AN INVESTIGATION INTO mRNA EXPRESSION IN THE RAT PUP OLFACTORY BULB AFTER ODOR PREFERENCE LEARNING

by

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ABSTRACT

Neonate rat pups rely on odor cues to identify their mother for food and warmth. Olfactory learning modifies behavioral, neural and metabolic responses to odor after a subsequent presentation with 24 h memory being dependent on CREB phosphorylation (pCREB). However the mRNA dependent changes that take place after olfactory learning have not been studied. Our lab previously showed that there is increased pCREB localization in the dorsolateral quadrant of mitral cells of the learning pups (odor + tactile stimulation) compared to the non-learning pups (odor only). In the present study, odor + stroking and odor only groups of pups were used. Following 10 minutes of training, pups were killed, and mRNA from the dorsolateral and ventromedial quadrants of the mitral cell layer was isolated using the laser microdissection (LMD) system for analyses by microarray.

Thirteen genes with a minimum of a 1.5- fold difference in expression levels between groups were then analyzed using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Six of the 13 genes showed concordance between microarray and qRT-PCR. Seven genes showed opposite expression patterns. A second study compared gene expression between whole bulb and LMD samples and a third study utilized qRT-PCR in whole bulbs to analyze the expression of the 13 genes at various times post training. Several of the genes confirmed as changing with learning are known to be involved in plasticity related activities, but were not previously associated with olfactory learning and memory formation. These findings will form an important basis for conducting further studies that will help us understand olfactory learning better.

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List of Abbreviations

2APV	-	2-amino -5-phosphonovaleric acid
5-HT	-	5-hydroxytryptamine`
6-OHDA	-	6-hydroxydopamine
AC	-	adenylate cyclase, adenylyl cyclase
AMPA	-	±-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATF-1	-	activating transcription factor-1
cAMP	-	cyclic adenosine monophosphate
CaMKII	-	calcium-calmodulin dependent protein kinase 2
CRE	-	cAMP responsive element
CREB	-	cAMP response element binding protein
CREM	-	cAMP response element modulator
CS	-	conditioned stimulus
cDNA	-	Complementary DNA
DL	-	dorsolateral
EPL	-	external plexiform layer
GABA	-	^{3}-amino butyric acid
GL	-	glomerular layer
GCL	-	granule cell layer
Gr	-	granule cells
I-1	-	Inhibitor -1
ITM-	-	Intermediate term memory

IPL	-	internal plexiform layer
Jg	-	juxtaglomerular cells
LC	-	locus coeruleus
LMD	-	laser microdissection
LTM	-	long term memory
MCL	-	mitral cell layer
Mi	-	mitral cell
NE	-	norepinephrine
NMDA	-	N-methyl-D-aspartate
NMDAR	-	N-methyl-D-aspartate receptor
O/O	-	odor only
OBP	-	odorant binding proteins
ONL	-	olfactory nerve layer
O/S	-	odor + stroking
OSN	-	olfactory sensory neuron
pCREB	-	phosphorylated CREB
PKA	-	protein kinase A
PND	-	post natal day
PP1	-	protein phosphatase 1
RIN	-	RNA Integrity Number
rRNA	-	ribosomal RNA
qPCR	-	quantitative polymerase chain reaction
qRT-PCR	-	quantitative reverse transcription polymerase chain reaction.

STM	-	short term memory
UCS	-	unconditioned stimulus
VM	-	ventromedial
WB	-	whole olfactory bulb.

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Chapter One

1.0 Introduction

Olfactory learning in rat neonates is a typical classical conditioning paradigm (Sullivan and Leon, 1987). Pups need to learn the odor of the dam in order to survive and this learning can be acquired in a single 10 min. trial (Sullivan and Leon, 1987). Woo and Leon in 1987 showed that the first week of life of a pup (PND1-8) is the most sensitive period for olfactory learning. During this period the pup does not have the ability to differentiate between appetitive and aversive stimuli, however during the second week differentiation begins (Camp and Rudy1988).

Hofer et al, 1975 showed that there are substances on a dam's ventral skin that provide olfactory cues for rat pups' orientation and attachment to nipples. Stimulation of pups by mother (includes dam vigorously licking the pups, stepping on them and picking them up) occurs frequently right from birth and this may form the basis of conditioned learning in pups (Pedersen et al, 1982; Hofer, 1975). Bilateral olfactory bulbectomy in rat pups causes changes in nursing behavior leading to weight loss and eventually death (Singh and Tobach, 1975).

The present study is concerned with identifying changes in messenger RNA expression that occur in the olfactory bulb in rat pups forming an odor preference memory in the first week of life. This work takes advantage of evidence that changes in the olfactory bulb

are necessary and sufficient for odor preference learning and that critical mRNA transcription is required for learning in the first hour after a single training trial.
Prior to outlining the present experiments, a review of the olfactory circuitry and odor perception will be presented. Following this I will briefly review the neural underpinnings of olfactory conditioned learning. Finally, I will consider the role of CREB, a transcriptional factor implicated in early odor preference learning, in learning and memory in general as well as provide a brief discussion of what is known about the dependence of early odor preference learning on protein synthesis.

1.1 History of studies on the olfactory bulb

The first work on the description of the general structure of the mammalian olfactory bulb using the procedure of bichromate-silver impregnation was done by Dr. Camillo Golgi in 1875 (Golgi, 1875 translated by Shepherd et al., 2010). This all-important piece of work laid the foundation of modern neuroscience because work by other histologists built on the work done by Golgi. Santiago Ramon y Cajal was able to produce an elaborate illustration of the olfactory nerve pathway and its numerous cells by using Golgi's method in the late nineteenth century. He published an extensive discourse on the olfactory nerve fibers and their distinct peripheral origin in 1890 (Cajal 1890, translated by Levine et al., 2008).

Calleja in 1893, Kolliker in 1896 and Blanes (1897) together with Cajal and Golgi were the histologists who worked most extensively on the cellular structure and distribution of the neurons in the olfactory bulb (as cited by Allison A.C., 1953). Further work was done

by histologists such as Potter and Winker, Read, Smith, Humphrey and Crosby, all in the nineteenth century, on other aspects of the structure of the olfactory bulb, but they did not add much detail to what had been previously described by Cajal (as cited by Allison, 1953). In the early 20th century Allison investigated the cell- types of the olfactory bulb and their relationship to the olfactory nerves and olfactory tracts and concluded that the lateral olfactory tract and anterior commissure are composed of axons from both tufted and mitral cells of the olfactory bulb (Allison, 1953).

1.2 The olfactory circuitry

The olfactory system in the rat is vital for a number of processes including reproductive and maternal functions, aggression and neuroendocrine regulation (Shipley et al., 1996). The olfactory system is also an important model for studying the neural mechanisms involved in learning and memory formation from the intracellular to the behavioral level (Kosaka and Kosaka, 2005).

The olfactory bulb of the rat is made up of the following layers: olfactory nerve layer, glomerular layer, external plexiform layer, mitral cell layer, internal plexiform layer, granule cell layer and ependymal zone.

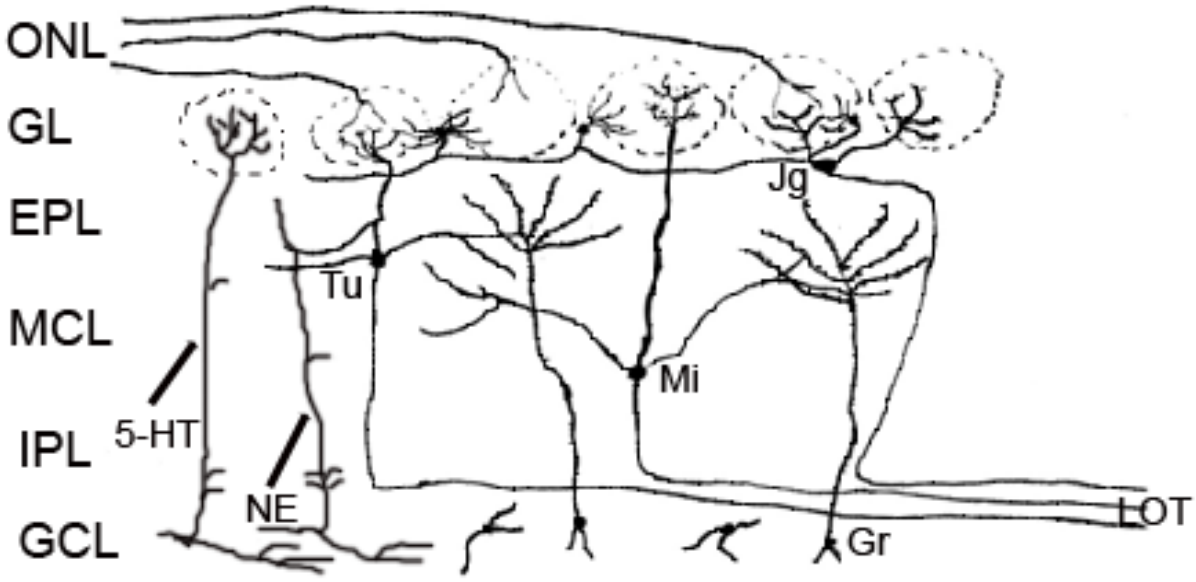

Figure 1: A schematic diagram showing the cytoarchitecture of the main olfactory bulb. The mitral cell layer, specifically the major projection cell of the olfactory bulb, the mitral cell (Mi), is the focus of this thesis. Other major cells in the bulb include juxtaglomerular cells (Jg), tufted cells (Tu) and granule cells (Gr). Centrifugal afferents relevant to this thesis include serotonergic (5-HT) and noradrenergic (NE) input. Other abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; IPL, internal plexiform layer; GCL, granule cell layer; LOT, lateral olfactory tract (formed by axons of bulbar output cells). The dotted lines represent the glomeruli in the GL.

1.2.1 Olfactory nerve layer

The olfactory nerve layer (ONL) is made up of olfactory nerves and glial cells. The olfactory nerves arise from the olfactory sensory/receptor neurons (OSN) in the nasal mucosa (Pinching and Powell, 1972). The OSN axons from the olfactory epithelium merge to form the olfactory nerve. At the olfactory bulb, the OSN axons form a plexus which is called the olfactory nerve layer (Akins and Greer, 2006). Axons reorganize within the ONL before reaching their respective glomeruli (Au et al., 2002).

1.2.2 Glomerular layer

The glomerular layer (GL) is the second deepest layer of the olfactory bulb and it is made up of spherical structures called glomeruli that contain the terminal axons of the OSN and their contacts with the apical dendrites of mitral cells, tufted cells and juxtaglomerular interneurons (Pinching and Powell, 1971a; Pinching and Powell, 1971b). Juxtaglomerular cells (Fig. 1) include periglomerular cells, short axon cells and external tufted cells (Pinching and Powell, 1971b) which are all interneurons that modify incoming OSN input from the nose or modify excitation of mitral cell dendrites. The glomeruli range in size from 60 - 80 μm in diameter. The number of glomeruli in the adult rat is about 3000 (Meisami and Safari, 1981). The main neurotransmitters in the juxtaglomerular interneurons of the GL are ^{3}-amino butyric acid (GABA) and dopamine (Gall et al., 1987).

1.2.3 External plexiform layer

The external plexiform layer (EPL) is made up mainly of dendrites of the mitral/tufted cells and granule cells and has a low cell density, it contains reciprocal synapses between the dendrites of the mitral/ tufted cells and the dendrites of granule cells (Price and Powell, 1972a). The EPL has the highest amount of GABA and glutamic acid decarboxylase activity in the olfactory bulb (Graham, 1973) most likely from dendrites of granule cells. The EPL is made up of three overlapping zones, the deepest zone, the intermediate zone and the superficial zone (Orona et al., 1984). The superficial layer of the EPL (closest to the glomerular layer) is made up of short dendrites of tufted cells (Orona et al., 1984). The intermediate zone of the EPL consists of secondary dendrites of middle tufted cells (Mouradian and Scott, 1988; Orona et al., 1984). The deepest zone of the EPL contains internal tufted cells and many basal dendrites of mitral cells (Mouradian and Scott, 1988; Orona et al., 1984). It is this deepest zone where the dendrodendritic interactions between mitral and granule cells take place.

1.2.4 The mitral cell layer

The mitral cell layer (MCL) is the next deepest layer of the olfactory bulb and the most important layer relevant to this thesis. It contains the cell bodies of mitral cells and granule cells as well as blood vessels and glial processes (Bailey and Shipley, 1993; Scott, 1986). One estimate suggests there may be two granule cells for every mitral cell in the mitral layer of older rats (McCollum et al, 1997). However, the mitral cells visually predominate this layer because of their large size, thus the name of the layer. The mitral cell bodies range in diameter from 25-35µm (Shipley et al., 1996) and the cells create,

essentially, a single cell layer (Scott, 1986). They are the largest cells in the olfactory bulb with their primary and secondary dendrites extending in to the EPL and the GL (Fig. 1); the primary dendrite extends into only one glomerulus (Price and Powell, 1970b). The smooth apical dendrites (about 200-300µm in length) extend to the glomerular layer and form axodendritic synapses with the OSN axonal input from the olfactory epithelium (Scott, 1986). Periglomerular cells (both GABAergic and dopaminergic types) make dendrodendritic synapses with mitral cell dendrites in the glomerului (Schoppa and Urban, 2003) and in the EPL, mitral cells also make dendrodendritic synapses with granule cells (Montague and Greer, 1999). There is a large literature base regarding physiological interactions of mitral cells with other cells in the olfactory bulb. However, such physiological information goes beyond the scope of this thesis so will not be examined in detail here.

1.2.4.1 Differences between Mitral Cells and Tufted Cells

The output cells of the olfactory system, mitral cells and tufted cells have some features in common however other morphological features distinguish them (Orona et al., 1984). The cell bodies of mitral cells lie in the mitral cell layer while those of the tufted cells are mainly found in the EPL (Orona et al., 1984). Many external tufted cells lack secondary dendrites but mitral cells have secondary dendrites that ramify in the deeper portion of the EPL (Macrides and Schneider, 1982).Tufted cells are generally much smaller than mitral cells (Macrides and Schneider, 1982). The mitral cells and tufted cells contain different types of neurotransmitters and neuropeptides: catecholamines, substance P and

encephalin have been found in the external tufted cells but not in mitral cells and internal tufted cells (Macrides and Schneider, 1982). Mitral cells contain glutamate, an excitatory neurotransmitter (Ottersen and Storm-Mathisen, 1984). There is a significant increase in the release of glutamate from mitral cells during olfactory learning (Brennan et al., 1998) which is one of several lines of evidence of mitral cell involvement in odor memory. Axons of tufted cells project mainly to the rostral part of the olfactory cortex near the lateral olfactory tract (Haberly and Price, 1977). Mitral cell axon collaterals project to the following areas: the anterior olfactory nucleus, anterior olfactory tubercle, lateral olfactory tract and anterior piriform cortex (Ojima et al., 1984). There is growing literature of secondary olfactory regions being involved in olfactory processing. However, that information is outside the scope of this thesis so will not be covered in detail here.

1.2.4.2 Receptors on the mitral cells

There appear to be several receptor interactions on mitral cells that are involved in early olfactory memory formation. Work by Yuan et al. (2003) showed the presence of ² 1-adrenoceptors and 5-HT_{2A} receptors on mitral cells. The co-localization of these receptors on mitral cells is hypothesized to be important in olfactory learning as discussed in Section 1.4 (and Figure 3). There are also NMDA, AMPA (±-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate receptors on mitral cells (Ennis et al, 1996). The NMDA receptors on mitral cells associate presynaptic OSN input (glutamate release) with post synaptic mitral cell depolarization caused by isoproterenol activation, during olfactory learning (Lethbridge et al, 2012). There is an increase in membrane

AMPA receptors following olfactory learning, infusion of an interference peptide against AMPA insertion disrupted memory formation showing that AMPA insertion is necessary for memory formation (Cui et al, 2011). Besides the ² 1-adrenoceptors on mitral cells, there are alpha 1 and 2 (±1 and ±2) adrenoceptors (McCune et al, 1993). NE, via ±1 adrenoceptors, depolarizes mitral cells and this causes an increase in mitral cell responsiveness to ON input (Ciombor et al, 1999; Hayar et al, 2001). Thus, these receptors have been linked to odor perception. Olfactory learning can also be achieved via activation of ±2-adrenoceptors (Shakhawat et al, 2012). In summary, a number of receptor types are on mitral cells and some of them have been linked to memory formation. While short lasting phosphorylation of receptors might mediate some of the learning plasticity (Cui et al., 2011; Lethbridge et al., 2012), it is also possible that formation of memory may result in changes to transcription of these receptors in order to sustain memory.

1.2.5 The internal plexiform layer

The internal plexiform layer (IPL) is found between the mitral cell layer and the granule cell layer (Fig. 1). The IPL is mainly made up of dendrites of granule cells and axons of mitral and tufted cells. The IPL also contains axons from other parts of the brain including serotonergic axons from the raphe nuclei (McLean and Shipley in 1987(b)) and noradrenergic axons from the locus coeruleus (McLean et al., 1989). The presence of cholecystokinin-like immunoreactivity in the IPL has also been described (Seroogy et al., 1985).

1.2.6 The granule cell layer

The granule cell layer (GCL) is made up mainly of granule cells (Fig. 1), which are a group of inhibitory interneurons (Scott, 1986). The granule cells are small in size with diameter of about 8-10µm, they are the most numerous cells in the olfactory bulb (Shepherd, 1972; Scott, 1986). Granule cells are bipolar cells whose dendrites either extend up into the EPL or deeper into the GCL, granule cells have no axons (Jackowski et al., 1978). The dendrites of the granule cells extending into the EPL have spine-like appendages called gemmules that interact with mitral cell secondary dendrites as mentioned in Section 1.2.3 (Price and Powell, 1970c).

1.2.7 Subependymal zone (SEZ)

This is the deepest layer of the olfactory bulb. The subependymal zone cells line the ventricle and provide progenitors of most olfactory bulb cells during development (Kishi et al., 1987). The stem cells in the SEZ generate neurons that migrate along the rostral migratory stream to the olfactory bulb and differentiate into various types of interneurons (Whiteman and Greer, 2009). Neural stem cells (NSC) also generate oligodendrocytes (Menn et al., 2006). NSCs are affected by different neurological diseases: in Huntington's disease there is increased proliferation of progenitor cells, in Parkinson's disease there is a decrease in the progenitor cell production and in Alzheimer's disease the A-² proteins are toxic to progenitor cells (Curtis et al., 2007).

1.3 Odor perception in the olfactory bulb

Olfactory perception begins in the olfactory sensory neurons (which can also be referred to as olfactory neurons or receptor neurons) located in the olfactory epithelium (Buck, 1996). In the rat, the olfactory epithelium is located on the medial surface on the endoturbinate bones (Scott and Brierley, 1999). Olfactory neurons are usually short lived and are replaced every 30-60 days (Graziadei and Monti-Grazaiadei, 1979; Farbman, 1990; Buck, 1996). It is a bipolar nerve cell that has its apical pole extending a dendrite to the epithelial surface and from its basal pole it extends an axon to the olfactory bulb (as reviewed in Buck, 2000). On the cilia of the olfactory neuron dendrite are odorant receptors (Buck, 1996). The main function of the olfactory neuron is to detect odorants, code information about their intensity, quality and duration, and change this information into electrical signals that can be processed by the brain (Getchell et al., 1984).

1.3.1 Odorants and odorant binding proteins

Odorants are small and mostly lipid soluble molecules that pass into the nasal mucosa and are detected by odorant receptors in the cilia of the olfactory neurons (Buck, 1996). They are small molecules with molecular weights of less than 1 KDa, odorants are light enough to be breathed, but complex enough that they have unique features (Bell, 1996).Odorant binding proteins (OBPs) are soluble proteins found in the nasal mucosa that are responsible for concentrating, transporting and removing odorants (Pevsner et al., 1986; as reviewed in Buck, 2000; Avanzini et al., 1987). OBPs belong to a family of proteins

called lipocalins, which bind to different odorants (Bell, 1996). The olfactory mucosa contains different OBPs, which detect distinct classes of odorants (Dear et al., 1991).

1.3.2 Odorant receptors

Odorant receptors were first cloned by Linda Buck and Richard Axel in 1991(Buck and Axel, 1991). A multigene family that was initially identified in the rat codes for 1,000 different odorant receptors (Buck, 2000, Buck and Axel, 1991). Odorant receptors are G-protein coupled receptors (Dohlman et al., 1991) and are organized such that each olfactory neuron expresses one type of odorant receptor. Olfactory sensory (receptor) neurons with the same type of odor receptor are generally found in the same zone of the olfactory epithelium (Buck, 2000). Odorant receptors are selective but rather non-specific and single odorants activate more than one receptor and one odorant receptor responds to multiple odorants (Breer, 2003).

