MOLECULAR CORRELATES OF MULTIDAY MEMORY IN AN APPETITIVE CONDITIONING MODEL: INSIGHTS INTO MEDIATORS OF MEMORY EXTENSION

by

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ABSTRACT

These experiments provide insight into potential mediators implicated in the maintenance of memory duration using an olfactory preference learning paradigm. Neonatal rats were trained in a single training session by pairing odor (CS) with pharmacological agents (UCS) to promote odor preference of different durations (24h, 4-Day and 5-Day). For the first time in olfactory preference learning we characterize the pCREB expression profile as biphasic with peaks at 10min and 2h post-conditioning in 24h and 5-Day olfactory preference memory. Second, we show histone acetylation is enhanced by HDAC inhibition via NaB in both learning and non-learning conditions. Finally, q-PCR reveals CREB target genes *Nr4a1* and *Egr-1* are differentially expressed across 24h and 5-Day models, consistent with a possible role in memory extension. These experiments support CREB as a possible initiator in mediating downstream events leading to the changes in synaptic plasticity that accompany extension of long-term memory.

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TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGEMENTS iii
LIST OF FIGURES ix
LIST OF ABBREVATIONS x
LIST OF APPENDICES xii
CHAPTER 1 – INTRODUCTION 1
1.1 Overview 1
1.2 Olfactory Processing 2
1.3 Olfactory Bulb Circuitry 3
1.3.1 Layers of the Olfactory Bulb 3
1.3.2 Olfactory Nerve Layer 4
1.3.3 Glomerular Layer 4
1.3.4 External Plexiform Layer 6
1.3.5 Mitral Cell Layer 6
1.3.6 Internal Plexiform Layer 7
1.3.7 Granule Cell Layer 7
1.3.8 Subependymal Layer 7
1.4 Classical Conditioning 7
1.5 Attachment Learning in Neonatal Rats 9
1.6 Olfactory Preference Learning 10
1.7 Advantages of Olfactory Preference Learning Model 11
1.8 Neuromodulators in Learning and Memory 12
1.8.1 Neurotransmission in the Olfactory Bulb 12
1.8.2 Norepinephrine 12
1.8.3 Serotonin 13
1.8.4 Acetylcholine 14
1.8.5 Dopamine 15

1.8.6 Glutamate 15
1.8.7 NMDA/AMPA 16
1.8.8 GABA 17
1.9 Stages of Memory Duration 17
1.10 Cyclic-AMP Response Element Binding Protein (CREB) 19
1.11 cAMP/PKA/CREB Pathway 19
1.12 Histone Involvement in Learning and Memory 21
1.13 Pharmacological Models of Olfactory Preference Learning 23
1.13.1 24 h Isoproterenol 23
1.13.2 4-Day Isoproterenol+Cilomilast 23
1.13.3 5-Day Iso+Cilo+NaB 25
1.14 CREB target genes 26
1.14.1 NR4A 27
1.14.2 CCAAT/Enhancer Binding Protein 29
1.14.3 Jun and Fos 31
1.14.4 Brain-derived neurotrophic factor (BDNF) 32
1.14.5 Egr-1/zif268 33
1.14.6 Objectives 33
CHAPTER 2 – METHODS 36
2.1 Animals 36
2.2 Drugs 36
2.3 Olfactory Preference Training 37
2.4 Experiment 1 38
2.4.1 Experimental Groups for Western Blotting 38
2.4.2 Tissue Collection 38
2.4.3 Sample Preparation 38
2.4.4 Protein Determination 39
2.4.5 Gel Electrophoresis 39
2.4.6 Protein Transfer 40
2.4.7 Antibody Application 40
2.4.8 Antibodies 40

