracellular signaling pathways (assumed to be the calcium/calmodulin mediated phosphorylation of CREB). We then co-infused the LTCC antagonist nimpodipine along with gabazine, to determine if preventing LTCC function would block the gabazine-induced learning. If learning had been blocked by nimodipine,

this would have demonstrated that LTCCs were contributing to odor learning independent of β-AR activation.

Our results indicated that nimodipine infusion was insufficient to block the gabazine-induced learning (Figure 12). We do not believe, however, that this implies natural early olfactory learning is LTCC-independent. MC disinhibition via an intrabulblar infusion of gabazine is not reflective of any physiological process likely to be involved during *in vivo* early olfactory learning. Gabazine-induced learning was employed not as a model of natural early olfactory learning, but in an attempt to stimulate LTCCs without the co-activation of β-ARs. Furthermore, this does not necessarily indicate that LTCCs are inactive without β-AR co-activation. LTCCs have been implicated in GABA release from PG cells (Jerome et al., *in press*; Murphy et al., 2005). The application of nimodipine during gabazine-induced learning would have had the effect of inhibiting GABA release from PG cells. Nimodipine-suppression of PG cell GABA release would have enhanced the gabazine-induced disinhibition on the ON-MC synapse since the gabazine would be competing with less GABA for the available GABA-A sites. This effect of the enhanced disinhibition may have been sufficient to mask the effect of any parallel suppression of MC-bound LTCCs. Given the unnatural over-excitation of mitral cells in this circumstance, calcium influx via other channels (NMDARs, mGluRs, or calcium-induced calcium release from intracellular stores) may have been sufficient to initiate the calcium/calmodin phosphorylation of CREB in these circumstances. This later theory is supported by the fact that the co-infusion of the NMDAR antagonist D-APV along with nimodpine did successfully block gabazine-induced learning (Figure 12). This is inline with findings from Lethbridge et al., (2012)

who demonstrated that the co-infusion of D-APV into the lateral OB was sufficient to block gabazine-induced learning.

Further work is needed in order to expand upon our results presented here, and clarify the role β-ARs in the processes of early olfactory learning.

4.4 LTCC Activation Does Not Appear to be Sufficient to Induce Early Odor Preference Learning

Once it had been established that natural early odor preference learning was dependent on LTCC activation (Figure 9), we were interested in testing if LTCC activation itself was sufficient to induce an early odor preference learning. In a similar study by Lethbridge et al. (2012) on the role of MC-bound NMDARs on early olfactory learning, the group first demonstrated that NMDAR activation on MCs was necessary for early olfactory learning, and then supported this finding by demonstrating that stimulation of NMDARs at the ON-MC synapse was sufficient to induce the early odor preference. In our experiments, we infused the LTCC agonist BayK-8644 into the lateral olfactory bulb to test if LTCC activation was sufficient to induce learning. Pups that received an intrabulbar infusion of BayK-8644-only did not develop an odor preference (Figure 11). This suggests that LTCC activation by itself is not sufficient to induce early odor preference learning. In order to understand this potentially surprising result, it is necessary to consider that BayK-8644 infusion into the dorsolateral OB would activate all the LTCCs present in the area.

Our immunohistochemistry results indicate that in this region, LTCCs are present on MC apical dendrites, and on PG cells (Figure 7; Section 4.3). Previous work by

Murphy et al., (2005) demonstrated that LTCCs are present on PG cells and that they are involved in dendritic calcium spikes, which trigger the release of GABA from PG cells. They demonstrated that dendritic calcium spike in PG cells were inhibited by the LTCC antagonist nimodipine, and enhanced by the LTCC agonist BayK-8644.

In our work, infusion of the LTCC agonist BayK-8644 into the lateral OB was expected to activate MC-bound LTCCs. It is possible, however, that the drug activated both MC-bound LTCCs and PG-bound LTCCs. Activation of the MC-LTCCs, if it occurred, would support MC depolarization. Activation of PG-LTCCs, however, would lead to an increased release of GABA onto the ON-MC synapse, which would both inhibit the release of glutamate by presynaptic ONs and hyperpolarize the postsynaptic MCs. Indeed, Western blot analysis has confirmed that BayK-8644 infusion into the dorsolateral OB promotes vesicle release, potentially from PG cells (Jerome et al., *in press*). The increased inhibition of the MCs due to PG-LTCC activation may have been sufficient to block the depolarizing effect of MC-LTCC activation.