1.3.3 Odorant information in the olfactory bulb

The axons of olfactory neurons, which carry sensory information from the nose, form synapses within the glomeruli (Buck, 1996). In the glomerulus, the olfactory sensory neuron axons form synapses with the periglomerular cell axons, the tufted cell axons and the mitral cell axons (Buck, 1996). In rodents, there are about 2400 glomeruli and each glomerulus contains axons from about 1000 to 3000 olfactory receptor neurons, which then synapse onto about 20-50 mitral and tufted cells (Meisami, 1990; Buck, 1996).

Olfactory neurons that project to the same glomerulus can be found in populations in the olfactory epithelium (Jastreboff, 1984). In the glomeruli olfactory axons make excitatory synapses onto dendrites of mitral cells (Mori, 1987), tufted cells and periglomerular cells (Mori, 1987). Mitral cells and tufted dendrites also make dendrodendritic synapse onto PG cells, which express GABA to inhibit the mitral/tufted cell dendrites (Mori et al., 1987). Activation of mitral/tufted cells causes the release of glutamate, which depolarizes the dendrites of granule cells and granule cells in return release GABA, which hyperpolarizes the mitral cells (Jacobson et al., 1986). The excited granule cells are known to cause lateral inhibition in the nearby mitral/tufted cells which helps in the process of enhancing the mitral cell output (olfactory information) sent to the other brain areas (Shepherd et al., 2004).

Odorants typically show combinatorial profiles of glomerular activation (Su et al, 2009) and glucose uptake in response to an odor input can be characterized as a specific spatial pattern on the surface of the olfactory bulb (Johnson and Leon, 2007). Thus odorants are selectively associated with mitral cell regions and this association is typically similar across animals.

1.3.4 Information processing in the olfactory cortex

Although this thesis concentrates on odor learning within the olfactory bulb, olfactory information is eventually relayed to other regions of the brain. These connections were especially investigated in the 1970's and 1980's when modern neuroanatomical tracing

techniques became available. The olfactory cortex is made up of the areas of the brain that receive direct input from the olfactory bulb (Price, 1973). The main olfactory bulb projects axons to the following areas of the olfactory cortex: the piriform cortex, lateral entorhinal area, agranular insula, olfactory tubercle, anterior olfactory nucleus pars externa, anterior olfactory nucleus pars principalis, ventral portion of the tenia tecta, anterior cortical amygdaloid nucleus, posterolateral cortical amygdaloid nucleus and peduncular cortex (Neville and Haberly, 2004; Shipley and Adamek, 1984; Price, 1973). Axons of mitral and tufted cells (Figure 1) extend to the olfactory cortex through the lateral olfactory tract (Haberly and Price, 1977). The anterior olfactory nucleus is responsible for mediating information transfer between symmetrical regions of the olfactory bulb (Scott et al, 1985; Schoenfeld and Macrides, 1984). Olfactory information from the amygdala, piriform cortex and olfactory tubercule projects to the hypothalamus while olfactory information from the entorhinal area projects to the hippocampus (as reviewed in Buck, 2000; Buck, 1996). Information through the thalamus to the orbitofrontal cortex (from the amygdala, piriform cortex, olfactory tubercle and entorhinal area) is involved in perception and odor discrimination (Buck, 1996; as reviewed in Buck, 2000). The piriform cortex has also been shown to be involved in olfactory learning, pairing ^{2}-adrenoceptor stimulation and odor in the piriform cortex has been shown to lead to odor preference learning (Morrison et al., 2013).

1.4 Early olfactory learning

1.4.1 Basis of olfactory conditioned learning

Olfactory conditioned learning is a classical conditioned learning in which the conditioned stimulus (CS) is associated with an unconditioned stimulus (UCS) to produce a conditioned response. A conditioned stimulus is an initially neutral stimulus (example- peppermint odor) that after repeated association with the unconditioned stimulus (example- tactile stimulation) triggers the conditioned response (example- olfactory learning).

Pedersen and Williams (1982) paired a novel odor (lemon odor) with tactile stimulation (stroking with soft artist brush) in 3-day old albino rats and their results showed that these pups developed a conditioned preference for the novel odor. Sullivan and Hall (1986) showed that tactile stimulation is as reinforcing as milk in a classical conditioning paradigm where each can act as UCS. This learning model became an attractive means to study mechanisms of learning because of its simplicity and ability to identify the areas involved in the learning. Some other related studies of an unconditioned stimulus paired with a novel odor include: odor of dam's saliva (Sullivan et al., 1986), mild foot shock of 0.1- 0.5mA (Camp and Rudy, 1988), high humidity (100% relative humidity; Do et al., 1988), tail pinch (Sullivan et al., 1986), brain stimulation using implanted stimulating electrodes in the medial forebrain bundle/lateral hypothalamus (Wilson and Sullivan, 1990), and increasing ambient temperature (33°C; Pedersen et al., 1982).

1.4.2 Receptors within the olfactory bulb and neurotransmitters involved in early olfactory preference learning

Work by Lincoln et al., (1988) showed that pups injected with 2APV (2-amino -5-phosphonovaleric acid), an N-methyl-D-aspartate (NMDA) receptor blocker had less 2-deoxyglucose uptake in the glomerular area of the olfactory bulb and also spent less time over peppermint odor after the training session than normally trained pups. The results of their work show that activation of NMDA receptors (NMDAR) is needed for the behavioral response to olfactory training. NMDAR is a type of inotropic glutamate receptor that plays a role in many brain functions including learning and memory. NMDAR are down-regulated during normal memory formation (Lethbridge et al., 2012). NMDAR on mitral cells associate presynaptic glutamatergic ON input with postsynaptic mitral cell depolarization. These receptors on mitral cells therefore act as coincidence detectors (Lethbridge et al., 2012) for the CS (the presynaptic release event) and the UCS the modulation of the mitral cell dendrites). NMDAR have also been shown to interact with L-type calcium channels on the mitral cell (Jerome et al., 2012). During synaptic stimulation there is evidence that NMDAR (found on the distal dendrites of mitral cells in the GL) activation increases calcium transients (Yuan and Knopfel, 2006) and depolarizes the dendritic tuft. The NMDAR-mediated depolarization is thought to trigger the opening of L-type calcium channels along the apical dendrite leading to the entry of calcium closer to transcription sites. If L-type calcium channels are blocked learning does not occur. These transcription effects may be mediated by activation of the calcium–

calmodulin pathway (for a discussion of these ideas see Dolmetsch et al., 2001; Jerome et al., 2012; Satin, 2011).

McLean and Shipley (1987a) showed that serotonergic fibers begin entering the olfactory bulb of rats after the first 4 days following birth and by the eighth day all layers of the olfactory bulb to receive 5-HT are innervated. McLean and Shipley's work showed that the glomeruli (point of information transfer from the nose to the olfactory bulb) have the densest serotonergic innervation in the olfactory bulb. Depleting bulbar 5-HT prevents normal early odor preference learning (McLean et al. 1993) and, similarly, blocking 5-HT_2 receptors in the olfactory bulb prevents normal learning (McLean et al. 1996). However, olfactory learning can be achieved in pups which are depleted of serotonin if they are administered high doses (between 4mg/kg and 6mg/kg) of the ² - adrenoceptor agonist isoproterenol (Langdon et al., 1997). This finding suggests that ² -adrenoceptor activation serves as a UCS for olfactory memory formation and that 5-HT acts as a helper or primer, but its role is not essential for olfactory learning (see Figure 3 for hypothesized interactions).

1.5 Role of norepinephrine and locus coeruleus in neonate olfactory learning.

1.5.1 Locus coeruleus

The locus coeruleus (LC), also called nucleus locus coeruleus, is located in the dorsolateral pons. In the rat it has 1600 neurons and all of these neurons are noradrenergic (Swanson L.W, 1975). The LC projects to several brain areas and it is involved in many

functions, such as maintaining the sleep–wake cycle (Chu et al., 1973), analgesia (Bodnar et al., 1978), stress (Corrodi et al., 1971) and olfactory learning (Sullivan and Wilson, 1994). Approximately 40 % of all LC neurons, in the rodent, project to the olfactory bulb and the projections are approximately 10 times greater in the olfactory bulb than to any other part of the cerebral cortex (Shipley et al., 1985).

1.5.2 Norepinephrine and locus coeruleus in olfactory learning

Norepinephrine (NE) is a catecholamine also known as noradrenaline. It is derived from tyrosine and serves as a neurotransmitter in both the central nervous system and in the autonomic system (Hoar, 1983). At birth rat pups have a significant number of neurons (approximately 200) from the locus coeruleus projecting into the olfactory bulb (McLean and Shipley, 1991). Work by Wilson and Leon (1988) showed that NE modulation of olfactory bulb excitability starts in the first week of life and may play an important role in the pup's response to olfactory cues, with the NE system playing a critical role in conditioning-induced plasticity in the rat pup (Sullivan et al., 1989). Bilateral 6-OHDA (6-hydroxydopamine) infusions into the locus coeruleus on PND4, before olfactory conditioning on PND6, resulted in a significant depletion (83-91%) of olfactory bulb NE and blocked odor preference learning. This suggests that NE from the LC projection to the olfactory bulb is necessary for olfactory learning (Sullivan et al., 1994). Odor cues activate LC and cause the release of NE (as stated by Nai et al., 2010). However, NE is not needed for the expression of the learned response because work by Sullivan et al. (1991b) showed that injecting trained pups with a ^{2}-adrenoceptor antagonist (timolol or

propranolol) 1 hr before 24 hr olfactory memory testing did not block olfactory memory. Pairing a systemic injection of an NE receptor agonist (isoproterenol) with an odor produces preference for that odor. This means that NE acts as a UCS through stimulation of ^{2} -adrenoceptors (Sullivan et al., 1991a). Figure 3 shows the hypothesized interactions of the UCS mediated by NE and supported by 5-HT and the CS mediated by glutamate AMPA and NMDA receptors.

The ability of NE to act as a UCS is dose dependent; overstimulation (4-6 mg/kg body weight of isoproterenol) or under stimulation (1mg/kg body weight of isoproterenol) does not produce odor preference learning, however the optimal dose of isoproterenol (2mg/kg) produces odor preference learning when paired with an odor (Sullivan et al., 1989). Thus, the dose response produces an inverted U-curve (Figure 2).

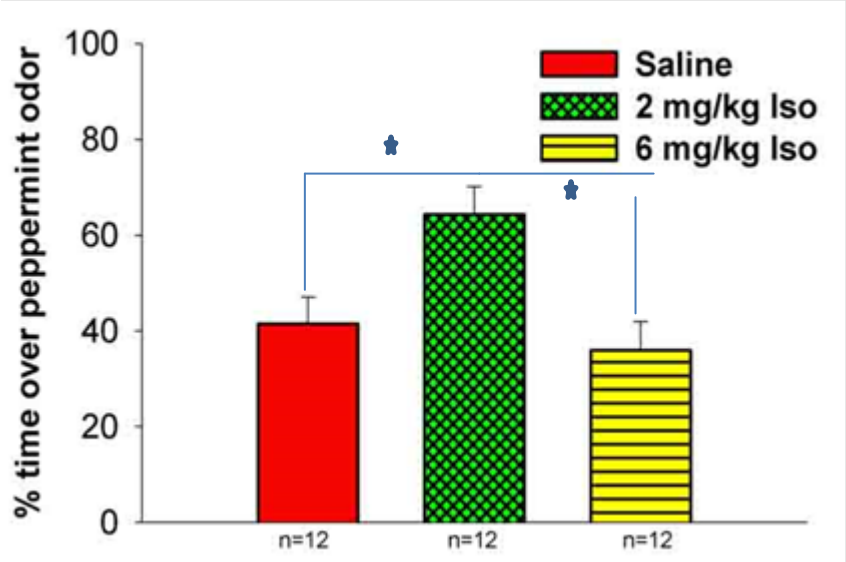

Figure 2: Behavioral experiment for odor preference memory showing the inverted U-curve that is typical when varying concentrations of the UCS, isoproterenol (Iso, β-adrenoceptor agonist), is given before a 10 min odor presentation. The odor memory test was performed by me 24 hr after training. One way ANOVA ($p = .0044$, $F_{(2,33)} = 6.417$), Post hoc analysis using the Tukey –Kramer test confirmed significant difference between saline and 2mg/kg Iso and 2mg/kg Iso and 6mg/kg Iso groups. *$p<0.05$).

Sullivan et al. (1989) showed that a combination of a suboptimal dose of isoproterenol with a suboptimal intensity of another UCS (such as stroking) when paired with an odor also produces odor preference learning. Pups injected with propranolol (a ² -adrenoceptor antagonist) immediately or 1 hr after training showed no odor preference but pups injected with propranolol 4 hr after training showed odor preference for the CS. The results of this study show that NE is critical for the consolidation, as well as the acquisition of olfactory memory, and this takes place less than 4 hr after the training session (Wilson et al., 1994). The critical involvement of β-adrenoceptors as the UCS for odor preference learning implicates the cAMP-PKA-CREB pathway in this model. We now turn to a consideration of the role of CREB and the CREB pathway in odor preference learning.

1.6 Role of CREB in olfactory memory formation.

1.6.1 CREB

cAMP response element binding protein (CREB) is a family of nuclear transcription factors that bind to sites on a gene's promoter called cAMP responsive element (CRE; Silva et al., 1998). The mammalian CREB family includes 3 homologous genes CREB, cAMP response element modulator (CREM) and activating transcription factor-1(ATF-1; Alberini, 2009). CREB and AFT-1 are expressed in all tissues but CREM is expressed mainly in the neuroendocrine system (Barco et al., 2003). CREB was identified through studies on the neuropeptide hormone gene somatostatin (Montminy and Bilezikjian, 1987). CREB has a molecular mass of 43 kDa and it is phosphorylated *in vitro* when

incubated with the catalytic subunit of cAMP dependent protein kinase (Montminy and Bilezikjian, 1987). CREB is phosphorylated *in vivo* in the nucleus by protein kinase A at Ser 133 followed by the recruitment of CREB binding protein (CBP) and p300; this makes CREB's transcriptional activity functional (De Cesare and Sassone-Corsi, 2000; Mayr and Montminy, 2001).

1.6.2 CREB in olfactory memory formation.

Work by Dash et al. (1990) indicated that phosphorylated CREB (pCREB) is essential for long-term memory formation in *Aplysia*. This finding has been confirmed in many other studies from several species including Drosophila (Yin et al., 1994), mice (Hummler et al., 1994) and rats (Guzowski and McGaugh, 1997). In 1999, McLean et al. showed that after odor learning there was a significant increase in pCREB staining in the nuclei of mitral cells, via immunohistochemistry, in the dorsolateral quadrant of the learning group (odor +stroking) compared to the non-learning groups (stroking only, odor only and naïve). In addition the results also showed that the ventromedial quadrant of mitral cells of the learning group (odor +stroking) showed less pCREB staining density and there was no significant difference among groups for this quadrant. In these studies, peppermint odor was used and peppermint has been shown to primarily enhance metabolism (visualized by 2-deoxyglucose uptake) in the dorsolateral quadrant of the olfactory bulb (Johnson and Leon, 2007; Woo et al., 1987). The McLean study also showed that pCREB levels were highest 10 min after the training session and had returned to normal levels by 60 min. Additionally, serotonin and NE (mimicked by isoproterenol in the experimental model) together interact in the olfactory bulb during odor preference

learning to promote cAMP increases in the cells activated by odor and this in turn promotes CREB phosphorylation (Yuan et al., 2000). Both CREB and pCREB have been shown to have causal roles in rat pup olfactory learning (Yuan et al., 2003). The hypothesized intracellular pathways leading to CREB phosphorylation are shown in Figure 3 in Section 1.7.

1.6.3 CREB regulated genes

The binding of CREB to the TGACGTCA sequence in most cAMP responsive genes is the basis of cAMP induced gene expression (Johannessen et al., 2004). CREB's important role in cellular function is shown by the large number of CREB related genes and the diverse functions of the proteins that are encoded by the genes (Johannessen et al., 2004).

Changes in behavior associated with learning are generally assumed to require functional and/or anatomical changes in neural connections. In their review of genes controlled by CREB, Mayr and Montminy (2001) identified ten functional groups where there was evidence of CREB control. Of these groups at least half could be involved in the support or initiation of changes in neural connectivity. Specifically, genes controlling receptor production or neurotransmitter production might be up- or down-regulated, those involved in cell survival, as well as those involved in supporting the growth of processes, could be part of structural change. Genes encoding structural proteins themselves might be altered. Since delivery of material is also likely to change, genes involved in transport

are another class of functional CREB genes that might be of interest. Metabolic genes could be recruited if activity patterns were altered.

1.7 The cAMP/PKA/CREB Pathway

The hypothesized signalling pathways involved in the process of olfactory preference learning are shown schematically in Figure 3. This figure illustrates the pairing of a CS (peppermint odor) with an UCS (e.g., stroking, norepinephrine, or isoproterenol) in a mitral cell.

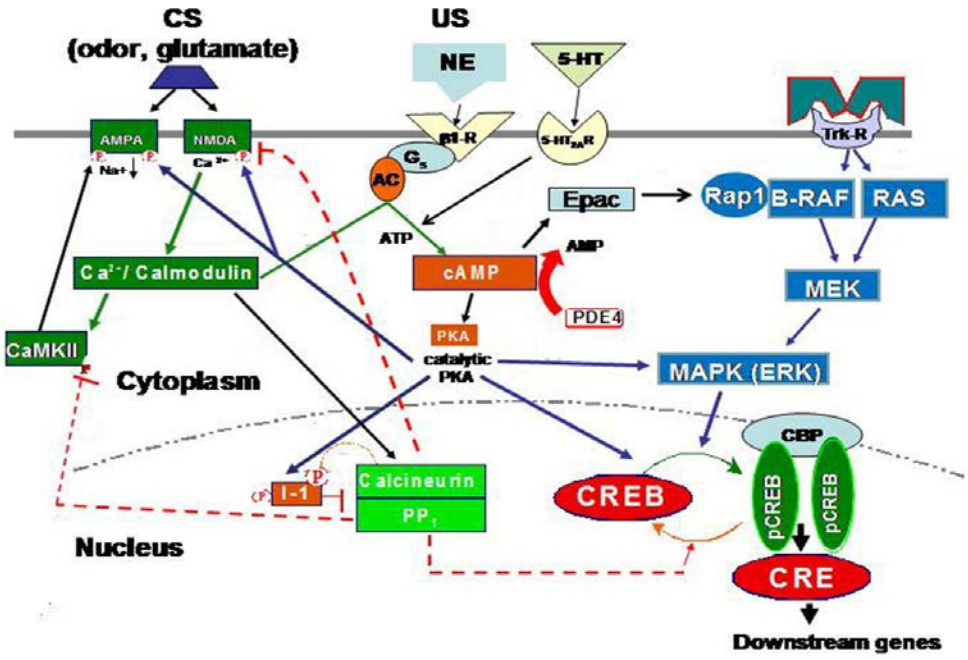

Figure 3: Hypothesized intracellular pathways involved in memory formation that take place in the mitral cells of the olfactory bulb (original figure by John McLean).

NE released by stroking, for example, acts as the UCS to activate a ² 1-adrenoceptor causing the G-protein coupled to this receptor to be active. This results in increased adenylyl cyclase (AC) activity (Bockaert et al., 1977; see McLean and Harley, 2004, for review). As well, a synergistic interaction between NE and 5-HT through ² 1-adrenoceptor and 5-HT_{2A} receptors co-localization on mitral cells (see Fig. 3) further facilitates cAMP production (Yuan et al., 2003; Madison and Nicoll, 1986). The peppermint odor CS, mediated by glutamate release at the olfactory receptor cell axonal terminals in the glomerular layer, activates AMPA and NMDA receptors (Yuan et al., 2003). Additionally, Ca^{2+}/Calmodulin acts on Ca^{2+}/Calmodulin-sensitive AC to modulate

cAMP following the peppermint representation (Mons et al., 1999; Yovell et al., 1992). Optimal patterns of cAMP production leads to its binding and activating PKA (Protein kinase A), this is known to increase CREB phosphorylation. PKA phosphorylates CREB at Ser-133 (Gonzalez and Montminy, 1989; Kaang et al., 1993; Mayr and Montminy, 2001). CREB has been shown to bind to cAMP response element (CRE) sites to either decrease or increase transcription of downstream genes (Silva et al., 1998; Mayr and Montminy, 2001).

There are secondary pathways that lead to dephosphorylation of CREB. These pathways include the PKA activation of phosphodiesterase 4 (PDE4), which causes the breakdown of cAMP into AMP (Sample et al., 2012; Barad et al., 1998). PKA also activates inhibitor-1(I-1) which dephosphorylates/activates protein phosphatase 1 (PP1). Calcineurin also dephosphorylates I-1 to increase the activity of PP1 (Nimmo and Cohen, 1978). Calcineurin (protein phosphatase 2B) is a Ca^{2+}/Calmodulin dependent serine/threonine phosphatase (Florio et al., 1996). Christie-Fougere et al. (2009) showed that inhibition of calcineurin increased the duration of pCREB phosphorylation and increased the duration of olfactory memory. This suggests that the duration of CREB phosphorylation may modulate the duration of memory.