2.4.9 Enhanced Chemiluminescent Exposure ... 41
2.4.10 Statistical analysis ... 42
2.5 Experiment 2 ... 42
2.5.1 Real-time Polymerase Chain Reaction (q-PCR) ... 42
CHAPTER 3 – RESULTS ... 46
3.1 CREB phosphorylation plays a role in long-term memory and memory extension ... 46
3.1.1 24 h long-term early odor preference training induces biphasic increases in CREB phosphorylation ... 46
3.1.2 CREB phosphorylation in the 4-Day olfactory memory training paradigm is unchanged by training ... 47
3.1.3 CREB phosphorylation in the 5-Day olfactory memory paradigm displays a biphasic pattern of post training increases ... 47
3.2 Histone acetylation may contribute to memory extension ... 48
3.2.1 24 h memory training is not associated with changes in histone acetylation ... 49
3.2.2 4-Day memory training is not associated with changes in histone acetylation ... 49
3.2.3 5-Day memory reveals robust increases in histone acetylation immediately after learning ... 49
3.2.4 Sodium butyrate produces rapid increase in histone acetylation ... 50
3.3 Downstream CREB target differences in expression provide insight into potential mechanisms of memory extension ... 50
3.3.1 NR4a family of nuclear receptors ... 51
3.3.2 CCAAT-enhancer binding protein (C/EBPβ) ... 53
3.3.3 Jun ... 53
3.3.4 Fos ... 53
3.3.5 Egr1 ... 54
3.3.6 BDNF ... 54
CHAPTER 4 – DISCUSSION ... 55
4.1 Summary of major findings ... 55
4.2 Expression pattern of pCREB across long-term olfactory preference learning models 56
4.3 4-Day long-term memory does not show evidence of pCREB enhancement ... 61
4.4 NaB initiates histone 3 acetylation ... 62

4.5 Differential gene expression suggests CREB regulated genes are implicated in the promotion of memory duration 66
4.6 NR4A gene expression is differentially expressed across long-term and extended long-term olfactory preference learning 67
4.7 C/EBPβ is significantly enhanced in the OB after long-term and extended long-term odor preference training 69
4.8 Jun, Fos and Bdnf expression are not enhanced after olfactory preference learning 71
4.9 Egr-1 expression is enhanced in extended long-term olfactory preference learning 71
4.10 Conclusions 73
4.11 Future directions 75
GRAPHICAL RESULTS 911
APPENDIX A: Layers of the Olfactory Bulb 103
APPENDIX B: 5-Day Odor Preference 104
APPENDIX C: Role and function of pharmacological agents used for 24 h, 4-Day and 5-Day olfactory memory models 105
APPENDIX D: Cilomilast concentration adjustment sample equation 106
APPENDIX E: Schematic representation of olfactory preference training protocol 108
APPENDIX F: Western Blotting Recipes 109
APPENDIX G: q-PCR Sample Calculation ΔCT Method 112

LIST OF FIGURES

Figure 1. Western blotting pCREB profile 91

Figure 2. Western blotting Acetyl H3 profile 93

Figure 3. q-PCR relative fold change of Nr4a1 gene... 95

Figure 4. q-PCR relative fold change of Nr4a2 gene... 96

Figure 5. q-PCR relative fold change of Nr4a3 gene... 97

Figure 6. q-PCR relative fold change of C/EBPβ gene 98

Figure 7. q-PCR relative fold change of Jun gene 99

Figure 8. q-PCR relative fold change of Fos gene 100

Figure 9. q-PCR relative fold change of Egr1 gene 101

Figure 10. q-PCR relative fold change of BDNF gene 102

LIST OF ABBREVATIONS

2-DG	2-deoxyglucose
5-HT	serotonin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	Ammonium persulphate
AR	adrenoceptor
BDNF	brain derived neurotrophic factor
C/EBP	CCAAT/enhancer binding protein
Ca^{2+}	calcium
cAMP	cyclicAMP
CBP	CREB binding protein
cilo	cilomilast
Cl^-	chloride
CNS	central nervous system
CR	conditioned response
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
CS	conditioned stimulus
DA	dopamine
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
ECL	enhanced chemiluminescent
EDTA	ethylenediaminetetraacetic acid
Egr-1	early growth response factor 1
EPL	external plexiform layer
GCL	granule cell layer
GL	glomerular layer
H	histones
h	hour
HAT	histone acetyltransferase
HDAC	histone deacetylase
IEG	Immediate early gene
IPL	internal plexiform layer
Iso	isoproterenol
JG	juxtaglomerular neuron
LC	locus coeruleus
LOT	lateral olfactory tract
LTCC	L-type calcium channel
LTF	long-term facilitation
LTP	long-term potentiation
MCL	mitral cell layer
Na^+	sodium