Electrophysiology techniques are able to apply small volumes of pharmacological agents in extremely small areas, such as through a puff pipette, or releasing a caged substance with focal laser activation so that only cells in a targeted area will be significantly affected by their release. During electrophysiology experiments, it may be possible to activate or inhibit MC-LTCCs without influencing the activity of PG-LTCCs. Further electrophysiology experiments will therefore be helpful in determining if activation of MC-LTCCs, without the co-activation of PG-LTCCs, is sufficient to depolarize the MC and initiate LTP of the ON-MC synapse.

4.5 LTCC Activation Serves to Amplify Calcium Influx Following NMDAR Activation

Recent studies have demonstrated that NMDAR activation is critical for odor preference learning (Lethbridge et al. 2012; Yuan & Harley, 2012). These findings are supported by the results of our experiments in which the NMDAR antagonist D-APV blocked both isoproterenol-induced (Figure 11) and gabazine-induced learning (Figure 12). Our results confirm that odor preference learning is NMDAR-dependent, and suggests that LTCCs can serve to amplify the effect of NMDAR activation by providing a secondary path by which extracellular calcium can enter MCs.

Isoproterenol-induced learning, which involves an intrabulbar infusion of isoproterenol, is successfully blocked by the co-infusion of either the NMDAR antagonist D-APV, or the LTCC antagonist nimodipine. Since both NMDARs and LTCCs are calcium channels, this suggests that significant calcium influx is required for early olfactory learning, and that simultaneous activation of both NMDARs and LTCCs are required for early olfactory learning. This co-activation is necessary to produce a signal strong enough to counter the inhibitory effect of PG-released GABA on the MC (Murphy et al., 2005).

A BayK-8644 infusion did, however, rescue isoproterenol-induced learning from a D-APV block. A strong LTCC stimulation is therefore insufficient to overcome PG inhibition and induce learning. This suggests that LTCCs may act downstream of NMDARs in the MC response to incoming odor information. This hypothesis is supported by the activation mechanisms of the two calcium channels. Activation of NMDARs via glutamate could reasonably trigger the subsequent activation of the

voltage-gated LTCCs, but LTCC activation could not lead to the activation of glutamate-activated NMDARs.

4.6 Our Findings Support the MC Potentiation Model of Early Odor Preference Learning

The GC-MC disinhibition model (Wilson & Sullivan, 1994) and the MC potentiation model (Yuan et al., 2003b) are two competing theories of the physiological processes that underlie early olfactory learning. Recent studies have provided growing evidence in support of the MC potentiation model. Yuan (2009) demonstrated that stimulation of the ON with TBS produces LTP of the glomerular fEPSP in the OB, and that TBS in the presence of isoproterenol induces a significant calcium response in the cell bodies of MCs. Yuan & Harley (2012) demonstrated that early olfactory learning involves a change in postsynaptic AMPA/NMDA ratio at the ON-MC synapse. And Lethbridge et al., (2012) provided further evidence that NMDARs located on postsynaptic MCs are phosphorylated during odor memory formation, and down-regulated 3 hours following odor preference training. All of these findings indicate that the active physiological processes that lead to early olfactory formation take place within the MC, as proposed by the MC potentiation model.

Our study provides fresh evidence in support of the MC potentiation model of early odor preference learning. Our experiments have demonstrated that learning is dependent on LTCCs located in the lateral OB, far away from the MC-GC synapse. Infusion of the LTCC antagonist nimodipine is sufficient to block isoproterenol-induced learning (Figure 9). We suggest that this indicates LTCCs are functioning downstream of

MC-bound NMDARs. Just as LTCCs allow calcium influx into the dendrites of PG cells, which leads to GABA release from those cells (Murphy et al., 2005), we suggest that in our experiments MC-bound LTCCs allow an influx of calcium along the MC dendrite. We suggest that this calcium influx activates the calcium/calmodulin pathway, which leads to the phosphorylation of CREB, which in turn facilitates the production of plasticity related proteins. These proteins could be responsible for the changes in the AMPA/NMDA ratio and NMDAR subunit composition observed by Yuan & Harley (2012) and Lethbridge et al., (2012). In this case, LTCCs would be contributing to the CS within the MC potentiation model, since they would be activated downstream of the glutamate-induced activation of NMDARs.