1.8 The role of protein transcription in early olfactory preference memory

It has been proposed for decades that memory formation has a biochemical basis and depends on the formation of molecules such as proteins and nucleic acids (as stated by Flexner et al., 1962). Flexner et al. (1963) showed that puromycin, an antibiotic and

protein synthesis inhibitor blocked memory in mice. Studies using inhibitors of protein synthesis such as cyclohexamide and anisomycin have been shown to inhibit long term memory, but not short term memory, when they were applied just before or after training (Flood, 1975; Squire et al., 1974). While these earlier findings are consistent with more recent evidence for the importance of transcription in learning and memory (Alberini, 2009) there are alternative interpretations of these data. See the 2008 issue of the Neurobiology of Learning and Memory (Volume 89) for discussions of the pros and cons of the ability of protein synthesis inhibitors to address the role of de novo transcription and translation in learning and memory.

Work by Grimes et al., (2011) looked at the effect of inhibiting translation and transcription on early olfactory preference learning. In this study actinomycin-D (a transcription inhibitor) and anisomycin (a translation inhibitor) were used. Rat pups were cannulated on PND5, trained for olfactory learning on PND6, followed by olfactory bulb infusion of actinomycin, anisomycin or saline. Pups were tested 1, 3, 5 or 24 hr after olfactory learning. Results showed that blocking protein translation by anisomycin disrupts 5 hr (intermediate term memory, ITM) and 24 hr (long term memory, LTM) memory but does not affect 1 hr and 3 hr (short term memory, STM) memory. When anisomycin was infused either at 1 hr or 3 hr after the olfactory training, results showed that protein translation for 24 hr memory occurs within the first hour after olfactory learning. The pups that had anisomycin 3 hr post training showed significant olfactory memory after 24 hr; demonstrating that the proteins required for 24 hr memory are produced before 3hr post training. Additionally, when actinomycin–D was infused

immediately after training and its effect on 5hr and 24 hr memory was examined the experiment showed protein transcription is required for LTM (24 hr memory) but not ITM (5hr memory). The results of the study showed that LTM is both translation and transcription dependent and ITM is translation dependent. Since a translation inhibitor disrupts LTM when given 1 h after training, I infer that critical 24 h memory-supporting mRNAs are present for at least one hour after training, but not three hours.

1.9 mRNA changes during learning and memory formation.

Studies using methods such as microarray and RNA fingerprinting have shown that during memory formation there are gene expression changes that are shown by up and downregulation of mRNA (Cavallaro et al, 1997; Cavallaro et al, 2001). There is also evidence for changes in mRNA at synapses during learning; this is shown, for example, by an increase in GAT-1 mRNA in synapses after learning an avoidance task (Ferrara et al, 2009). Of particular relevance to the present investigation are the results of a large microarray study of over 12,000 genes assessed in the mouse hippocampus at three time points after a single inhibitory avoidance training trial, including an initial one-hour time point (Levenson et al., 2004). Changes were seen in only about 30 genes (~10 at each time point) in each of the two hippocampal sub-regions (Levenson et al., 2004) assayed. There was little overlap in the genes up- and down- regulated in the two sub-regions or at the three time points. The one-trial inhibitory avoidance model they used produces a memory lasting many days and is thought to undergo multiple rounds of consolidation and transcription events.

One of the objectives of the Levenson et al. (2004) study was to identify common regulatory factors in the genes modified by learning. Surprisingly, for the authors, there was no enrichment of CREB-related genes among those genes modulated by learning, despite the considerable evidence implicating CREB phosphorylation in this form of learning. The functional categories of genes changed included those involved in the regulation of transcription, signalling, growth, metabolism, extracellular matrix and cell structure. They validated six of the sixty or so genes regulated using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

There is also an increase in the expression of Arc mRNA in the brain 15 and 45 minutes after learning in the mouse brain (Montag Sallaz and Montag, 2003). Arc is an acronym for 'activity-regulated cytoskeleton' immediate early gene that is rapidly activated by neural activity. Work on Arc mRNA has shown an increase in nuclear transcription within minutes of new learning, followed by translocation of Arc mRNA to the cytoplasm by ~20 min (Marrone et al., 2008) and then likely degradation by nonsense mediated RNA degradation pathways (Bramham et al., 2008). Homer1a is typically also up-regulated following Arc up-regulation (Marrone et al., 2008). However, Homer1a was not up-regulated with inhibitory avoidance learning in the Levenson et al., 2004 microarray study. They suggested that was the case because of the later time points they sampled and that even transcription of Homer would have stopped before they sampled. Arc was not included in their microarray. All plasticity-associated immediate early genes would be expected to up-regulate within minutes of learning-associated activation and their

transcription may be transient as appears true for Arc and Homer1a, but less is known about the transcription events they may in turn initiate.

No studies have yet been directed at mRNA synthesis initiated in association with early odor preference learning. A look at the proposed intracellular pathways for olfactory learning (as described in Section 1.6-1.7 and Figure 3) shows some of the proteins and pathways necessary for the initiation of olfactory learning, however the mRNA changes that support the subsequent memory formation and memory itself have not been studied and hence my project.

1.10 Overview of the project

The background given thus far indicates that there are likely to be many genes that will be differentially expressed in the consolidation period leading to memory. As discussed in Section 1.8, it is likely that much of the transcription changes after early preference learning will occur within the first hour or so after training. Thus, the transcription/translation changes must take place very quickly within the olfactory bulb in order to produce 24hr memory. The plasticity related changes in mRNA levels that take place in the olfactory bulb following olfactory learning and the genes that are turned on during the consolidation period are not known. My focus in this thesis is to identify the transcriptional changes in the mitral cell layer within the critical time frame for consolidation.

Since mitral cells are the critical link in processing information from the olfactory nerve and sending it on to the brain and since memory changes from olfactory learning can be initiated solely within the olfactory bulb, I wanted to begin to investigate the transcriptional changes in mitral cells that are associated with learning. To do this I focused on the peppermint-encoding region of the olfactory bulb and on the MCL using laser microdissection to specifically isolate this area for analysis. I compared similar olfactory bulb tissue dissected from odor conditioned pups versus non-conditioned pups using a microarray analysis, followed by qRT-PCR.

I hypothesize that plasticity-related changes in olfactory bulb mRNA species after olfactory training will be related to the genes turned on in the MCL of the olfactory bulb during the time window in which protein synthesis is critical for learning and after the time point at which transcription factors, such as pCREB, are activated in the learned odor-encoding mitral cell. In brief, the project employed the use of the laser microdissection system to isolate MCL quadrants with minimal contamination from neighboring cells. Although cell types other than mitral cells, such as blood vessels and glia (Bailey and Shipley, 1993) are also located in the MCL, the LMD approach should help increase the signal of learning responses and differential changes responding to learning should be detected, regardless of the cell type.

The extracted mRNAs were analyzed by a 27,000+ gene rat microarray to determine which mRNAs differ between conditions in the critical dissected layers. The results of the microarray analysis were followed by investigation using qRT-PCR to assess selected

candidate transcripts found in microarrays. Pups were trained and tested to confirm learning prior to laser microdissection. Finally, targeted mRNAs extracted from the whole olfactory bulb of trained pups and of control pups sacrificed at different time points (0 min to 3 h) after training were assessed with qRT-PCR to examine the time course of transcription of selected genes and the similarity among MCL and whole bulb transcription patterns.

Chapter Two

2.0 Materials and Methods

2.1Animals

Post-natal day (PND) 6 Sprague Dawley (SD) rats were used for the study, with the day of birth considered PND0. The study was approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (Protocol number 12-01-M). The litters were culled on PND1 to a maximum of 12 pups per litter, with one male and one female pup per litter for each condition (odor only and odor +stroking) was used for the study. The rats were on a reverse light/dark cycle (12 hr. each, lights off at 12 noon) and had access to food and water *ad libitum.*

Twenty seven litters were used for the entire study. Six pups from 3 litters provided the tissue for microarray analysis, 6 pups from another 3 litters provided tissue for qRT-PCR to confirm the microarray results and tissue from 24 pups from12 litters were used in the time line qRT-PCR experiment.

2.2 Procedures

2.2.1 Summary of procedures

The flow chart shown in Figure 4 is a summary of all the procedures I used in this study.

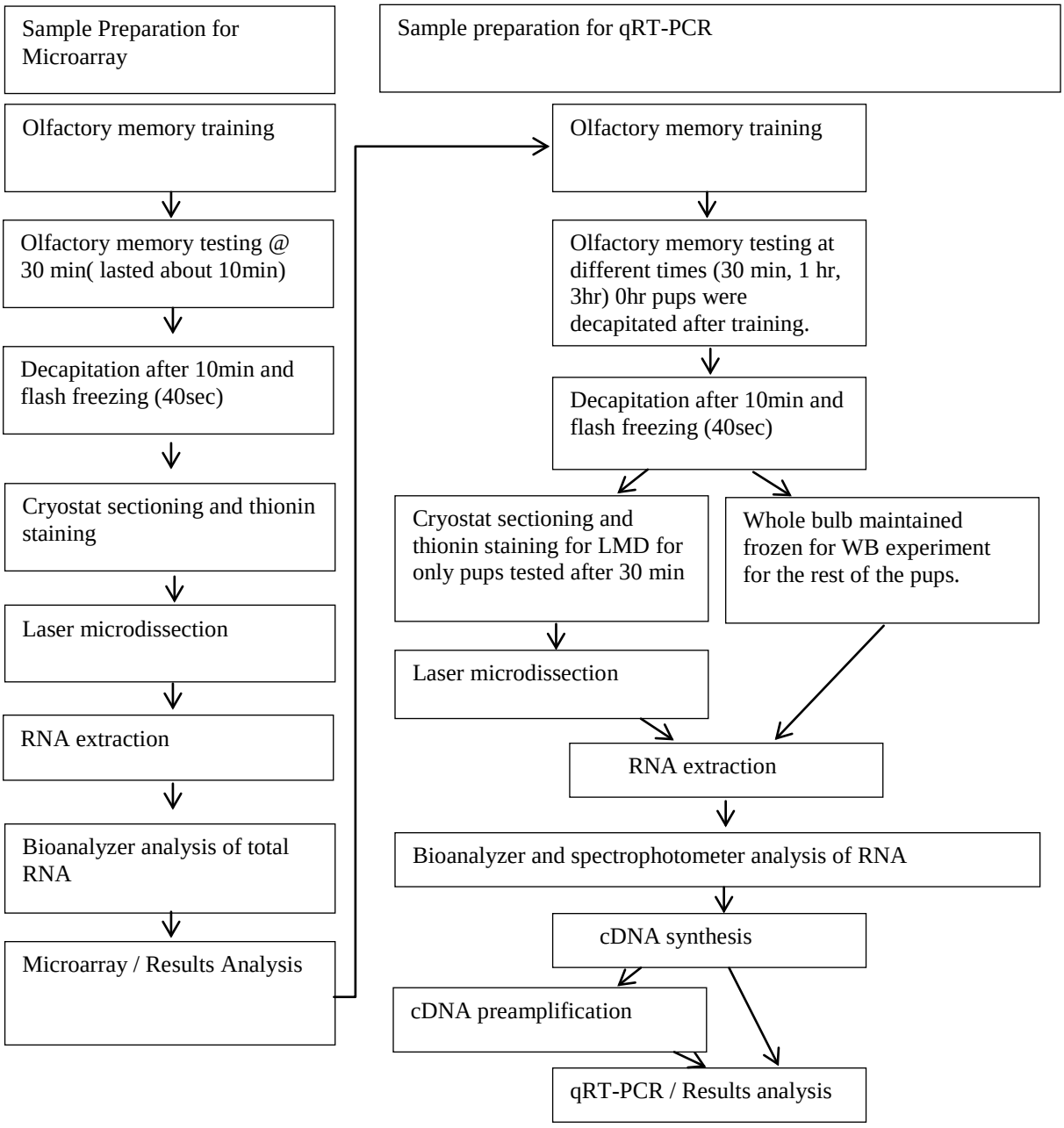

Figure 4: Flow chart showing summary of experimental procedures.

2.2.2 Olfactory memory training

On PND 6, dam and litter were brought to the behavior room in their home cage. Pups were labelled and then placed back into their home cage 10 min before the training started.

In a fume hood, peppermint scented bedding was prepared by mixing 0.3 ml of peppermint extract with 500 ml of fresh bedding; this was mixed and left in a closed polyethylene bag for the peppermint to penetrate the bedding. The bedding was then spread into a mouse cage and placed in the fume hood for 10 min to evaporate the alcohol in the peppermint. Each pup to be trained was placed on fresh bedding for 10 min to enable the pup to recover from handling by the dam. After 10 min the pup was placed on the peppermint scented bedding and stroked with a soft brush vigorously on its rump. It was stroked for 30 sec every other 30 sec for 10 min. The odor only pups were placed on the scented bedding for 10 min with no stroking. After the 10 min odor exposure period, pups were returned to the home cage with the dam and other littermates.

2.2.3 Olfactory memory testing

Pups were tested by a two-choice odor discrimination test 30 min, 1 hr or 3 hr after the training session in a manner described previously (McLean et al., 1993). Peppermint-scented bedding was prepared as outlined previously and poured into a tray (18 cm x18 cm); another tray of the same size was filled with 500 ml of fresh bedding. A stainless steel testing box with a 1 cm^2 wire mesh bottom was placed on the 2 trays and the trays were positioned 2 cm apart (neutral zone). A fine polypropylene mesh (1 mm^2 spacing)

was placed in the testing box over the wire mesh to allow easy movement of the pups. The testing box and the polypropylene mesh were cleaned with 70% ethanol and allowed to dry prior to use and after testing each pup.

The odor preference test consisted of five 1 min trials. During the testing the pup was placed in the neutral zone and the timer was started when the pup's head and one paw crossed to either the control or peppermint area. At the end of the minute, the pup was removed from the testing box, the peppermint bedding tray was closed and the times the pup spent in each area were recorded. The pup was placed in the opposite direction (in the neutral zone) for each trial. Total time spent testing each pup was approximately 10 min. The percentage time over peppermint for each pup was calculated by dividing the time spent in the peppermint area over the total activity time (total time spent in both the peppermint and control area). This provided results that normalized litter or individual pup effects so that the level of pup activity did not factor into the calculations. The results of the trials were analyzed using unpaired t-test.

2.2.4 Decapitation and cryostat sectioning

Pups used for microarrays or qRT-PCR were first tested for odor preference at 30 min, 60 min or 180 min after training (those killed immediately after training were not tested). At 10 min after the testing session, one male and female were decapitated from each litter in the odor/stroke learning group (>50% over peppermint= learning) and the odor non-learning group (<30% of test time spent over peppermint) . Their brains were then

removed from the skull within 2 min, embedded in freezing medium (OCT Tissue-Tek®), then immediately flash frozen with 2-methyl-butane slurry (described in Appendix1A) for 40 sec and stored at -80 ^{o}C. Thus, brains from the 30 min testing group were frozen by 52 min post training, the 1 h test groups by 82 min post training and the 3 h test groups by 202 min post training. All instruments for decapitation and the working area were cleaned with RNase Away (Molecular BioProducts), to prevent contamination of the brain tissue by RNases and DNA from the instruments and working area.

Brains were held at -20^{o}C for 24h prior to cryostat sectioning. The cryostat (Thermo Scientific, Microm HM 550), brushes and chucks used for sectioning were all cleaned with RNase Away; the brains were allowed to acclimatize to the cryostat temperature for 30 min. Empirical preliminary studies (Appendix 5) were performed to determine conditions of sacrifice (fixed or unfixed, freezing conditions), sectioning (temperature, section thickness) and LMD (maximum time required to preserve RNA quality). Brains were sectioned at 30 µm thickness using the cryostat set to a temperature of -12^{o}C. The duration of sectioning was limited to one hour to preserve RNA integrity (Appendix 5, Trial 7 & 8, Table 7 & 8). The olfactory bulb sections were put on nuclease and human nucleic acid free PEN (polyethylene naphthalate) membrane slides (Leica).

2.2.5 Quick thionin staining and laser microdissection

After cryostat sectioning, the slides were held at 4^{o}C for 10 min before quick thionin staining. The staining was a quick procedure as suggested by Leica for RNA work with

a few modifications. A solution containing 0.5% thionin dissolved in RNase free water was applied and the slides were incubated for 15 sec. Slides were rinsed twice in RNase free water for 15 sec each, once in 70% ethanol for 1 min and once in 90% and 100% ethanol respectively for 30 sec each. Slides were then air dried for 10 min at 40^{o}C (on the slide warmer) for 10 min then taken immediately to the laser microdissection microscope (Leica, AS LMD) for cutting of the MCL. All the solutions were prepared with RNase and DNase free water (Life Technologies) and stored in RNase free glass bottles.

The dorsolateral quadrant and ventromedial quadrant of the MCL of 2 pups, one from the learning group (odor + stroking) and one from the control group (odor only) from the same litter, were isolated using the laser microdissection microscope (Figure 5). The dorsolateral quadrant was used because this region was shown previously to contain increased pCREB levels when pups were conditioned to peppermint odor on PND 6 (McLean et al., 1999). Phosphorylated CREB is critical in odor preference learning (Yuan et. al., 2003). The ventromedial region of the MCL was chosen as a region that does not respond significantly during peppermint odor preference learning (McLean et al., 1999), thus serving as a non-learning internal control region. The samples were collected by gravity into 0.5 ml VWR microcentrifuge tubes, certified RNase and DNase free.

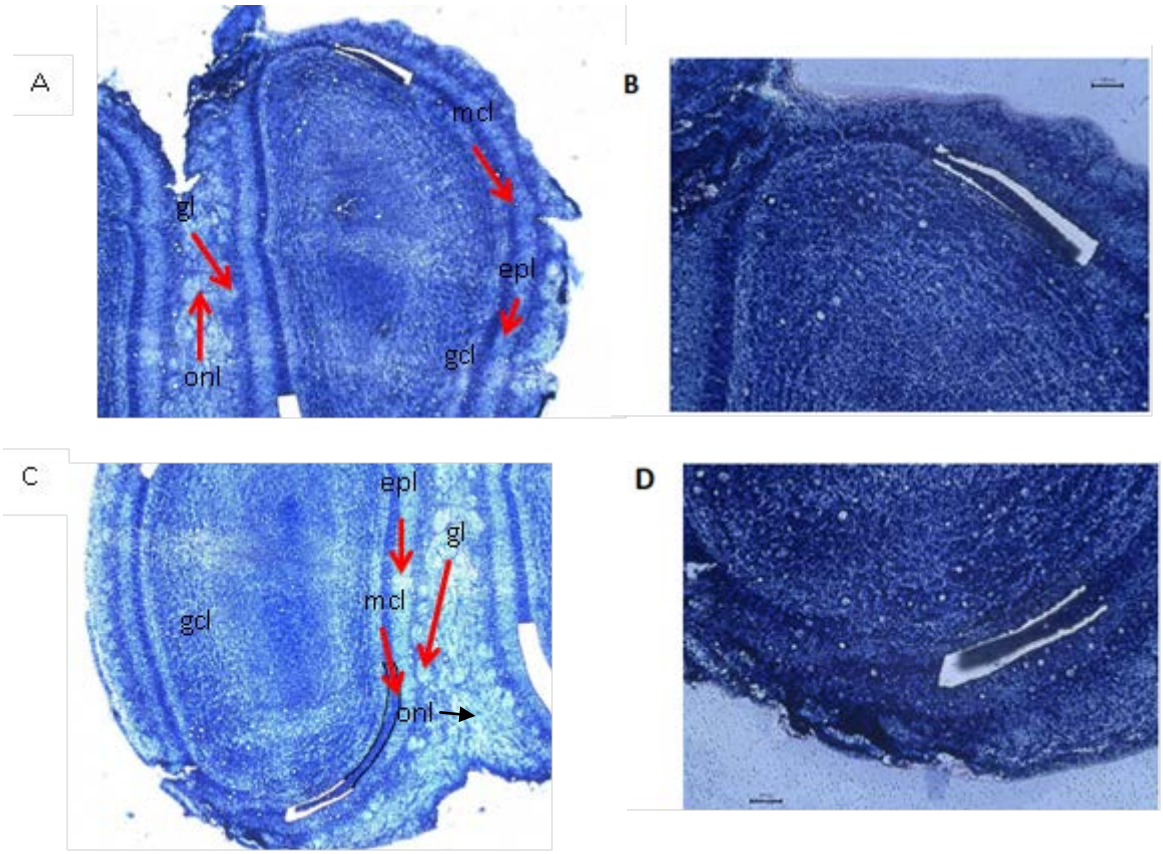

Figure 5: A labelled thionin stained olfactory bulb cryostat section showing: A. laser microdissected dorsolateral region viewed at 4 x magnification. The lasered region is about to fall into the cap of microcentrifuge tube. B. The same dorsolateral area as in A except at 10x magnification. C. The ventromedial region viewed at 4x magnification. D. Same ventromedial area as in C except at higher magnification

Each of the tubes contained 65µL of buffer RLT (RNA stabilizing solution and lysis buffer; Qiagen). The total time for laser microdissection was limited to 1 hr for each slide. This is because longer cutting times resulted in crystallizing of the buffer RLT and RNA of poor quality. Samples were obtained from 20 cryostat sections from both bulbs and included rostral to caudal levels of the bulb, except for the most rostral area, which had poorly defined MCL. The microdissected tissue samples suspended in buffer RLT were stored at -80^{o}C until further isolation of RNA.