NaB	Sodium butyrate
Narp	neuron activity regulated pentraxin
NE	norepinephrine
NMDA	N-methyl-D-aspartic acid
NR4A	nuclear receptor
OB	olfactory bulb
ON	olfactory nerve
ONL	olfactory nerve layer
ORN	olfactory receptor neuron
PD	post-natal day
PDE4	phosphodiesterase 4
PKA	protein kinase A
PMSF	phenylmethanesulfonyl Fluoride
RGF136	[N-(6-(2-amino-4-fluorophenylamino)-6-oxohexyl)-4-methylbenzamide]
q-PCR	real-time polymerase chain reaction
s.c.	Subcutaneous
SDS	Sodium dodecyl sulphate
Ser-133	Serine-133
SVZ	subventricular zone
TBST	tris buffered saline with tween
TSA	trychostatin A
UCS	unconditioned stimulus
β-ME	β-mercaptoethanol

LIST OF APPENDICES

APPENDIX A: Layers of the Olfactory Bulb..103

APPENDIX B: 5-Day Odor Preference...104

APPENDIX C: Role and function of pharmacological agents used for 24 h, 4-Day and 5-Day olfactory memory model……...105

APPENDIX D: Cilomilast concentration adjustment sample equation.....................................106

APPENDIX E: Schematic representation of olfactory preference training protocol..................108

APPENDIX F - Western Blotting Recipes...109

APPENDIX G - q-PCR Sample Calculation ΔCT Method...112

CHAPTER 1 – INTRODUCTION

1.1 Overview

The first behavioural studies that assessed the cellular and molecular correlates of memory used simple systems to study the most basic forms of learning and memory. This is best exemplified by the pioneering work of Eric Kandel and his colleagues through the study of *Aplysia californica* that showed there is much to be gained through the study of simple systems. Studies of memory have since grown and expanded into a variety of species using models directed at all forms of learning, with the goal of gaining a better understanding of the behavioural, cellular and molecular aspects of memory. The purpose of this study was to examine potential mechanisms involved in long-term memory extension with the use of neonatal olfactory preference learning models.

This thesis focuses on determining the cellular and molecular correlates involved in long-term memory extension. Specifically, the experiments in this study focus on two central aspects of long-term memory signalling. First, I investigate the effects of post-translational modifications on integral components of the long-term memory signalling cascade, the phosphorylation of cyclic AMP response element binding protein (CREB) and the acetylation of histones, through the use of three memory models of different durations (24 h, 4-Day and 5-Day memory). How these modifications vary across our models is explored. Second, I focus is directed to the genetic targets of CREB mediated transcription and how the expression of CREB target genes differs across these models. I will discuss olfactory information processing and the anatomy of the olfactory bulb and its cellular structure to provide an overview of the processes involved in

olfactory preference learning. I then provide a review of classical conditioning models followed by an introduction to neonatal olfactory preference learning and the advantages of using this model to study memory. A discussion of the neuromodulators implicated in learning and memory is presented to provide insight into mediators of the cellular pathways involved in learning and memory. I then describe the role of the cAMP/PKA/CREB signaling cascade as it pertains to learning and memory and how phosphorylation and acetylation are implicated in this pathway. The olfactory preference models used in this study will be discussed in detail. This is followed by the study of CREB target genes and how they relate to learning and memory.

1.2 Olfactory Processing

Olfaction is the process that defines our sense of smell. Olfactory processing involves the conversion of volatile chemicals (odorants) from the external environment and transducing them into electrical signals the brain can interpret. These signals can initiate action potentials that can lead to the encoding of odor specific maps allowing for discrimination of explicit odors within the environment (Bakalyar and Reed 1991; Reed 1992).