Finally, our results suggest that β-ARs may play an additional role in the MC potentiation model of early olfactory learning. While previous studies have focused on the contribution of β-ARs to the UCS within this model, we observed evidence that β-AR activation may also be crucially involved in regulating calcium influx during the CS. Our observations that LTCC stimulation is not sufficient to induce a learning event (Figure 11) is in contrast to the previous findings that stimulation of MC-bound NMDARs was sufficient to induce an early odor preference (Lethbridge et al., 2012). This suggests the possibility that MC-bound LTCCs are under the regulation of β-ARs, as has been suggested in myocetes (Hell, 2010) and in some neurons (Davare et al., 2001). Further work is necessary to determine what role, if any, β-ARs contribute to LTCC regulation and the CS within the MC potentiation model.

4.7 Discussion of fEPSP Experiments

Despite significant effort, I was not able to achieve regular or reliable induction of LTP at the ON-MC synapse in control slices. We were therefore unable to test for the effect of inhibiting LTCC function on the induction of LTP. The protocol used in these experiments has been used successfully before (Yuan, 2009), and so the inability to achieve LTP in control slices is likely due to the skill level of the operator.

Preparing and performing electrophysiology recordings is an advanced technical procedure. In our experiments described here, we were able to reliably obtain baseline recordings in which glomerular fEPSPs demonstrated a strong response to stimulation of the incoming ON. This suggests that I had developed sufficient skill in performing the electrophysiology recordings. My error was not likely due to incorrect placement of the recording pipette within the glomerulus or the stimulation electrode on the appropriate section of ON. This also suggests that the electrophysiology apparatus itself was functioning properly, and our lack of results were not due to faulty equipment or to background interference.

Rather, the lack of successful LTP induction suggests that the cells were not healthy enough to undergo LTP. The area in need of improvement is therefore likely my skill in slice preparation. At each stage of slice preparation (harvesting, cooling, cutting and incubation) there is risk of damaging the cells in the slice. One significant risk is of physically damaging the cells during the harvesting and cutting stages, or from handling the samples during any stage of the process. Another significant concern is the effect of oxygen deprivation. Samples are kept in oxygenated solution at most points throughout sample preparation, but during the harvesting stage and when the sample is being

mounted at the beginning of the cutting stage, the samples are at greater risk of oxygen deprivation. As an inexperienced technician, I performed slice preparation slower than an experienced technician (increasing the periods of oxygen deprivation) and I handled slices more than an experienced technician would have, which likely damaged or killed cells in the test slices.

An unhealthy cell could respond to an incoming signal and achieving an action potential, so long as the cell's membrane was intact and the membrane-bound channels still functioned. As such, unhealthy cells could still be capable of establishing a fEPSP in a glomerulus in response to ON stimulation, as we observed. An unhealthy cell is much less likely, however, to be able to undergo LTP, as this process involves the recruitment of much more cellular machinery involved in intracellular signaling, and protein transcription, translation, and trafficking to the membrane.

4.8 Conclusion and Future Directions

This study has provided, for the first time, a cell-specific map of LTCC expression within the neonate rat olfactory bulb. We have demonstrated that LTCCs play an important function in allowing calcium into MCs as part of the CS during early olfactory learning. LTCC activation was determined to be necessary for early olfactory learning to occur. LTCC activation does not seem to be sufficient to induce an early olfactory preference, though this may be due to the unspecific way in which LTCCs were activated in the region of the OB glomerulus. It remains unclear if LTCC activation depends on the co-activation of β-ARs, as has been demonstrated in other areas of the brain.

Future work should focus on clarifying the functional relationship between LTCCs and β-ARs in neurons of the OB. Further electrophysiology experiments will be helpful in determining if MC-LTCC activation is necessary for ON-MC LTP. Other electrophysiology experiments will be able to test if activation of MC-LTCCs, without the co-activation of PG-LTCCs, is sufficient to depolarize the cell. Optogenetic tools may also be used to selectively suppress or increase the activity of specific neurons *in vivo*. Amongst other possibilities, this will provide an opportunity to study MCs free from the influence of PG cells.

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