2.2.6 Tissue disruption and homogenization

The laser microdissected samples in RLT buffer were homogenized by vortexing for 30 sec. The whole olfactory bulb was homogenized using the procedure below (this includes modifications to the procedure described in the RNeasy® Micro handbook):

1. The bulb was placed in 1.5 ml Precellys® ceramic bead tube kept on ice.
2. The tube with the bulb was taken out of the ice and 350 µL of buffer RLT was immediately added.
3. The tube was placed in a Precellys® 24 tissue lyser and homogenizer (Bertin Technologies) and homogenized for 20 sec at 5500 rpm
4. The tube was then held at room temperature for 1 hr (to settle all the foam and bubbles formed as a result of the homogenization process).
5. The tube was centrifuged at maximum speed of 13,300 rpm for 3 min and the supernatant transferred to a new microcentrifuge tube by pipetting.

2.2.7 RNA extraction

RNA was extracted from the microdissected samples using the RNeasy Micro Kit from Qiagen. For details see Appendix 1B.

2.2.8 Spectrophotometer analysis

The extracted RNA from the whole olfactory bulb samples was analyzed for quality using the Thermo Scientific Nanodrop® ND 1000 UV-Vis spectrophotometer. The pedestal of the equipment was cleaned with RNase free water and set up to detect RNA. First, the blank solution (RNase free water) was measured and then 1 µL of the sample was placed on the pedestal. The arm of the equipment was closed and the measurement was taken. The surfaces were cleaned with lint–free lab wipe and the rest of the samples were measured in the same way. The RNA concentration, 260/280 ratio and 260 /230 ratio of each sample was recorded. The RNA concentration was measured in nanogram per microliter (ng/µL). The ratio of absorbance at 260 nm to absorbance at 280 nm is used to measure the quality of RNA. The 260/230 ratio is also a measure of purity of nucleic acids. (Thermo Scientific- Nanodrop 1000 spectrophotometer V3.7 user manual, available online). Absorbance at 260 nm is used to quantify the nucleic acid in a sample. Absorbance at 280 nm detects aromatic amino acids; evaluate the amount of protein contamination in the sample. Absorbance at 230 nm is used to detect the presence of contaminants such as guanidine thiocyanate (commonly used in the RNA extraction procedure) and proteins. A260/A280 ratio of 1.9- 2.2 is accepted as high purity RNA and A260/A230 ratio above 1.7 means the RNA sample is not contaminated with either phenols, carbohydrates or guanidine hydrochloride.

2.2.9 Bioanalyzer analysis

The concentration of RNA extracted from the laser microdissected samples was below the detection range of the Nanodrop, so the bioanalyzer system (Agilent, 2100 Bioanalyzer) was used to evaluate both quantity and quality. The bioanalyzer works on the basis of gel electrophoresis although it is on a chip. The chip is made up of wells for samples, gels and an RNA molecular size standard (called a ladder). The bioanalyzer measures the concentration and integrity of each sample on the chip. The integrity involves the measurement of the ribosomal RNA ratio (the ratio of 28S:18S rRNA) and the relative integrity number (RIN). The RIN is an algorithm built into the software of the equipment that determines the quality of a sample based on the electrophoretic profile of the sample and the presence and absence of degradation products. The RIN has a numeric scale of one to ten with one being RNA of very poor quality and ten being RNA of the highest quality (Agilent, Bioanalyzer 2100 Expert User's Guide, available online). The Agilent RNA 6000 Pico kit was used and the procedure outlined by the manufacturer were followed (details of this procedure are in Appendix 1C).

2.2.10 Microarray

The microarray analysis of the laser microdissected samples was performed at the microarray facility in the Centre for Applied Genomics, Hospital for Sick Children (Sick Kids) in Toronto, Ont., Canada. The RNA samples were preamplified with the WT-Ovation One-Direct System (NuGEN Technologies, Inc.) at the microarray facility. Amplification was required because microdissected samples do not have enough RNA for

direct microarray. The process of microarray assessment involved three main steps: washing, scanning and analysis. The Rat Gene 1.0 ST Array chip was used. On the Rat Gene 1.0 ST Array chip the probes are designed to every exon of all the transcripts on the chip, there are 27,342 genes on the chip. One sample was run on one chip, in total 12 chips were used for the experiment. Three chips each for O/ODL, O/OVM, O/SDL and O/SVM. 500 picograms (pg) of RNA from each of the samples were used to produce cDNA, which was amplified and 5.5 micrograms (µg) of the cDNA was used for the microarray analysis.

2.2. 10.1 Microarray data analysis

The gene differential analysis of the microarray data was done by the Statistical Analysis Core Facility of the Center for Applied Genomics, Hospital for Sick Children Research Institute, Toronto. The raw data were normalized using a robust multi-array average (RMA) algorithm (Irizarry et al., 2003). A local-pooled-error test (LPE; Jain et. al., 2003) was used to identify differentially expressed genes. The statistical significance of all genes was evaluated using a false discovery rate (FDR; Benjamini and Hochberg, 1995). The following groups were analyzed; the first group was treated as the case (experimental) group and the second group as the control group.

1. Difference of Odor only dorsolateral (O/O DL) vs. Odor only ventromedial (O/O VM).
2. Difference of Odor +stroking dorsolateral (O/S DL) vs. O/O DL.
3. Difference of O/S DL vs. O/S VM.
4. Differences of O/S VM vs. O/O VM

2.2.11 Quantitative real time polymerase chain reaction

2.2.11.1 cDNA synthesis

Complementary DNA (cDNA) was prepared from RNA extracted from the laser microdissected samples and from the whole olfactory bulb samples. For the microdissected samples a total of 11 ng of RNA was used from each sample. A total of 2 microgram (µg) of RNA from the whole olfactory bulb samples was used. The SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies) was used. This is because the kit can be used for very low and very high amounts of RNA (1 pg to 2.5 µg of total RNA). The procedure in the kit's manual was followed. See details in Appendix 2A.

2.2.11.2 cDNA preamplification

The quantity of cDNA produced from the laser microdissected samples was not enough for all the qRT-PCR reactions therefore they were preamplified using the TaqMan® PreAmp Master Mix. A TaqMan® total of 13 gene expression assays were pooled together. The procedures in the TaqMan® PreAmp Master Mix Kit protocol were followed. Each preamplification reaction was prepared in a 0.2 ml microcentrifuge tube. For details see Appendix 2 B.

2.2.11.3 Preamplified cDNA dilution

For 14 cycles preamplification PCR, the dilution below was used as stated in the protocol of the TaqMan® PreAmp Master Mix Kit:

Table 1: Dilution factor of preamplified cDNA

Number of preamplification cycles	Dilution factor of preamplification products with 1X TE Buffer	Final volume of preamplification product
14	1: 20	1 ml

2.2.11.4 Taqman® gene expression assays

qRT-PCR reactions were performed using TaqMan® Gene Expression Assays (Applied Biosystems AB) and procedures in the TaqMan® Gene Expression Assays protocol (Applied Biosystems, USA) were followed. TaqMan® Gene Expression Assays have been pre-designed by the Applied Biosystems (Applied Biosystems, USA) with optimized parameters, such as melting temperature (T_m), low secondary structure, % guanine-cytosine content and amplicon length, to ensure that all assays have consistently high amplification efficiency. This chemistry uses a fluorogenic probe that detects a specific PCR product as it accumulates during PCR cycles. TheTaqMan® gene expression assays are built on 5' nuclease chemistry and consist of a fluorescent dye/MGB-quencher labelled probe (250 nM, final concentration), and two unlabelled PCR primers (900 nM each, final concentration). All probes contain a fluorescent reporter dye at the 5' end and an MGB non-fluorescent quencher at the 3' end. All TaqMan® Assay components are quality-control (QC) tested and formulated into a single 20X mix (Taqman Gene expression assay product Brochure available online). The gene expression assays used in this study were FAM/MGB-NFQ labelled probes. The qPCR target information can be found in Appendix 2C.

2.2.11.5 Endogenous control

All qRT-PCR data needs to be normalized and one of the common methods employed is the use of one or more endogenous controls (reference genes). An endogenous control gene is normally expressed stably within the samples being compared despite differences in treatment, experimental conditions or differences in tissue (Walker et al., 2009). The use of an endogenous control corrects for: variation in RNA content, variation in reverse-transcription efficiency, possible RNA degradation, presence of inhibitors in the RNA sample and differences in sample handling. The endogenous control used in this study was eukaryotic 18S (VIC/MGB-NFQ, primer limited.)

2.2.11.6 PCR reaction

The procedure outlined in the TaqMan® Fast Advanced Master Mix protocol was followed for the PCR reaction. For details see Appendix 1D.

2.2.11.7 qRT-PCR data analysis

The results of the PCR reaction were analyzed using a variation of the comparative Ct method (" " Ct method). The Ct values obtained from two different experimental RNA samples are directly normalized to an endogenous control. This method assumes that the amplification efficiencies of the gene of interest and the endogenous control are close to 100%. 2^-" Ct is calculated for the endogenous control and the experimental sample.

Their mean and standard deviation are also calculated. A Student's t-test was performed on this data to determine if difference between the experimental and control sample were statistically significant. The fold-change in expression of the gene of interest between the two samples was calculated by dividing the mean of the experimental group by that of the control group (Schmittgen and Livak, 2008).

Definition: ” Ct = Ct gene of interest – Ct reference (endogenous control). Ct values were obtained from the Viia7 software and the ” Ct was calculated in Excel®.

2.2.11.9 Statistical analysis on qRT-PCR

The relative expression calculation and standard deviation calculations were done using Excel®. Unpaired t- tests were performed using Graph Pad Instat 3 and $p < 0.05$ was considered significant. All data were expressed as vertical bars with standard error of the mean using SigmaPlot version 9 Software.

Chapter Three

3.0 Results

3.1 Olfactory testing results

To increase the likelihood that trained pups had learned an odor preference, only trained pups that had a 50% or better preference score in testing were used for microarray and qRT-PCR analyses, while odor only exposed pups were selected that showed 30% or less preference at test. Figure 6A shows the behavioral results for pups used in the microarray analysis, which were trained and then tested after 30 min. Figure 6B, shows the behavioral results for pups that were used in the qRT-PCR analyses to confirm the microarray results with testing again at 30 min after training. In Figure 6C, the behavioral results for pups that were trained and then tested at different times (30 min, 1hr and 3hr), for use in the whole bulb qRT-PCR time course experiment are shown. Unpaired t-test revealed significant differences between O/S and O/O conditions in the 3 groups as expected based on the performance selection criteria.

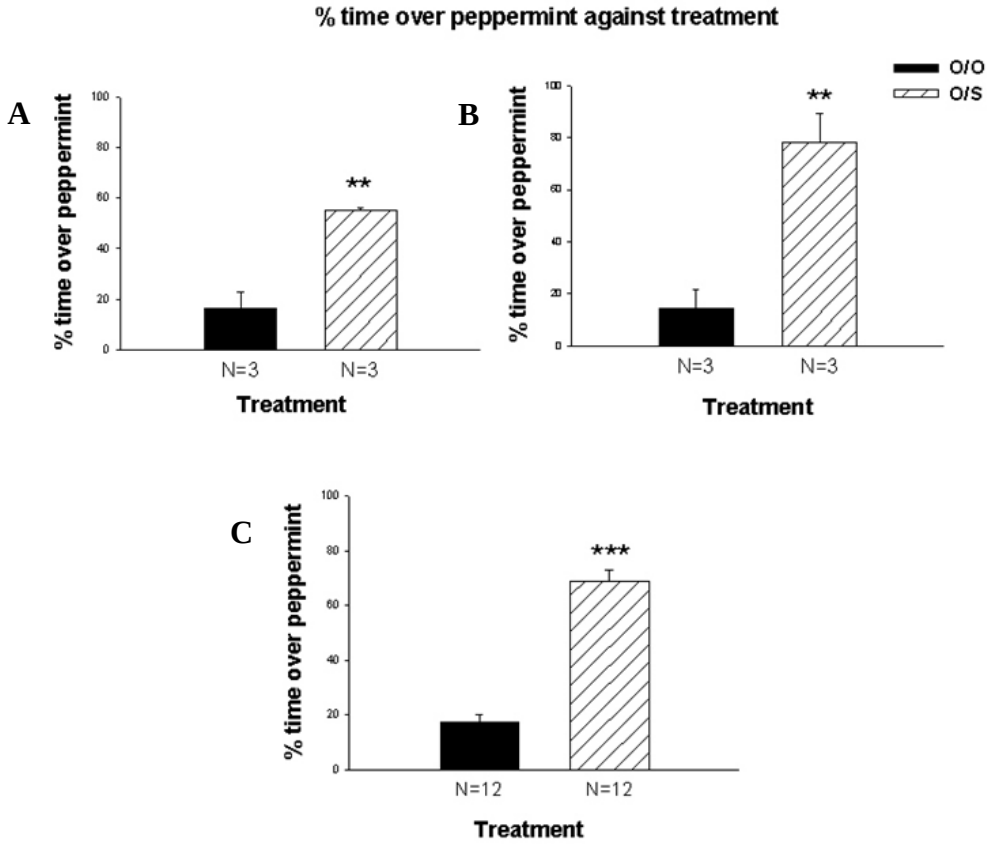

Figure 6: Percentage of time spent over peppermint in odor only (non-learning) and odor + stroking (learning) conditions. Testing was performed 30 min after training. A) Results for pups used for LMD followed by microarray analysis. p= 0.0043. B) Olfactory learning results of pups used for LMD followed by qRT-PCR analysis p=0.0094. C) Olfactory learning results for pups used in qRT-PCR time course investigations p value is < 0.0001.

3.2 Microarray results

3.2.1. Statistical overview

Comparisons of differentially expressed genes in the mitral cell layer were identified using the Local-Pooled -Test (LPE; Jain et. al., 2003). The statistical significance of all genes was evaluated using a false discovery rate (FDR; Benjamini and Hochberg, 1995). The FDR was preset at .05 and fold change at 1.5. Genes with adjusted p-values <0.05 and fold changee 1.5 (up-expressed genes) and genes with adjusted p-values <0.05 and fold changed -1.5 (down-expressed genes) were selected for further qRT-PCR analysis. These cut –offs were chosen because of the small sample size (n=3) that was used in each group in the microarray experiment. The adjusted p-value is the smallest significant level at which a comparison can be deemed statistically significant when it (that significance level) is applied to a group of comparisons (Hochberg and Benjamini, 1990).

3.2.2 Genes altered by associative learning in the odor encoding region

The main microarray analysis of interest for this thesis is a comparison for the DL quadrant MCL that encodes peppermint between odor + stroking pups that had a preference prior to sacrifice and odor only pups that did not (the non-learning condition due to an absence of UCS stimulation). This DL region is strongly reactive to peppermint odor and shows increased activity in peppermint trained pups as visualized by 2-DG uptake (Sullivan et al. 1991a) or CREB phosphorylation (McLean et al. 1999). Thus, samples in this comparison would both be expected to be stimulated by peppermint odor but only the odor + stroke group would be expected to have genes related to associative

learning. The analysis showed 16 genes differentially expressed of the 27,342 genes of the microarray (Table 2).

Table 2: Expression changes of genes in early olfactory learning (O/S DL vs. O/ODL). Each comparison had n=3 for each condition. Fold changes indicated by – sign indicated decrease expression of the gene in the O/S DL group.

Fold change	Adjusted p-value	Gene Assignment
4.629244941	0.000326619	NM_001108593 // **Sec23b** // Sec23 homolog B (S. cerevisiae) // 3q41 // 362226 ///
2.704962264	0.000584597	NM_001170534 // **Mcc** // mutated in colorectal cancers // 18q11 // 307449
-2.325436737	0.001230015	NM_001106902 // **Sema4c** // sema domain, immunoglobulin domain (Ig),transmembrane
2.809571403	0.001359133	NM_001108372 // **Rpp14** // ribonuclease P 14 subunit (human) // 15p14 // 361020 //
-2.690051339	0.002394506	NM_033499 // **Scrg1** // stimulator of chondrogenesis 1 // 16p11 // 64458
2.235157313	0.003294716	NM_001106941 // **Magee2** // melanoma antigen, family E, 2 // Xq31 // 302392 /// EN
2.818962031	0.008566502	NM_001137626 // **E2f3** // E2F transcription factor 3 // 17p12 // 291105 /// ENSRNO
7.928200211	0.013431564	XM_002729803 // **RGD1565493** // similar to DKFZP434I092 protein // 7q22 // 500853
2.637188653	0.013431564	NM_001106430 // **Mgst2** // microsomal glutathione S-transferase 2 // 2q26 // 29503
2.611973557	0.022826599	NM_001009651 // **Clic2** // chloride intracellular channel 2 // 20p12 // 294141 ///
1.549887367	0.022826599	ENSRNOT00000015637 // **RGD1308023** // similar to CG5521-PA // 3q41 // 296211 /// E
-2.15805599	0.029708204	NR_031826 // **Mir23b** // microRNA mir-23b
2.412030613	0.029708204	ENSRNOT00000000462 // **Dcbld1**
-1.529775065	0.042305647	ENSRNOT00000055954 // Slfn4 //**schlafen 4**
2.067762607	0.042305647	ENSRNOT00000003188 // **RGD1566265**
-1.651124821	0.042305647	ENSRNOT00000007403 // **Gng12** // guanine nucleotide binding protein (G protein), g

The microarray pattern found that of the more than 27000 genes compared only eleven genes were up-regulated in the odor+stroke MCL and 5 genes were down-regulated. Of the eleven up-regulated genes, six (Mcc, Rpp14, Magee2, E2F3, Dcbld1, Clic2) are known to regulate cell differentiation and development (Liu et al., 2012; Takano et. al., 2012; Julien et al., 2013; also see http://ghr.nlm.nih.gov/gene; http://www.ncbi.nlm.nih.gov/gene?cmd=Retrieve&dopt=full_report&list_uids=285761) and are enriched in the nervous system. This is consistent with the general hypothesis that genes involved in growth and development of neural circuits are likely to be involved in learning-related modification. Three genes including Sec23b, RGD1308023 and RGD1566265 (see http://ghr.nlm.nih.gov/gene; http://www.ncbi.nlm.nih.gov/gene/?term=RGD1566265) are involved in vesicle formation, transport and receptor regulation which are also processes implicated in the support of synaptic change. Of the remaining two, one is known to negatively regulate the production of cAMP through modulation of adenylate cyclases (RGD1565493: see http://www.ncbi.nlm.nih.gov/gene/?term=RGD1565493). Such negative-feedback might be expected in a cAMP-driven learning model. The final gene is likely involved in energetics but less is known of its role in the central nervous system (Mgst2). Among the five down-regulated genes, three (Schlafen4, Sema4c, Scrg1) appear to reduce the probability of differentiation or to promote neuronal cell death (van Zyulen et. al., 2011), consistent with a general picture of increased differentiation, and possibly stabilization, of circuitry in the associative learning sub-region (Jan and Jan, 2010). Scrg1 has also been found enriched in dense cored vesicles in the brain (Dandoy-Dron et al., 2003) suggesting a role in neural transmission. A fourth gene, Gng12, is involved in G protein signalling

(Morishita et al., 1995) suggesting this may relate to the importance of G protein signalling in mediating the UCS (see Figure 3). One of the most intriguing genes in the down-regulated set was that for microRNA 23b. This microRNA is known to support the plasticity involved in axonal wiring (Carmeliet and Tessier-Lavigne, 2005) and in the increase of GABAergic inhibition (Salama-Cohen et. al., 2006). Thus the down-regulation of MicroRNA 23B in the MCL with learning may reduce local GABA modulation, consistent with increased mitral cell activity (Yuan and Knopfel, 2006). Unfortunately, the LMD tissue lysate material was insufficient amount to permit cross-checking of the microarray outcome with qRT-PCR.