The sense of smell is governed through the interaction of odor molecules with odor receptors in the nasal epithelium of the olfactory system. There are roughly 1000 odorant receptors within the mammalian olfactory bulb (OB) and each individual olfactory receptor neuron (ORN) expresses only one type of odorant receptor (Young and Trask 2002). At the basal end of an ORN is an axon that projects to the OB. The interaction between an odorant and an ORN is initiated when odorants bind to the cilia at the apical tip of ORNs and elicit olfactory signal transduction through a second-messenger cascade leading to depolarization of the neuron.

Binding to olfactory receptors activates G-proteins that stimulate adenylyl cyclase, leading to an increase in cAMP stores (Boekhoff and Breer 1990; Breer et al. 1990; Kaupp 2010). This in turn leads to an influx of sodium (Na^+) and calcium (Ca^{2+}), activating Ca^{2+}-gated chloride (Cl^-) channels and depolarization of the neuron (Bradley et al. 2005; Liman and Buck 1994; Nakamura and O'Leary 1989; Prasad and Reed 1999; Reisert et al. 2005). Axons from ORNs converge to form the olfactory nerve (ON) that passes through the cribriform plate and forms the olfactory nerve layer (ONL) on the surface of the bulb. Olfactory receptor axons synapse at individual glomeruli in the adjacent glomerular layer of the OB (Pinching and Powell 1971a).

The site of synaptic contact between the ON and the OB is on individual glomeruli. A high degree of specificity is seen in the OB as each ORN can synapse onto one glomeruli, and each glomerulus can receive information from many ORNs of the same type (Adam and Mizrahi 2010). The human OB is small in comparison to the rest of the brain whereas rat OBs are relatively large, representative of the extent to which they rely on their sense of smell to navigate their environment.

1.3 Olfactory Bulb Circuitry

1.3.1 Layers of the Olfactory Bulb

The OB is a highly organized laminar structure that forms the most anterior portion of the forebrain. The most superficial layer is the ONL followed by the glomerular layer (GL), the external plexiform layer (EPL), the mitral cell layer (MCL), the internal plexiform layer (IPL), the granule cell layer (GCL) and the deepest layer, the subependymal layer (Appendix A).

1.3.2 Olfactory Nerve Layer

The ONL is the most superficial layer of the OB and is composed primarily of the axons of ORNs as they head to the GL where they terminate, and glial cells (Shipley and Ennis 1996).

1.3.3 Glomerular Layer

The GL lies adjacent to the ONL on the surface of the OB and is the first site of synaptic integration. The GL is comprised of roughly 3000 individual glomeruli (Meisami and Safari 1981) that are spherical shaped (80-160 µm diameter) neuropil-rich structures surrounded by a shell of juxtaglomerular (JG) neurons and glial cells (Pinching and Powell 1971b). JGs are a collective set of neurons comprised of external tufted cells, periglomerular cells, and short-axon cells (Hayar et al. 2004).

External tufted cells (10-15 µm) are excitatory neurons that lie deep within the periglomerular region of the GL. They typically have a single primary dendrite that extends into individual glomeruli where it branches in a triangular pattern on the opposite side of entry (Pinching and Powell 1971a). Electrophysiological studies have shown that external tufted cells receive direct monosynaptic input from ON axons. This input excites external tufted cells resulting in coordinated firing patterns within each glomerulus. It is this correlated activity that summates and produces monosynaptic glutamatergic input to periglomerular and short-axon interneurons, an indirect link to ON input (Hayar et al. 2004).

Periglomerular cells are small (5-8 µm), ovoid shaped cells, that constitute part of the neuronal shell that encircle glomeruli (Pinching and Powell 1971a). Periglomerular cells are GABAergic and/or dopaminergic inhibitory cells (Maher and Westbrook 2008) and comprise a

high cell density in the GL (Puopolo and Belluzzi 1998). Periglomerular cells are believed to have long dendrites that extend into glomeruli with short dendrites extending into the periglomerular region (Pinching and Powell 1971a). The periglomerular region separates individual glomeruli (Pinching and Powell 1971c). Roughly 20% of periglomerular cells receive direct excitatory input from the ON. Input from the ON initiates inhibitory input from periglomerular cells onto mitral cells. Periglomerular cells also receive monosynaptic excitatory input from external tufted cells, which in turn produces inhibitory feedback onto external tufted and mitral cells (Wachowiak and Shipley 2006).