The second analysis (Table 3) shows those genes with significant changes in microarray when comparing O/O DL and O/O VM, that is, genes expressed in the MCL in peppermint versus non-peppermint coding areas with no UCS. I hypothesized that there would be differential expression of genes coding for the odor but these genes might not be expected to be involved in odor preference. O/O DL was from the experimental group and O/O VM was from the control group. In this comparison, 10 genes were differentially expressed. Since the focus of this thesis is associative learning-related genes regulated by protein synthesis in the odor coding area after training, I will not discuss this comparison in detail. However, it may be of interest that in the odor encoding region, when habituation to the odor, rather than preference learning for the odor, was likely to have occurred, Sema4c, a gene involved in inhibitory granule cell development was up-regulated, while it was down-regulated with associative learning.

Table 3: Expression changes of genes in early olfactory learning (O/O DL vs. O/O VM). For each condition, the n value was 3 pups. Fold changes indicated by – sign indicated decrease expression of the gene in the O/O DL group.

Fold Change	Adjusted p-value	Gene Assignment
-1.797781422	0.005945604	NM_001106585 // **Lsm8** // LSM8 homolog, U6 small nuclear RNA associated (S. cerevi
2.624239788	0.005945604	NM_138849 //Syt17 // **synaptotagmin XVII**
-3.101966156	0.005945604	ENSRNOT00000013044 // **Samd9l**
3.150950575	0.010198144	NM_001107882 // **March8** // membrane-associated ring finger
-3.517025726	0.012720925	NM_001025016 // **Chac2** // ChaC, cation transport regulator homolog 2 (E. coli) //
-3.027101239	0.016852926	NM_012614 // Npy // **neuropeptide Y** //
-2.324466605	0.018279617	NM_001109163 // **Fam111a** // family with sequence similarity 111, member A // 1q43
1.720381397	0.01948962	NM_001106902 // **Sema4c** // sema domain, immunoglobulin domain (Ig),transmembrane
-1.865717019	0.032012465	NM_001134695 // **LOC100188933** // hypothetical protein LOC100188933 //
-4.010973919	0.032012465	ENSRNOT00000005428 // **Rnf135** // ring finger protein 135 // 10q26 // 303350

The next comparison (Table 4) shows expression changes of genes in different regions of the early olfactory learning (O/S DL vs. O/S VM) bulbs. In this case, the tissue samples were from different portions of the same olfactory bulb, but in regions that show peppermint odor8 specific coding (DL) versus the VM region which apparently does not, since it shows neither increased uptake of 2-DG (Sullivan et al., 1991a) nor increased pCREB (McLean et al., 1999) after learning. Thus, differential changes in this comparison would likely identify genes that activated due to the learned odor and not just

to the UCS. Nine genes were identified by microarray as being significantly differentially expressed in the comparison. As previously seen for the associative learning comparison across learning and nonlearning groups, there is a down-regulation of microRNA23b in the associative odor encoding region of the MCL.

Table 4: Microarray results showing the expression changes of genes in early olfactory learning (O/S DL vs. O/S VM). N=3 pups per condition. Fold changes indicated by – sign indicated decrease expression of the gene in the O/S DL group.

Fold Change	Adjusted p-Value	Gene assignment
-2.632250693	0.000157231	ENSRNOT00000039318 // **LOC314942**
-9.156897273	0.000501773	ENSRNOT00000032204 // **Ttll11** // tubulin tyrosine ligase-like family, member 11 /
5.103916414	0.002623591	NM_001025139 // **Zpbp** // zona pellucida binding protein // 14q21 // 498415 /// BC
-2.745075659	0.003486364	NM_001109510 // **Gpr171** // G protein-coupled receptor 171 // 2q31 // 688737 /// E
-2.172054702	0.00671436	NM_001100666 // **Schip1** // schwannomin interacting protein 1 // 2q31 // 295105
-3.366533385	0.008454614	NR_031826 // **Mir23b** // microRNA mir-23b
-2.086906406	0.013717131	BC081845 // **Fth1** // ferritin, heavy polypeptide
-4.302274247	0.044754558	ENSRNOT00000004904 // **Rnf213** // ring finger protein 213 // 10q32.3 // 303735 ///
-1.845904528	0.048432983	ENSRNOT00000061287 // **LOC679547** // hypothetical protein LOC679547 // 10q12 // 67

The final comparison performed on the microarray is shown in Table 5. This compares O/S VM vs. O/O VM. Thus, it would most likely identify genes changed due to stroking without the influence of odor since the peppermint non encoding region in the learning group was compared to the same region in the non-learning group. O/OVM was the

control group and 3 pups were used in both O/OVM and O/SVM groups. Fourteen genes out of 27,342 genes probed were identified as being differentially expressed.

Table 5: Microarray results showing the expression changes of genes in early olfactory learning (O/S VM vs. O/O VM). Fold changes indicated by – sign indicated decrease expression of the gene in the O/S VM group.

Fold Change	Adjusted p-value	Gene Assignment
3.454544441	0.000250491	ENSRNOT00000061287 // **LOC679547** // hypothetical protein LOC679547 // 10q12
2.454829498	0.002085319	NM_138849 // Syt17 // **synaptotagmin XVII** // 1q35 // 192189 /// ENSRNOT0000002309
9.828814531	0.00368233	ENSRNOT00000032204 // **Ttll11** // tubulin tyrosine ligase-like family, member 11 /
2.438546297	0.00368233	BC081845 // **Fth1** // ferritin, heavy polypeptide 1 // 1q43 // 25319 /// BC081845
2.016729188	0.006930084	NM_145777 // **Olfm3** // olfactomedin 3
1.726899716	0.008066601	ENSRNOT00000039318 // **LOC314942** // similar to CUB and Sushi multiple domains 3
2.22853887	0.008455328	ENSRNOT00000064242 // **Anks1b** // ankyrin repeat and sterile alpha motif domain co
3.455867302	0.008455328	ENSRNOT00000015637 // **RGD1308023** // similar to CG5521-PA // 3q41 // 296211 /// E
-3.883067779	0.013360199	NM_001025016 // **Chac2** // ChaC, cation transport regulator homolog 2 (E. coli) //
3.417383139	0.02524731	NM_001109510 // **Gpr171** // G protein-coupled receptor 171 // 2q31 // 688737 /// E
2.786594805	0.027678249	NM_147207 // **Vof16** // ischemia related factor vof-16 // 8q22 // 259227 /// AB089
-1.214772843	0.030231874	NM_001025739 // **Rpl11** // ribosomal protein L11 // 5q36 // 362631 /// ENSRNOT0000
1.688697546	0.049286592	NM_001106726 // **G2e3** // G2/M-phase specific E3 ubiquitin ligase // 6q22 // 29900
3.454544441	0.000250491	ENSRNOT00000061287 // **LOC679547**

3.3 qRT-PCR results

Since my limited micro-dissected tissue made it impossible to properly assess the validity of my MCL-selected microarray data with qRT-PCR in the normal way, I decided to try to repeat my original experiments, including the laser microdissection component going directly to qRT-PCR for a readily available subset of the genes implicated in the original microarray. I also chose to repeat experiments with still other animals and do a similar analysis on the whole bulb where gene amplification was less of an issue. Taking advantage of the ease of gathering material, I carried out a time sequence analysis on gene products with the whole bulb. As already discussed, only the O/S DL and O/O DL comparisons were of particular interest for this thesis and so these areas were laser micro-dissected for qRT-PCR. Similarly only odor+stroking and odor only groups were used in the whole bulb analyses.

Thirteen differentially expressed genes from the O/S DL vs. O/O DL group shown in Table 2 (Mcc, Clic2, Dcbld1, E2f3, Gng12, Magee2, Mgst2, RGD1566265, RGD1308023, Rpp14, Scrg1, Sec23b, Sema4c) were selected to investigate the results from the microarray experiment. Three other differentially expressed genes in the O/S DL vs. O/O DL group microarray analysis (RGD1565493, Mir23b and Slfn4) were not analyzed further because there were no ready-made gene expression assays for them.

All the qRT-PCR experiments were done with RNA from 3 pups in each condition (n=3). The results are presented as fold change in expression between the O/S DL (experimental

group) and O/O DL (the control group) which represents the difference in expression of a target gene between O/S and O/O. This analysis was designed in a manner similar to that used by others (Schmittgen and Livak, 2008; Vecsey et al., 2007). However, my qRT-PCR experiment did not show any significant differences in the targeted gene expression levels. The qRT-PCR results of LMD material are shown graphically in Figure 7 and in Table 6.

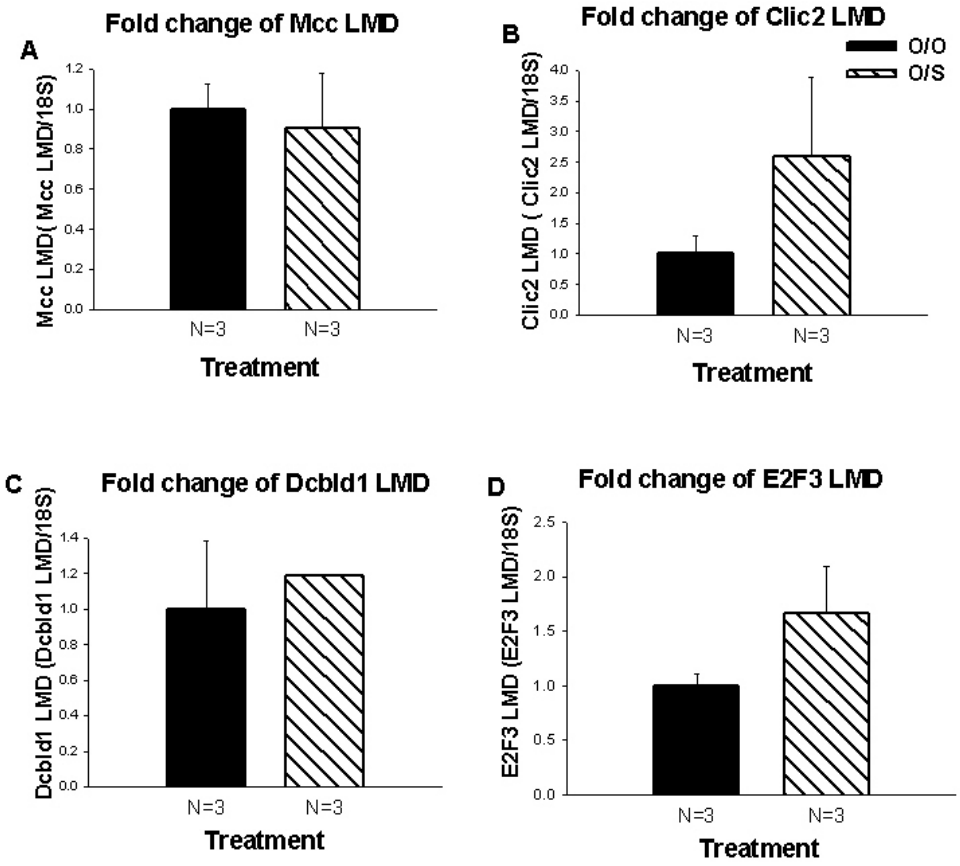

Figure 7A-D: These graphs show the qRT-PCR results for Mcc, Clic2, Dcbld1 and E2F3 in microarray validation experiments. None of the genes showed any significant change in expression between the odor only dorsolateral (O/O DL) and odor/stroke dorsolateral (O/S DL).

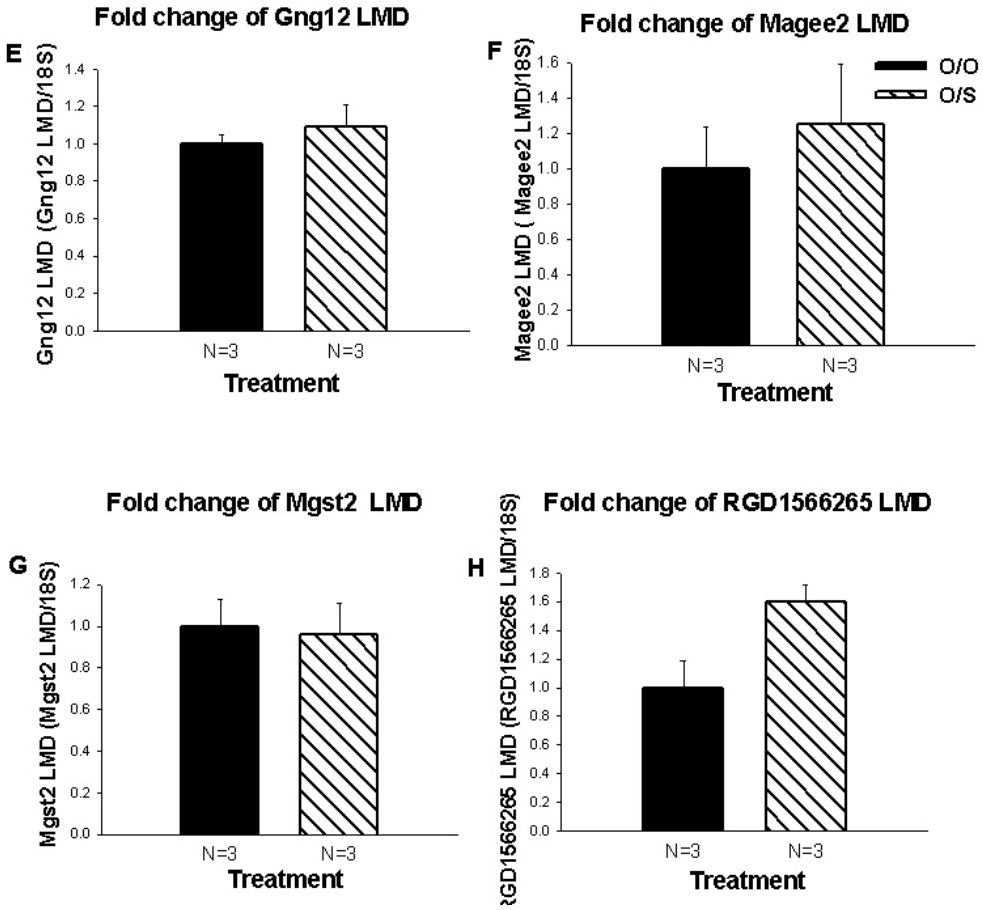

Figure 7E-H: The qRT-PCR results for Gng12, Magee2, Mgst2 and RGD1566265 in microarray confirmation experiments. None of these genes showed a significant difference in their expression in the odor /stroke dorsolateral (O/SDL) compared to odor only dorsolateral (O/ODL).

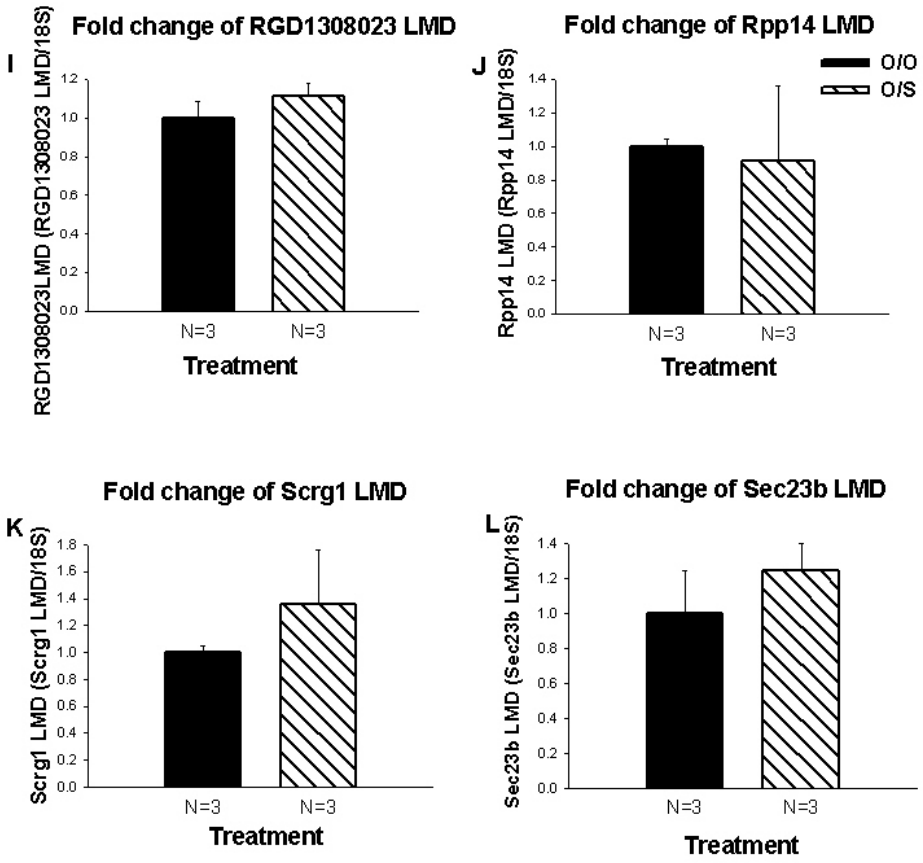

Figure 7I-L: qRT-PCR results for RGD1308023, Rpp14, Scrg1 and Sec23b. None of the genes showed any significance difference between odor /stroke dorsolateral (O/SDL) and odor only dorsolateral (O/ODL).

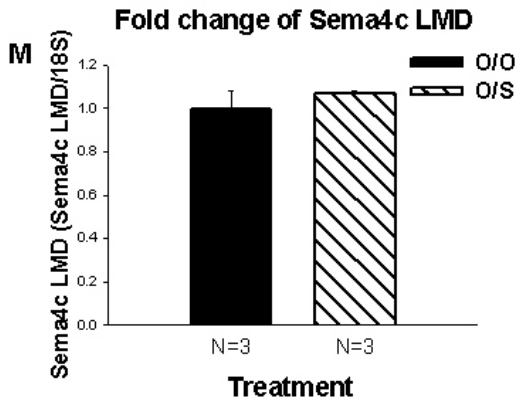

Figure 7M: qRT-PCR results for Sema4c. This does not repeat the pattern seen in the microarray where less Sema4c was seen in the learning condition. Sema4c did not show any significance difference between odor /stroke dorsolateral (O/SDL) and odor only dorsolateral (O/ODL).

3.4 Rationale for subsequent qRT-PCR experiments

The first set of qRT-PCR experiments was carried out to investigate the microarray results of LMD tissue. I decided to perform the same experiments on whole bulb samples from the same behavioral treatment conditions as the laser micro-dissected samples, 3 pups were used for each of the time points. I wanted to know if the whole olfactory bulb had similar gene expression profile changes as were seen in the mitral cell layer. Determining if expression profiles seen in the MCL are reflected in the whole bulb will be an advantage to our lab in subsequent studies because LMD, even though a very useful tool in isolating sections of a tissue with little contamination from neighbouring cell

population, is time consuming, requires some expertise, and is expensive since the RNA yield is low which means it has to be pre-amplified for both microarray and qRT-PCR. I first looked at the same time point (olfactory memory testing 30 minutes after olfactory learning) for both the laser microdissected samples and the whole bulb samples. The results for the 13 genes tested are shown in Figure 8A-M and Table 6.

In the 50 min sacrifice time WB (whole bulb) experiment, the O/S group was the experimental group and the O/O group was the control group. The results are presented as fold change in expression relative to the housekeeping gene between the O/S (experimental group) and O/O (the control group), which represents the difference in expression of a target gene between O/S and O/O. Similar examples are outlined by Livak and Schmittgen (2008) and Vecsey et al. (2007). There was significant up regulation of Clic2 and Dcbld1. The results for Mcc and E2F3 were not significant in the WB comparison (Figure 8A-D).

RGD1566265 was significantly up-regulated in the WB group (Figure 8E-H) and a similar direction of regulation was seen in the LMD qRT-PCR assessment. Gng12, Magee2 and Mgst2 were not significantly modulated in the WB preparation (Figure 8E-H).

None of the following genes, RGD1308023, Rpp14, Scrg1, and Sec23b, showed any significant difference between the odor/stroking and the odor only groups (Figure 7I-L).

Sema4C showed a significant difference between the O/S and O/O groups at 50 min in the whole bulb experiment (Figure 7I-L).

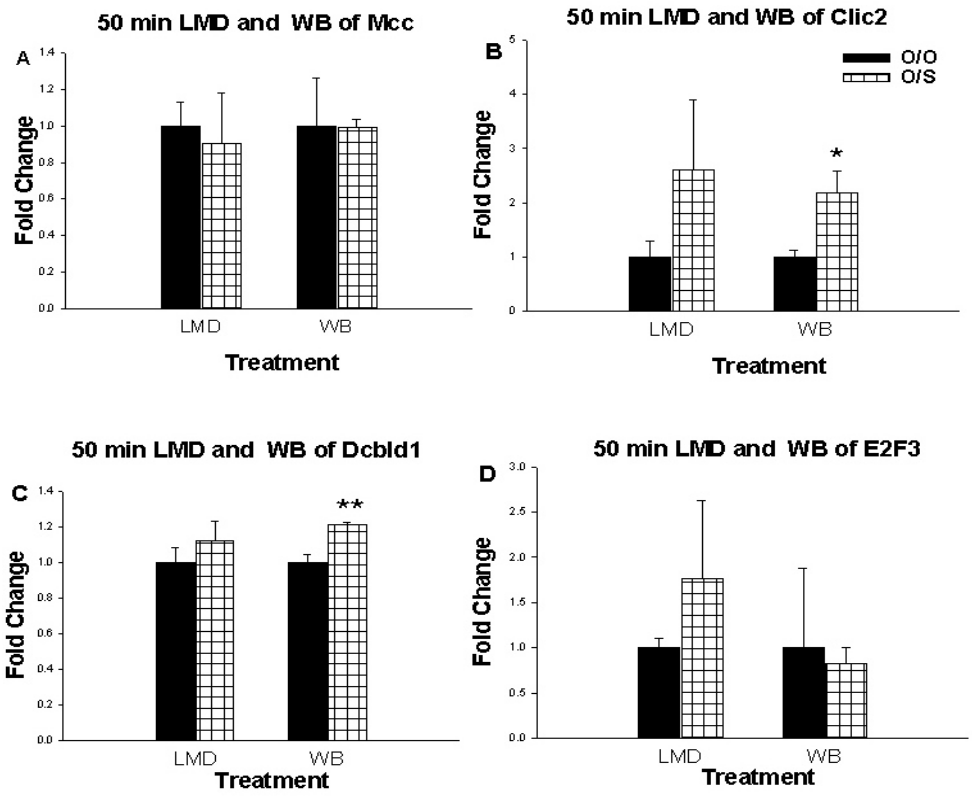

Figure 8A-D: Shows the results of the 50min laser microdissection (LMD) and whole olfactory bulb (WB) comparison of Mcc, Clic2, Dcbld1 and E2F3.There was a significant difference in the odor/ stroking (O/S) compared to the odor only (O/O) of 50 min whole bulb of Clic2 (*$p<0.05$) and Dcbld1(**$p<0.01$).

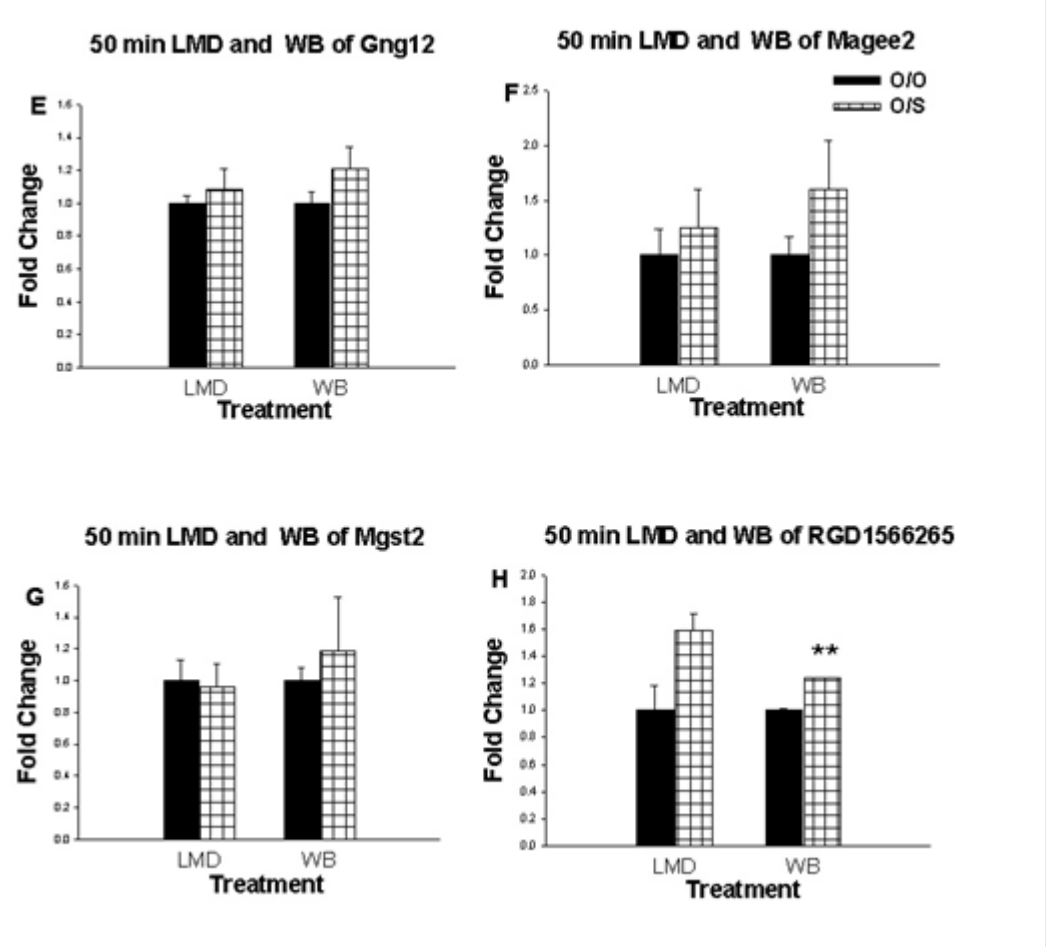

Figure 8E-H: Shows the laser microdissection (LMD) and whole olfactory bulb (WB) comparison results for Gng12, Magee2, Mgst2 and RGD1566265. RGD1566265 showed a significant expression in the whole bulb between odor only and odor/stroking, **$p<0.01$.

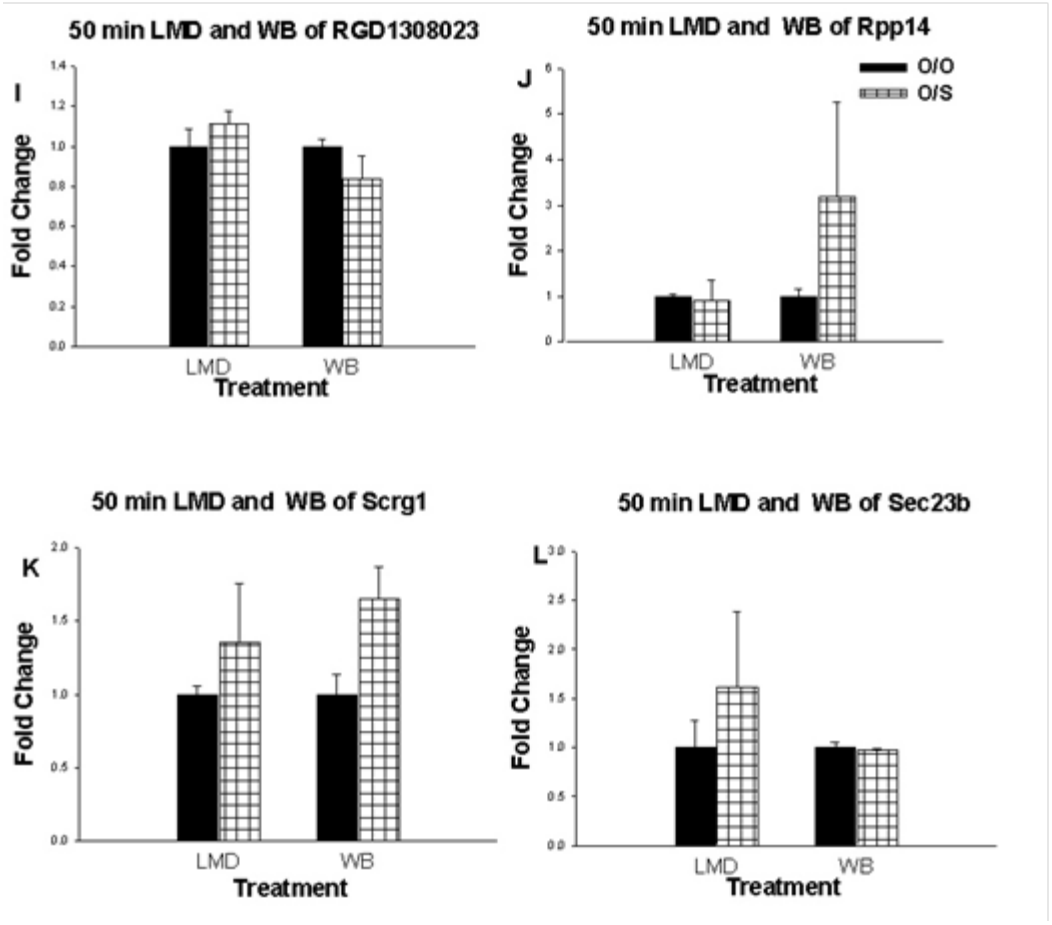

Figure 8I-L: Results for laser microdissection (LMD) and whole olfactory bulb (WB) comparison experiment for RGD1308023, Rpp14, Scrg1 and Sec23b. There were no significant expression differences for any of the genes.

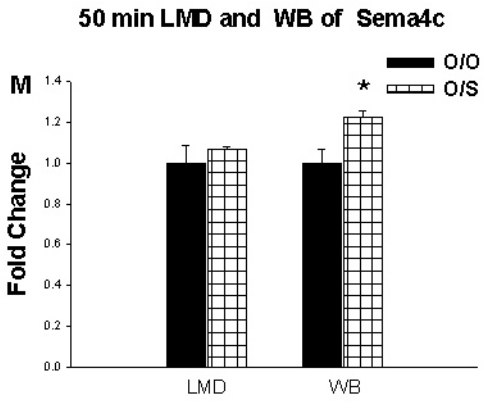

Figure 8M: Results for Sema4C laser microdissection (LMD) and whole olfactory bulb (WB) comparison. There was a significant difference in Sema4c expression in odor/stroking compared to the odor only in the whole bulb at 50 min (**$p<0.05$).

Table 6: qRT-PCR results comparison of 50 min laser microdissection and 50 min whole bulb experiment (n=3 pups/group)

Gene	Treatment	50 min LMD		50 min WB	
		Fold Change	±SEM	Fold Change	±SEM
Mcc	O/O	1	0.1270	1	0.2607
	O/S	0.9009	0.2770	0.9914	0.0408
Clic2	O/O	1	0.2998	1	0.1313
	O/S	2.6018	1.2860	2.1745*	0.4006
Dcbld1	O/O	1	0.0803	1	0.0439
	O/S	1.1179	0.1106	1.2125**	0.0091
E2F3	O/O	1	0.1028	1	0.0320
	O/S	1.7594	0.8729	0.8254	0.1708
Gng12	O/O	1	0.0494	1	0.0677
	O/S	1.0888	0.1185	1.2069	0.1384
Magee2	O/O	1	0.2398	1	0.1697
	O/S	1.2484	0.3456	1.5995	0.4420
Mgst2	O/O	1	0.1282	1	0.0805
	O/S	0.9574	0.1510	1.1833	0.3389
RGD1566265	O/O	1	0.1854	1	0.0144
	O/S	1.5959	0.1189	1.2423**	0.0011
RGD1308023	O/O	1	0.0861	1	0.0355
	O/S	1.1094	0.0692	0.8379	0.1128
Rpp14	O/O	1	0.0482	1	0.1467
	O/S	0.9112	0.4546	3.17963	2.080
Scrg1	O/O	1	0.0525	1	0.1373
	O/S	1.3576	0.4006	1.6470	0.2229
Sec23b	O/O	1	0.2767	1	0.0583
	O/S	1.6211	0.7609	0.9719	0.0229
Sema4C	O/O	1	0.0838	1	0.0506
	O/S	1.0690	0.0098	1.2220*	0.0320

After the previous set of analysis, I decided to run a time course experiment (sacrifice at 0 min, 80 min and 200 min besides the 50 min samples already obtained) on additional olfactory bulb samples. Three pups were used for each of the time points. The major question of interest was whether gene transcription altered by the associative learning condition was restricted to the first hour after training. Protein transcription critically related to learning appears over after the first hour.

Figure 9A-M and Table 7 shows the pattern of regulation for the 13 genes and includes again the 50 min time point data already reviewed. With one anomalous late exception, 50 min was the only time point at which genes were significantly altered by the training manipulation: Clic2, Dcbld1, RGD1566265 and Sema4C. While this suggests an up-regulation of transcription in the first hour after learning, a visual inspection of the graphs suggests only the Clic2 gene was up-regulated in the learning condition itself across time. However there was no comparison between time points using ANOVA.

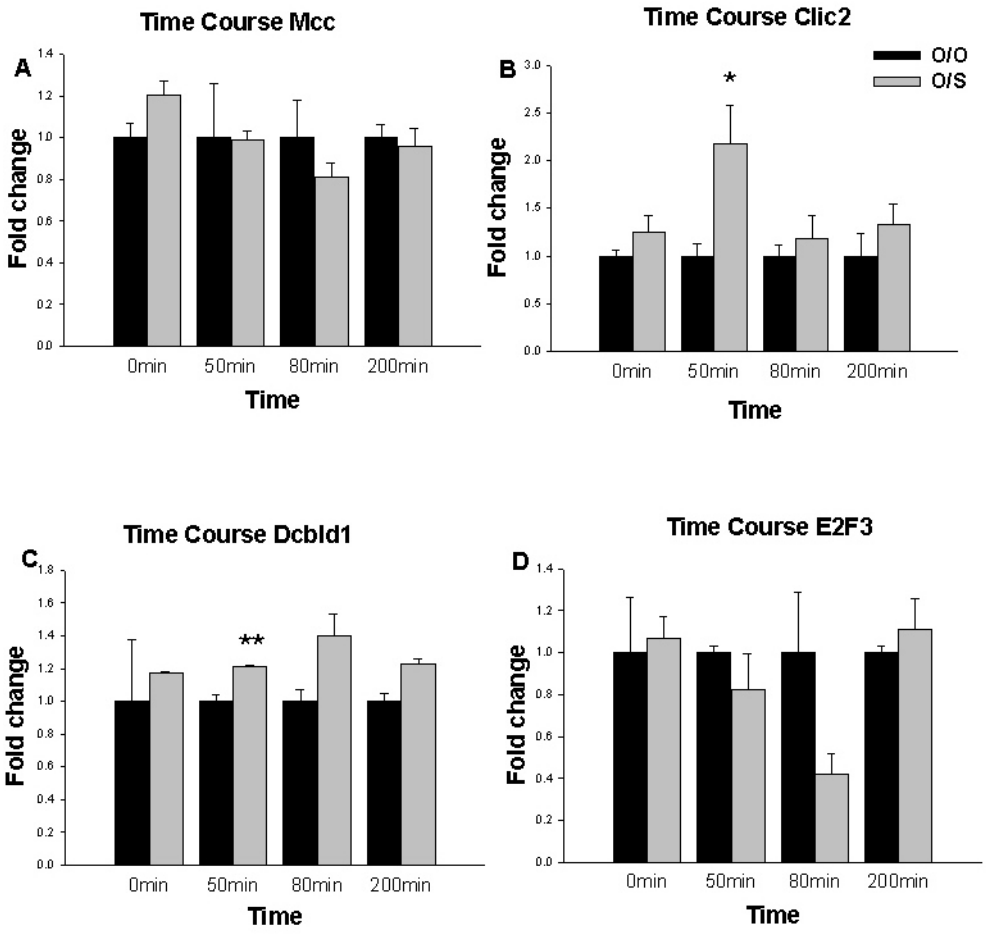

Figure 9A-D: qRT-PCR timeline experiment result for Mcc, Clic2, Dcbld1 and E2F3. The relative expression of Mcc, Clic2, Dcbld1 and E2F3 at 0min, 50min ,80min and 200 min after olfactory memory training. Mcc and E2F3 did not show any significant expression between odor/stroking and odor only. Clic2 was significant at 50min (*$p < 0.05$) and Dcbld1 showed significance at 80 min (**$p<0.01$).

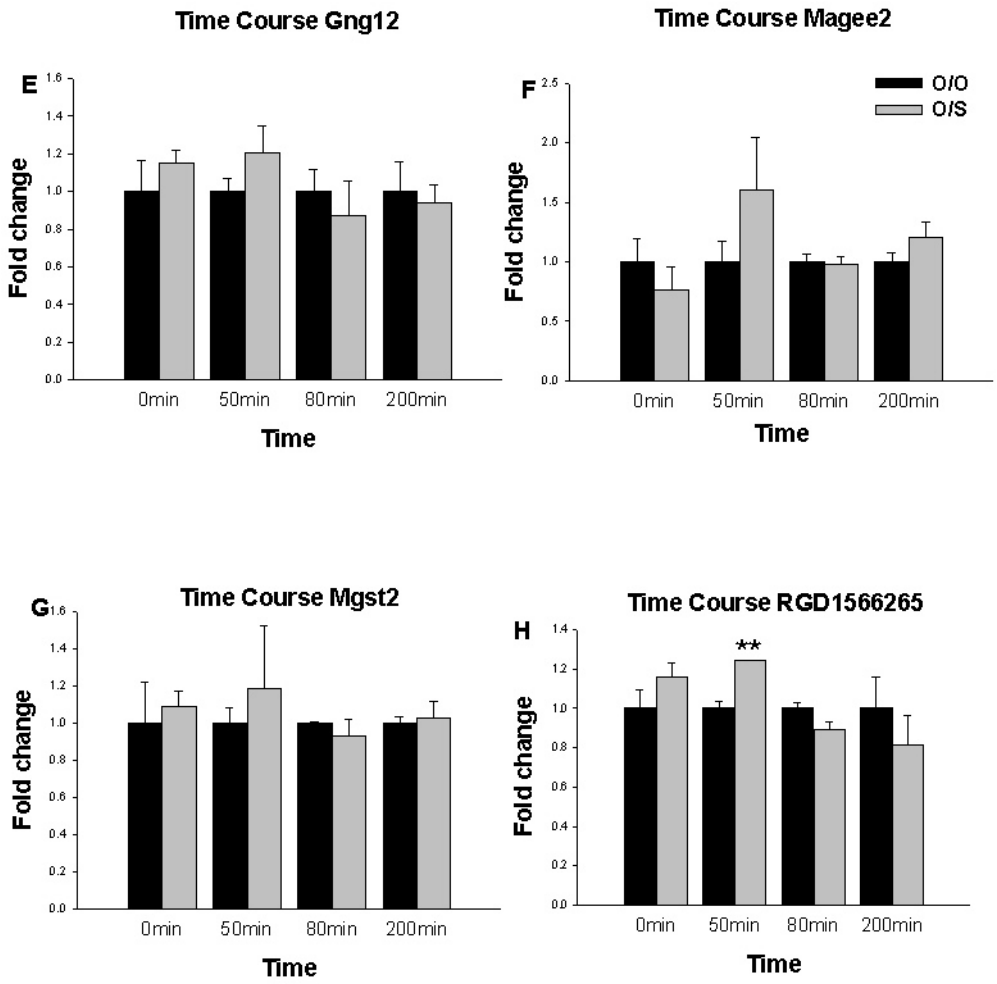

Figure 9E-H: qRT-PCR time course experiment results for Gng12, Magee2, Mgst2 and RGD1566265.The expression of Gng12, Magee2 and Mgst2 in pups used in the study did not show any significant difference between odor/stroking and odor only. However, RGD1566265 showed a significant difference in its expression at 50 min (*$p < 0.01$).

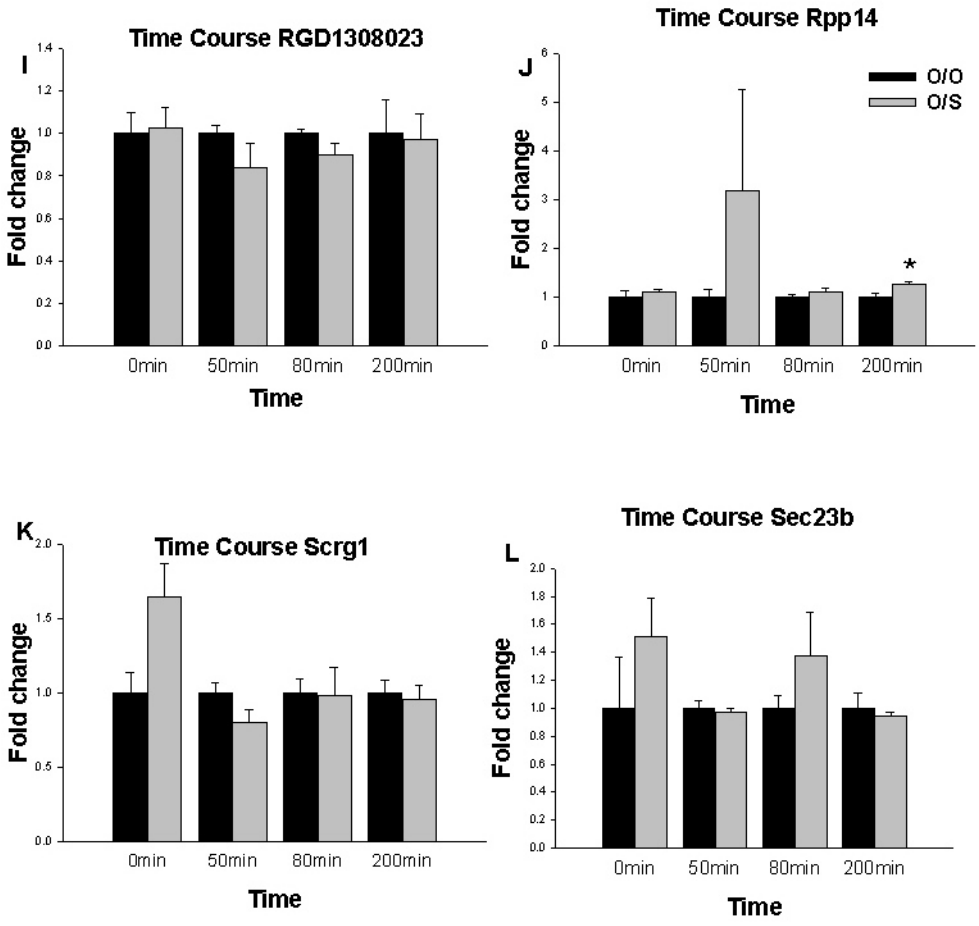

Figure 9I-L: qRT-PCR timeline study results for RGD1308023, Rpp14, Scrg1 and Sec23b. It might be noted that the Rpp14 increase at 50 min was the highest fold change seen in the qRT-PCR study, although only a much later time point was statistically significant (*$p<0.05$).

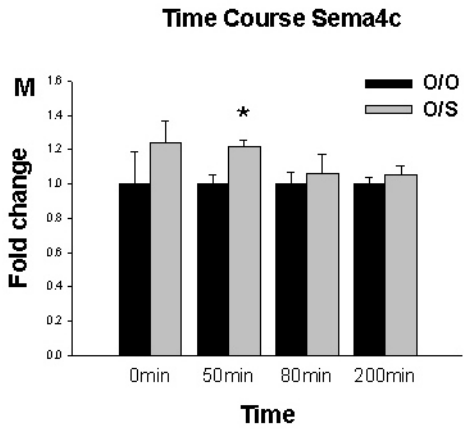

Figure 9M: qRT-PCR time line study results for Sema4c.Pups used in the experiment showed significant difference in the expression of Sema4c at 50 min between the odor/stroking and odor only groups (*$p < 0.05$).

Table 7: qRT-PCR results of WB time course experiments

Gene	Treatment	0 min WB		50 min WB		80 min WB		200min WB	
		Fold Change	SEM	Fold Change	SEM	Fold Change	SEM	Fold Change	SEM
Mcc	O/O	1	0.0675	1	0.2607	1	0.1773	1	0.0630
	O/S	1.2061	0.0666	0.9914	0.0408	0.8087	0.0729	0.9608	0.0846
Clic2	O/O	1	0.0632	1	0.1313	1	0.1152	1	0.2418
	O/S	1.2495	0.1716	2.1745*	0.4006	1.1772	0.3544	1.3296	0.2099
Dcbld1	O/O	1	0.3790	1	0.0439	1	0.0746	1	0.0450
	O/S	1.1718	0.0094	1.2125**	0.0091	1.3978	0.4854	1.2286	0.1389
E2F3	O/O	1	0.2655	1	0.0320	1	0.2894	1	0.0309
	O/S	1.0678	0.1020	0.8254	0.1708	0.4200	0.0973	1.1099	0.1499
Gng12	O/O	1	0.165	1	0.0677	1	0.1200	1	0.1555
	O/S	1.1498	0.0721	1.2069	0.1384	0.8723	0.1828	0.9374	0.1003
Magee2	O/O	1	0.1954	1	0.1697	1	0.0630	1	0.0793
	O/S	0.7686	0.1860	1.5995	0.4420	0.9820	0.0568	1.2016	0.1298
Mgst2	O/O	1	0.2203	1	0.0805	1	0.0094	1	0.0316
	O/S	1.0888	0.0798	1.1833	0.3389	0.9282	0.0919	1.0252	0.0926
RGD1566265	O/O	1	0.0946	1	0.0144	1	0.0280	1	0.1575
	O/S	1.16097	0.0690	1.2423**	0.0011	0.8924	0.0389	0.8150	0.1514
RGD1308023	O/O	1	0.0949	1	0.0355	1	0.0177	1	0.0175
	O/S	1.0264	0.0936	0.8379	0.1128	0.8959	0.0565	0.9692	0.1204
Rpp14	O/O	1	0.1301	1	0.1467	1	0.0506	1	0.0744
	O/S	1.0970	0.0666	3.17963	2.080	1.1023	0.0764	1.2719*	0.0451
Scrg1	O/O	1	0.1839	1	0.1373	1	0.0713	1	0.0898
	O/S	1.0194	0.3691	1.6470	0.2229	0.7981	0.0897	0.9851	0.1857
Sec23b	O/O	1	0.3695	1	0.0583	1	0.0893	1	0.1096
	O/S	1.5090	0.2795	0.9719	0.0229	1.3765	0.3088	0.9462	0.0254
Sema4C	O/O	1	0.1861	1	0.0506	1	0.0687	1	0.0356
	O/S	1.2376	0.1282	1.2220*	0.0320	1.0601	0.1151	1.0562	0.0475

Chapter Four

4.0 Discussion

4.1 Rationale for study

This project was a continuation of work done in the McLean laboratory. In 1999, McLean et al. showed that after the odor-stroke preference learning paradigm there was increased pCREB in the MCL of dorsolateral quadrant of the olfactory bulb in odor preference learning groups (odor +stroking) compared to non-learning groups (stroking only, odor only and naïve). Based on these results (McLean et al., 1999), it was assumed that significant learning-related transcription would take place in mitral cells of the peppermint-encoding dorsolateral quadrant. This assumption was consistent with ex vivo electrophysiology studies in the dorsolateral olfactory bulb quadrants from pups that had learned an odor preference unilaterally and showed enhanced olfactory nerve/mitral cell excitatory postsynaptic potentials 24 h after training relative to the untrained side (Yuan & Harley, 2012).

The McLean et al. (1999) study also showed that increased pCREB levels were highest 10 min after the training session, significantly elevated at 30 min, and had returned to near normal levels by 60 min. More recently, (Grimes et al.,2011) studies on protein translation and transcription in early odor preference learning indicated that new protein translation was still required 60 min after training, but not 3 h after training, although the transcription window itself was not assessed directly. Nonetheless, whether they represent newly transcribed mRNA or resident mRNA, learning-critical mRNA species must be

present at the time of translation for the 60 min memory window, while post-training transcription and translation was required for 24 h memory.

While it is well documented that immediate early genes are recruited in an early time window in associative learning paradigms (for review see Alberini, 2009), I was particularly interested in the effector proteins recruited by associative learning. Few studies have attempted to examine these directly. CREB phosphorylation itself does not trigger transcription unless supported by other synergistic transcription factors (see Alberini, 2009). Thus it is likely that early CREB transcriptional events are related to interconnected webs of immediate early gene transcription, that then result in appropriate effector gene transcription.

4.2 Summary and discussion of findings

4.2.1 Paucity of CREB-related genes and caveats

Because of the expense of microarray analysis on the samples from laser microdissected sub-regions of the olfactory bulb, I believed it was critical to confirm that learning had occurred with the O/S pups and that there was no preference in O/O controls. All pups used in the study (with the exception of the 0 min post training controls) were trained and tested for olfactory preference memory. To give the pups a rest prior to testing, a 30 min memory interval was selected. To be in the learning condition, pups had to have had exhibited odor preferences greater than 50% of the time, while non-learning pups in the

control odor only condition were chosen if they displayed the normal aversion to peppermint by staying in the peppermint odor area < 30% of the time. The testing and sacrifice meant that ~20 min elapsed from the onset of testing to freezing of the tissue. This meant that sacrifice occurred after CREB phosphorylation levels had returned to normal (McLean et al., 1999). Four genes with CRE elements were significantly altered in the microarray in the associative learning condition: Scrg1, Sec23B (seehttp://natural.salk.edu/CREB/search.htm), Rpp14 and E2F3 (Lesiak et. al., 2013, supplement Table 2). However this is less than I had anticipated. Interestingly, in another strong cAMP/PKA/CREB driven learning model (inhibitory avoidance), hippocampal learning related genes seen 1 hour after learning by microarray were not enriched in CREB promoted genes either (Levenson et al, 2004). As also discussed by Levenson et al a possible reason for the paucity of CREB-related genes in the microarray results may have been the relatively late time point of sacrifice. Tao et al., (1998) showed that CREB controlled gene transcription occurs in a short time window. Greenberg and Ziff also showed that *c-fos* transcription occurs just minutes after stimulation and it returns to baseline within 1hr of stimulation (Greenberg & Ziff, 1984). The Affymetrix array sampled a very large gene set and it included all of the normally targeted immediate early genes as well as BDNF. It could be that at the time of sacrifice the immediate early genes had returned to baseline levels so the microarray results did not show any significant expression of these genes.

A virtue of the present approach was that I was more likely to identify ultimate effector protein mRNA transcription since the later protein messages produced would not be

transcription factors, but should be those required to support circuitry changes, for example Sec23b. The 50 min time point was within the critical transcription window. It is therefore likely that the genes I identified are directly related to memory support.

The present results suggest CREB-related gene expression is likely to be relatively brief following a 10 min training event. This remains to be confirmed however. It should also be mentioned that despite the care taken to verify memory, I cannot be certain that pups that display preferences or aversions 30 min after training would display these same preferences or aversions at 24 h. No study with repeated testing of the same pups has been carried out to demonstrate that there is a consistent relationship in preference behavior. It is possible to have only short-term odor preference memory without long-term memory (Grimes et al., 2012) and there is some natural variability in testing behavior. But, under our training conditions, the McLean laboratory experience suggests reliable 24 h memory is likely to have been initiated.

It is also the case that re-exposure to peppermint odor during the olfactory testing period may modulate mRNA expression; For example, work on Arc mRNA using catFISH shows that the initial or repeated exposure of rats to effective environmental stimuli can cause immediate early gene expression in hippocampal pyramidal cells within 5 min (Guzowski et al., 1999). This does not appear to have occurred differentially in the present study since there was no evidence, in microarray analysis (even though Arc was on the array), of Arc mRNA at higher levels in learning versus non-learning bulbs or in the peppermint-encoding versus non-encoding quadrants. I also examined Arc with

immunohistochemical staining in learning pups versus controls. I saw no differences among quadrants or conditions, consistent with the microarray report (see Appendix 5).

4.2.2 Microarray findings

The results obtained from microarray showed sixteen learning-related genes in the comparison group of most interest to me (O/S DL vs. O/O DL). I focused on this comparison group because the DL quadrant in the O/S group is known to show increased pCREB in the nucleus of mitral cells, while there is no significant difference in nuclear pCREB levels in the different quadrants of the O/O group (McLean et al., 1999). No associative learning-related change should have occurred in the O/O group, although non associative habituation is likely.

Thirteen of the sixteen genes were analysed using qRT-PCR as an investigation method for the microarray, using another set of laser microdissected tissue samples. I did not analyse 3 of the genes because there were no ready-made gene expression assays for them (they are Schlafen 4, RGD1565493 and mir23b).

4.2.3 Laser microdissected qRT-PCR experiments used to investigate microarray.

As already noted the qRT-PCR experiments with laser micro-dissected tissue were carried out on separate groups of rat pups, since there was not sufficient RNA for both the microarray experiments and the normal confirmation studies. The whole bulb and time line experiments were also conducted on separate groups. From the second group of laser

microdissected tissues, none of the 13 genes analysed showed significant differences between the O/O and O/S group. The lack of significance may have been due to the low number of pups used (3). Inspection of the data indicated that one datum (animal) had values very different from the other two, which led to a large standard error. I did not contact the Hospital for Sick Kids to make a comparison of the primers used in the microarray with those I used for qRT-PCR. It is possible too that I was not examining the same site in all cases.

Differences in the pattern of gene expression between microarray and qRT -PCR are not uncommon (Liewk and Chow, 2006).The following are some of the reasons for the differences in gene expression: 1) biological variability between samples used for microarray and qRT-PCR makes same sample validation more appropriate for microarray confirmation (Chuaqui et al., 2002), 2) varying priming methods used in qRT-PCR and microarray, and 3) different efficiencies of reverse transcriptases used (Freeman et al., 1999). There is also a difference in the data normalization technique used in qRT-PCR and microarray (Dallas et al., 2005). Generally microarray is a less sensitive technique especially in detecting genes with low levels of expression (Bruder et al., 2006), hence the need for a validation of its results. qRT-PCR is a rapid, sensitive and reproducible technique that is more accurate for quantifying a particular mRNA (Walker, 2002), which is why it is used to validate results obtained by a microarray.

4.2.4 LMD and WB comparisons and WB timeline

Fifty min LMD and 50 min WB comparison experiments were run to determine if there were any differences using RNA from the whole olfactory bulb and RNA from the dissected mitral cell layer. Four of the genes in the 50 min WB showed significant difference between O/O and O/S (Clic2, Dcbld1, RGD1566265 and Sema4C). Three of these genes were up-regulated in the whole bulb at 50 min and in the LMD microarray at 50 min. This concordance was seen even though only 3 rat pups were used per sample for whole bulb. This is encouraging for further studies of transcription underpinnings of early odor preference memory. Two of these genes are in the semaphorin family (Sema4c and Dcbld1) and known to have roles in promoting neuronal connectivity (Nagai et al., 2007 and Ohoka et al., 2001). One is involved with intracellular calcium signalling (Clic2) and has been implicated in cognitive function since its abnormal modulation is associated with retardation (Takano et al., 2012). The fourth (Sec23b) is involved in intracellular transport (Lord et al., 2013). Importantly, in the time line experiment only the 50 min point was significantly different for these mRNA species. Clic2 in particular appeared to increase in levels at that time point. Thus two separate experiments confirm a role for Clic2, Dcbld1, Sema4c and RGD1566265 in learning-related plasticity in the first hour after training in the olfactory bulb. Of these four genes Dcbld1 has CRE motifs in its promoter region and may be regulated by CREB. It will be of interest to pursue the role of these mRNA species by using interference RNA infusion in the olfactory bulb of rat pups in the first hour post-training. It will also be of interest to use immunocytochemistry to look at the distribution of the presumed protein product in pups with olfactory preference

memories versus controls. The direction of the Sema4C change was opposite to that seen in the microarray suggesting a possible difference in remodelling effects within the peppermint-encoding area and in the rest of the olfactory bulb. Since Sema4C would be expected to enhance inhibitory modulation that might be quite reasonable, as one might predict less inhibition in the learning region and increased lateral inhibition in other areas. Or alternatively the mRNA is important for learning and the microarray direction is not relevant. In comparing the present experiments to the one large microarray study done earlier with learning (Levenson et al.,2004) it may be worth noting that both microarray sets (that for the olfactory bulb associative learning tissue and that for the dentate gyrus and CA1 region of the hippocampus) indicated an up-regulation of E2f transcription factor.

Overall this study showed that there is some correlation between what happens in the whole olfactory bulb compared to the mitral cell layer itself during learning. It could be that the largest changes occur in the learning regions and are seen but in a diluted fashion in the whole bulb, or there could be differential changes across many areas of the bulb. Because of the likelihood of dilution effects and thus a low signal-to-noise ratio for mRNA transcription, caution must be taken when using the whole olfactory bulb as a measure of what happens in the mitral cell or any other smaller region of the olfactory bulb.

4.2.6 Conclusion and future directions

This present study is the first to attempt to identify the mitral cell gene transcription that is responsible for 24hr olfactory memory in the rat pup. Previous studies that led to this research have showed that there are a number of changes that take place in the olfactory bulb following olfactory memory formation and the present data are consistent with changing glomerular circuitry.

Using an Affymetrix microarray, I identified 16 genes that were differentially expressed in the mitral cell layer of O/S DL quadrant compared to O/O DL quadrant. These 16 genes were not however confirmed by qRT-PCR when a cohort of samples was used. None of these genes have been previously associated with mammalian olfactory learning, but a number of them are similar to genes identified with dendritic remodelling. The functions of these genes include nervous tissue development, protein transport, interaction with PSD-95, increasing PKA signalling and increasing glutamate production. All of these could logically play a supporting role in mitral cell plasticity and the class of functions altered was similar to that reported in an earlier confirmed microarray analysis by Levenson et al. (2004). However, in the absence of proper validation, I would not attempt to publish the present data set. The four genes found in the whole bulb analysis do, however, merit pursuit.

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Appendix 1 – Sample Preparation

1A) Preparing 2- methyl –butane (isopentane) for flash freezing

1. 2- methyl- butane was pre-cooled in a beaker surrounded by dry ice (this prevented the 2-methyl-butane from bubbling over when the dry ice was added).
2. In a fume hood, crushed dry ice was added to the isopentane to form a slurry mixture
3. When bubbling stopped, the isopentane was at the correct temperature of approximately -90°C.

1B) Protocol for RNA extraction

1. One volume (65 µL for microdissected samples and 350 µL for whole bulb samples) of 70% ethanol was added to the homogenised lysate and mixed well by pipetting.
2. The sample was transferred into an RNeasy MinElute spin column placed in a 2 mL collection tube and centrifuged for 15 sec at e 8000xg. The flow-through was discarded.
3. 350 µL of RW1 (wash buffer) was added to the RNeasy MinElute spin column and centrifuged for 15 sec at e 8000xg. The flow-through was discarded.
4. 10 µL of DNase l stock solution (for DNase digestion) was added to 70 µL of buffer RDD (DNA digest buffer) and then mixed gently by inverting the tube.

5. The DNase l incubation mix was added directly to the RNeasy spin column membrane and incubated by placing on the bench top (20-30°C) for 15 min.
6. 350 µL of buffer RW1 was added to the RNeasy MinElute spin column and centrifuged for 15 sec at e8000xg. This was to wash the spin column membrane. Both the flow- through and the collection tube were discarded
7. The RNeasy MinElute spin column was placed in a new collection tube and 500 µL of buffer RPE (wash buffer) was added to wash the spin column. It was centrifuged for 15 sec at e 8000xg and the flow-through was discarded.
8. 500 µL of 80% ethanol was added to the RNeasy MinElute spin column and centrifuged at e8000xg for 2 min. Both the flow-through and the collection tube were discarded. The spin column was placed in a new collection tube. The lid was opened and centrifuged at full speed for 5 min. Both the flow-through and the collection tube were discarded.
9. The spin column was placed in a new 1.5 mL collection tube and 14 µL of RNase free water was added, the spin column was centrifuged for 1min at full speed.
10. The eluted RNA is stored at -80°C until ready for further analysis.

1C) Bioanalyzer Procedure outlined by Agilent in the Agilent RNA 6000 Pico kit guide

1. All the reagents were allowed to equilibrate at room temperature (30 min) while protected from light.

2. The RNA Pico dye (blue) concentrate was vortexed for 10 sec and spun down. 1 µL of the RNA Pico dye concentrate was added to 65 µL aliquot of the filtered gel (red). This was vortexed and spun for 10 min at 1300g.
3. The gel-dye mix was allowed to equilibrate at room temperature for 30 min away from light.
4. The electrodes of the Bioanalyzer were cleaned with RNase Zap and RNase free water for 5 min each.
5. 9 µL of the gel-dye mix was pipetted at the bottom of the well-marked G and dispensed using the chip priming station.
6. 9 µL of the gel-dye mix was pipetted into the rest of the wells marked G.
7. 9 µL of the RNA 6000 Pico conditioning solution (white) was pipetted into the well-marked CS.
8. 5 µL of the RNA 6000 Pico marker (green) was pipetted into the well-marked ladder and each of the 11 sample wells.
9. The samples and the ladder aliquot were heat denatured at 70°C for 2 min.
10. 1 µL of the ladder was pipetted into the well labelled with the ladder symbol and 1 µL of sample was pipetted into each of the sample wells.
11. The chip was placed horizontally in the adapter of the IKA vortex mixer and vortexed for one min at 2400 rpm.
12. The chip was put in the Bioanalyzer machine and allowed to run.
13. The concentration, RNA Integrity Number (RIN) and the rRNA ratio were recorded.

1D) PCR reaction procedure

The procedure outlined in the TaqMan® Fast Advanced Master Mix protocol was followed.

1. The TaqMan® Fast Advanced Master Mix was mixed thoroughly

2. Frozen cDNA and TaqMan® Assays were thawed on ice, re-suspended by vortexing and centrifuged briefly

3. The volumes for the total number of reactions were calculated.

4. The volume for each reaction is as shown in Appendix 2B .

5. All the components were added and mixed in a 1.5ml microcentrifuge tube.

6. The tube was capped and vortexed briefly.

7. The tube was centrifuged briefly to spin down the contents and to eliminate air bubbles.

8. 20 µl of the reaction mix was pipetted into the wells of a MicroAmp® fast optical 96- well reaction plate with barcode (Applied Biosystems).

9. The reaction plate was covered with an optical Adhesive cover (Applied Biosystems).

10. The plate was run using the ViiA™ 7 qPCR system. The thermal cycling conditions used are as shown in Appendix 2B Table 3.

Appendix 2 – cDNA Preparation

2A) Protocol for cDNA synthesis using the SuperScript® VILO™ cDNA Synthesis Kit

Table 8: Protocol showing single reaction for cDNA synthesis

Component	Volume (µl)
5X VILO™ Reaction Mix	4
10X SuperScript® Enzyme Mix	2
RNA (up to 2.5 µg)	x
DEPC- treated water	to 20

These were combined in a 0.2 ml PCR tube (Fisher Scientific) and mixed gently. The thermal cycling conditions were as follows: the tube was incubated at 25°C for 10 minutes, incubated at 42°C for one hr and the reaction was terminated at 85°C for 5 min. The cDNA was immediately put on ice and stored at -20°C until further use.

2B) Protocol for cDNA amplification

Table 9: Protocol for cDNA Preamplification

Component	Volume (µl/reaction)	Final Concentration
TaqMan PreAmp Master Mix(2X)	25	1X
Pooled assay mix(0.2X)	12.5	0.05X (each assay)
1-250 ng cDNA sample + nuclease free water	12.5	0.02- 5.0 ng/µL
Total	50	-

1. The tubes were capped, inverted gently to mix the reactions and the centrifuged briefly.
2. The tubes were loaded into the thermal cycler (BIO RAD, C1000 Touch™).
3. The thermal conditions were set up at shown in Table 12.

Table 10: Thermal conditions for cDNA Preamplification

	Enzyme Activation	Preamplification PCR	
	Hold	Cycle (10 or 14 cycles)	
		Denature	Anneal/Extend
Temp	95°C	95°C	60°C
Time	10min	15sec	4min

1. Fourteen cycles were run.
2. When the reaction was completely the samples were put immediately on ice.
3. The samples were diluted and stored at -20°C until the PCR reaction was started.

Appendix 3: qRT-PCR information.

3A) Information on gene expression assays used in the microarray confirmation experiment

Table 1: qPCR target Information

Gene Symbol	Amplicon Length	Assay ID	Sequence Accession Number	Exon Boundary	Assay Location
Magee2	108	Rn01473949_s1	NM_001106941.1	1-1	1855
Dcbld 1	66	Rn01433907_m1	XM_228172.5	8-9	815
RGD1308023	58	Rn01334485_m1	XM_002729225.1	26-27	3475
Clic2	144	Rn01767096_m1	NM_001009651.1	4-5	605
Mgst2	61	Rn01494938_m1	NM_001106430.1	1-2	224
E2f3	168	Rn01434981_m1	NM_001137626.1	-	1134
Scrg1	82	Rn00583743_m1	NM_033499.1	2-3	293
Rpp14	80	Rn01537702_m1	NM_001108372.2	4-5	521
Sec23b	68	Rn01415529_m1	NM_001108593.1	8-9	1054
Sema4c	57	Rn01452244_g1	NM_001106902.1	6-7	1215
Mcc	57	Rn01527265_m1	NM_001170534.1	2-3	310
RGD1566265	103	Rn01473904_m1	NM_00113589.1	2-3	279
Gng12	87	Rn01425123_m1	XM_57287.3	1-2	94

3B) TaqMan ®Fast Advanced Master Mix protocol

Table 2: Protocol for PCR reaction

Component	Volume (µl) for 1 reaction		Final Concentration
	384-well plate	96-well and 48-well plate(both standard and fast)	
Taqman® fast Advanced Master Mix(2X)	5.0	10.0	1X
Taqman® Gene Expression Assay (20X) or Custom Taqman® Expression Assay(20X)	0.5	1.0	1X
cDNA template	1.0	2.0	100ng to 1 pg
Nuclease free Water	3.5	7.0	
Total Volume per reaction	10.0	20.0	-

Table3: Thermal cycling conditions for PCR reaction

	Thermal-cycling profile				
Applied Biosystems Real-Time PCR System	Parameter	UNG incubation ǂ	Polymerase activation ǂ	PCR(40 cycles)	
		Hold	Hold	Denature	Anneal/Extend
	Temp (°C)	50	95	95	60
ViiA™ 7 system	Time (mm:ss)	02:00	00:20	00:01	00:20

ǂ Required for optimal UNG activity.
ǂ Required to activate the AmpliTaq® Fast DNA Polymerase

3C Fold change calculation

Three rat pups were trained for olfactory learning by stroking (O/S) and three pups were exposed to peppermint odor (O/O). cDNA was synthesized from RNA from the whole olfactory bulbs and triplicate PCRs were performed on each sample of cDNA. The mean CT (O/S pups) from the triplicate PCRs for the target (Magee2) and internal control (18S) gene are 27.982, 27.461, 27.827 (Magee2) and 13.257, 13.002 and 13.151 (18S). The mean CT (O/O pups) from the triplicate PCRs for the target and internal control gene are 27.858, 27.752 and 28.083 (Magee2) 13.240, 13.324 and 13.345 (18S).

What is the mean ± s.d. and the fold change in expression between the O/S and O/O pups.

Using the $2^{-\Delta CT}$ method

ΔCT = CT gene – CT internal control

O/S pups	O/O pups
$2^{-(27.982-13.257)}$= 3.69261E-05	$2^{-(27.858-13.240)}$ =3.97689E-05
$2^{-(27.827-13.002)}$ = 4.43974E-05	$2^{-(27.752-13.324)}$ =4.53564E-05
$2^{-(27.827-13.151)}$ =3.82106E-05	$2^{-(28.083-13.345)}$ =3.66033E-05

The mean ±s.d. = 3.98447E-05±3.9947E-06 (O/S) and4.05762E-05 ±4.43206E-06 (O/O)

The fold change in expression between O/S and O/O = 3.98447E-05/4.05762E-05

= 0.981971643

Appendix 4: Microarray Information

Table 2: Information on Samples Used for Microarray

	RNA Name	Used (pg)	Amp. Date	Reaction ID	cDNA used (ug)	Chip Lot#	Experiment File Name
1	O/SDL	500	27-06-12	XW7089	5.5	4168819	RaGene1_062812W_JMc01_O-SDL
2	O/SVM	500	27-06-12	XW7090	5.5	4168819	RaGene1_062812W_JMc02_O-SVM
3	O/S2DL	500	27-06-12	XW7091	5.5	4168819	RaGene1_062812W_JMc03_O-S2DL
4	O/S2VM	500	27-06-12	XW7092	5.5	4168819	RaGene1_062812W_JMc04_O-S2VM
5	O/S3DL	500	27-06-12	XW7093	5.5	4168819	RaGene1_062812W_JMc05_O-S3DL
6	O/S3VM	500	27-06-12	XW7094	5.5	4168819	RaGene1_062812W_JMc06_O-S3VM
7	O/ODL	500	27-06-12	XW7095	5.5	4150707	RaGene1_062812W_JMc07_O-ODL
8	O/OVM	500	27-06-12	XW7096	5.5	4150707	RaGene1_062812W_JMc08_O-OVM
9	O/O1DL	500	27-06-12	XW7097	5.5	4150707	RaGene1_062812W_JMc09_O-O1DL
10	O/O1VM	500	27-06-12	XW7098	5.5	4150707	RaGene1_062812W_JMc10_O-O1VM
11	O/O2DL	500	27-06-12	XW7099	5.5	4150707	RaGene1_062812W_JMc11_O-O2DL
12	O/O2VM	500	27-06-12	XW7100	5.5	4150707	RaGene1_062812W_JMc12_O-O2VM

Appendix 5: Preliminary Results

Determination of the best staining method for laser microdissection

Trial 1: Immunohistochemistry

We tried Arc immunohistochemistry as staining method however there was no distinction in the mitral cell layer labeling of all the quadrants and there was not difference between the learning group and the control.

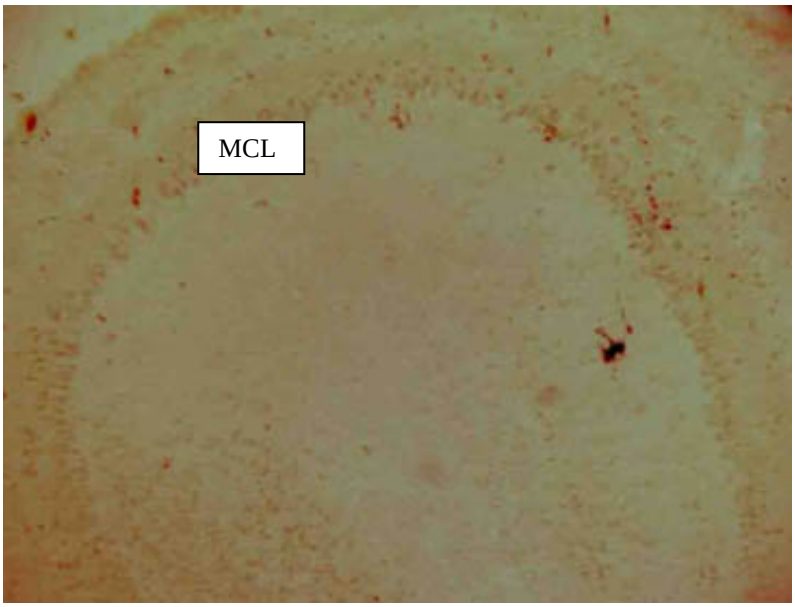

Fig 1: An olfactory bulb section showing Arc immunostaining at low magnification. Pups were treated with 2mg/kg Iso and peppermint odor and sacrificed 60 min after training. Label was observed in the mitral cell layer (MCL) in addition to other layers.

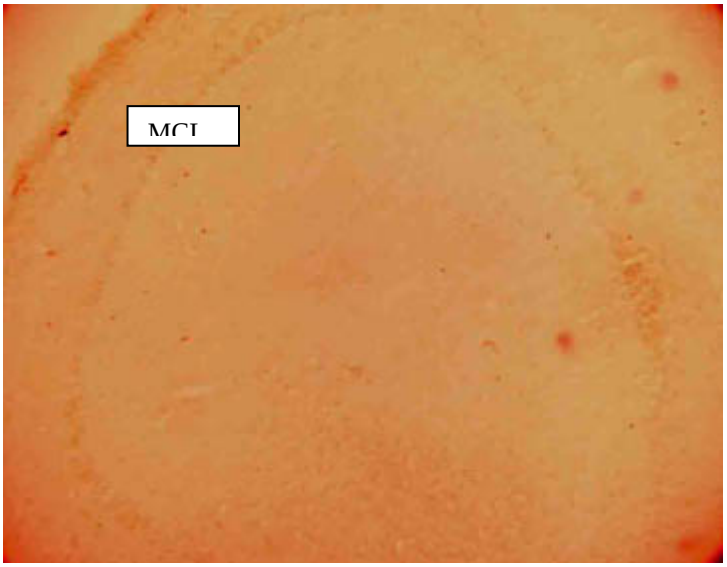

Fig.2: An olfactory bulb section showing Arc immunostaining at low magnification. Pups were treated with saline and peppermint odor and sacrificed 60 min after training.

Table 3: Relative Optical Density Image analysis of the mitral cell layer of cryostat sections immunostained with Arc. Results are from 2 pups treated with 2mg/kg Iso + and 2 pups given odor and saline + odor and sacrificed 1 hour after training.

Condition	dorsolateral	Ventral lateral	Dorsomedial	Ventral medial
saline	0.083	0.090	0.076	0.079
2mg/kg Iso	0.048	0.052	0.048	0.053

None of the methods above worked for this project because we needed a method that was not time consuming (necessary for maintaining RNA integrity and quality) and did not include the use of 4% PFA.

Quick thionin staining method (used in the main project) works best on fresh frozen brains and gave good morphology and maintained RNA integrity.

Determination of the best freezing method and cryostat sectioning conditions for good RNA quality

Trial 2: Dry Ice

Brain is frozen on dry ice, 8µm cryosections were cut at -18°C in RNase free cryostat, cryosections were thionin stained then laser microdissected.

Table 4: Spectrophotometer results of RNA extracted from brains flash frozen with dry ice

Sample ID	Concentration (ng/ul)	260/280	260/230
Naive 4-2 glomerular	16.89	1.50	0.37
Naive 4-1 mitral cell layer	6.36	1.47	0.29
Naive 4-2 mitral cell layer	11.70	1.56	0.46
Naive 4-2-2 mitral cell layer	2.76	5.97	0.01

Comments: The RNA obtained was highly contaminated as shown by the low 260/280 and 260/230. Pure RNA has a 260/280 ratio of between~ 1.8-2.0 and the 260/230 is slightly above 2.0. This could have been due to a lot of factors including time spent sectioning, time spent laser microdissecting, improper flash freezing. I decided to immerse the brain in an RNA stabilizing solution before flash freezing.

Trial 3: Immersing brain in buffer RLT

Brain was immersed in buffer RLT (RNA stabilising buffer) before freezing with dry Ice.

Results: The brain did not freeze. I decided to try flash freezing with liquid nitrogen and limit the time for cryostat sectioning (Trial 4).

Trial 4: Flash freezing with liquid nitrogen and cryostat sectioning reduced to 40 minutes. RNA was analyzed with the bioanalyzer.

Table 5: Bioanalyzer results of RNA from brains flash frozen with liquid nitrogen.

Sample	Concentration pg/µl	rRNA ratio	RIN
EPL	2,183	1.5	8.2
G1	1,645	0.4	5.3
MC	4,982	1.7	8.9
EPL	6,578	1.7	8.8
G2	2,116	2.1	8.6
MC	11,393	1.4	8.7

Comments: Results from this experiment was very good except for G1. RIN numbers above 6 is good for microarray and qPCR, and rRNA ratios for intact RNA must be close

to 2. This method was not used for the rest of the project because the brains broke easily once out of liquid nitrogen making it difficult to use this method for LMD.

Trial 5: Fixing brain with modified methacarn

Brains were fixed with modified methacarn (8 parts methanol: 1 part glacial acetic acid) and flash frozen with liquid nitrogen.

Results: Brains broke apart after taking out of liquid nitrogen

Fixing brain with modified methacarn (8 parts methanol: 1 part glacial acetic acid) and flash freezing with dry ice (ie. warmer temperature than liquid nitrogen)

Results: Brains thawed in cryostat were not cuttable at -18^{o}C. I decided not to use this approach in the future because flash frozen tissue does not show good morphology which is essential for laser microdissection.

Trial 6: fixing brain with 4% PFA

Table 6: Spectrophotometer results of RNA from brains perfused with 4% PFA

Sample	Concentration (pg/µL)	rRNA ratio	RIN
MC	110,864	0.4	-
EPL	7,254	0.5	-
GL	1,781	0	-

Comments: These sections had excellent morphology however the RNA quality was very bad so this approach could not be used for RNA analysis.

Trial 7: Quick freezing with dry Ice and 40minutes of cryostat sectioning and limiting LMD time to 1 hour

Table 7: Bioanalyzer results of samples obtained from sections laser microdissected in one hour.

Sample	Concentration pg/µl	RIN	rRNA ratio
O2	467	7.2	1.5
O20	996	7.6	1.5
O0	128	7.1	1.4
E2	314	7.4	2.1
E3	535	7.1	1.9
E30	243	6.9	1.9
G3	78	6.2	1.2
G20	166	6.9	1.9
G0	133	7.3	2.2

Comments: The RNA obtained using this procedure produced good RNA integrity however the concentrations were too low, and the cryostat sections showed poor morphology. In the next experiment (Trial 7) , I increased the thickness of the sections and reduced the cryostat temperature to -12 ^{o}C.

Trial 8: Fresh brain frozen with dry ice and sectioned at 25µm and 30µm, cryostat temperature reduced to -12 ^{o}C

Table 8: Bioanalyzer results of RNA obtained from brain flash frozen with dry ice and sectioned at 25µm and 30µm, cryostat temperature reduced to -12 ^{o}C.

Sample	Concentration (pg/µL)	rRNA ratio	RIN
ONL -25	1,566	1.3	7.4
GL-25	341	1.7	8.3
EPL-25	1,167	1.6	8.6
ONL-30	1,034	1.4	6.8
GL-30	296	1.3	-
EPL-30	2,839	1.4	5.1

Comment: This approach gave very good RNA as observed by RIN over 6 in most layers of the olfactory bulb. Further evidence showed that flash freezing with dry ice and isopentane slurry produced even better results for RNA integrity and concentration so that approach of freezing olfactory bulbs was used for the thesis.

Bioanalyzer Results for Microarray and qPCR

After LMD, RNA was extracted from the microdissected sections . Generally RNA extracted from tissue collected by LMD is very low in quantity therefore conventional methods of checking RNA quantity and quality such as gel electrophoresis and UV absorbance could not be used because these methods are not sensitive enough to accurately measure very low concentrations on RNA. The RIN showed in Table 1 is a measure of the integrity of the RNA samples. RIN numbers above 6 are good for microarray and qPCR analysis. Due to the low RNA quantity especially in O/O2VM AND O/S2VM the samples were preamplifed before further analysis.

Table 11: Results of samples used for microarray

Sample Name	Concentration (pg/µl)	RIN
O/S DL	10,889	8
O/S VM	6,157	7.9
O/S2 DL	1,598	7
O/S2 VM	524	7.2
O/S3 DL	1,143	7.1
O/S3 VM	13,233	7.1
O/O DL	2,793	8.3
O/O VM	4,914	8.4
O/O1 DL	325	7.8
O/O1 VM	3,156	8.2
O/O2 DL	16,905	6.8
O/O2 VM	178	7.1

The table above shows the RNA quantity and integrity of samples sent to the microarray facility at the hospital for sick kids for microarray analysis. The numbers after the O/O or

O/S is the animal number. Example O/O1DL and O/O1VM were collected from the same animal.

After the microarray experiment was done a new set of pups were trained, their bulbs were cryostat sectioned, their dorsolateral (DL) quadrants were isolated and the RNA extracted. Only the dorsolateral quadrants were isolated because I had decided to focus on the microarray comparison between the O/S (odor +stroking) DL and O/O (odor only) DL. Usually samples used for microarray are the same as the ones used to confirm the microarray results, however my samples were not available so I collected a new set of samples, that were treated the same as those used for microarray. Thus, strictly speaking these tests are not a confirmation of the microarray, but an attempted replication of gene results.

Table 12: Results of samples used for qPCR to confirm microarray data

Sample Name	Concentration (pg/µL)	RIN
O/O2 DL	10,590	8.10
O/O4 DL	9,862	7.8
O/O5 DL	11,410	8.10
O/S2 DL	2,817	8.10
O/S4 DL	9,951	8.10
O/S5 DL	8,499	7.8

The table above shows the RNA quantity and quality of samples used in the qPCR experiment to confirm the microarray results obtained. This suggests adequate RNA quality and quantity.

3.4 Spectrophotometer Results

After the microarray confirmation experiment, I trained and tested more pups for the whole bulb experiment; this experiment was to find out if the genes expressed in the mitral cell layer would also be reflected in whole olfactory bulb preparations and to probe the time course of any targeted gene expression that occurred. The pups were trained and tested at different times (30 min, 1hr and 3hr). A 0 min group was not tested; the pups were sacrificed immediately after the training session (this group was added to show the levels of the genes right after training). The spectrophotometer was used to check the quality and quantity of RNA since the whole bulb has enough RNA to be in the detection range (2ng/µl - 12,000ng/µl) of the spectrophotometer.

Table 13: Results showing RNA quality and concentrations of samples used for qPCR timeline experiment

Sample Name		Concentration (ng/µl)	260/280	260/230
O/S 1	0 min	1347.6	2.06	2.15
O/S 2	0 min	2289.4	2.00	2.06
O/S 3	0 min	1244.9	2.04	2.27
O/O 1	0 min	2508.2	2.00	2.17
O/O 2	0 min	2127.1	2.02	2.23
O/O 3	0 min	2046.9	2.01	2.23
O/S 1	30 min	1938.4	2.02	2.12
O/S2	30 min	1953	2.02	2.21
O/S 3	30 min	2081.1	2.02	2.16
O/O 1	30 min	2361.4	2.00	2.19
O/O 2	30 min	1833.2	2.03	2.23
O/O3	30 min	1773.9	2.05	1.87
O/S1	1 hr	1995.4	2.02	2.24
O/S2	1 hr	2563.1	2.01	2.19
O/S3	1 hr	2646	1.99	2.08
O/O1	1 hr	2148.5	2.02	2.17
O/O2	1 hr	2027.1	2.02	2.19
O/O 3	1 hr	2273.4	2.01	2.17
O/S 1	3 hr	2309.9	2.01	2.18
O/S2	3 hr	2151.1	2.02	2.12
O/S 3	3 hr	1486.8	2.04	2.16
O/O 1	3 hr	2693.3	1.98	2.05
O/O 2	3 hr	2672.2	1.98	2.18
O/O 3	3 hr	2069.8	2.03	2.25