Short-axon cells (8-12 μm) are a low density population of spherical cells found in the periglomerular region. Short-axon cells co-express markers for dopamine and GABA and have dendrites that are confined within the periglomerular region surrounding individual glomeruli (Liu et al. 2013; Pinching and Powell 1971a). Short-axon cell dendrites span 2-4 glomeruli with longer processes extending across multiple glomeruli. They are responsible for mediating the interglomerular circuit (Liu et al. 2013).

Mitral cells and tufted cells are the primary output cells of the OB (Aroniadou-Anderjaska et al. 1999; Christie et al. 2001; Johnson et al. 2013). The primary dendrites of these output cells, covered by a thin layer of glial cells, pass through the periglomerular region and enter glomeruli. Mitral cells can excite JG cells in the GL via glutamate (Aroniadou-Anderjaska et al. 1999). The GL comprises the first site of olfactory sensory input and thus plays a significant role in mediating olfactory processing. Olfactory information is transmitted to deeper layers of the OB by mitral and tufted cell axons.

1.3.4 External Plexiform Layer

Deep to the GL lies the EPL. The EPL is composed primarily of the granule cell dendrites and the secondary dendrites of mitral cells and tufted cells (Price and Powell 1970). External, middle and deep tufted cells comprise the neuronal types in the EPL with increasing size closer to the deepest part of the EPL (Macrides and Schneider 1982). Dendrites from granule cells and short-axon cells have peripheral processes that extend into the EPL. Dendrites from granule cells form reciprocal synapses with mitral cells in the EPL (Price and Powell 1970).

1.3.5 Mitral Cell Layer

Deep to the EPL is a thin layer of mitral cells that comprise the MCL. Mitral cells are the primary output cells of the OB and are the largest with a cell body diameter of 20-30 µm (Price and Powell 1970). Axons of mitral cells converge to form the lateral olfactory tract (LOT) which extends to the primary olfactory cortex. Mitral cells have primary dendrites that extend to the glomeruli of the OB where single mitral cell dendrites synapse within an individual glomerulus. Each glomerulus can contain dendrites from multiple mitral cells suggesting a divergence of information coming from an individual glomerulus. Secondary dendrites of mitral cells extend laterally within the EPL. Excitation of mitral cells is modulated by dendrodendritic synapses with granule cells where glutamate excites mitral cells, initiating GABAergic mediated inhibition by granule cells (Didier et al. 2001).

1.3.6 Internal Plexiform Layer

The IPL lies deep to the MCL and is densely innervated by axons and dendrites of OB cells including the axons of the main output cells, the mitral cells and the tufted cells as well as axons and dendrites from granule cells. The IPL also receives serotonergic (McLean and Shipley 1987a), noradrenergic (McLean et al. 1989) and cholinergic inputs (Nickell and Shipley 1988)

1.3.7 Granule Cell Layer

The GCL lies adjacent to the IPL. The GCL is comprised of small granule cells (8-10 μm) arranged in aggregates and coupled via gap junctions (Reyher et al. 1991). Granule cells are primarily GABAergic and form inhibitory synapses with mitral and tufted cell dendrites (Ribak et al. 1977).

1.3.8 Subependymal Layer

The deepest layer in the OB is the subependymal layer or the subventricular zone (SVZ). Activity in this layer occurs mostly during development and is the site from which granule cells and periglomerular cells are derived (Lledo et al. 2004).

1.4 Classical Conditioning

First identified in canines by Ivan Pavlov, classical conditioning has formed the premise for associative learning and memory models. In its traditional definition, classical conditioning involves associating two paired stimuli, an unconditioned stimulus (UCS) with a conditioned

stimulus (CS) where the CS comes to predict the onset of the UCS eliciting a conditioned response (CR). This form of associative learning permits animals to adaptively interact with and navigate their surroundings as these associations are used to predict events in the animal's environment. In the 1980's it was discovered that *Aplysia californica* can be classically conditioned in a withdrawal reflex response when tactile stimulation to the siphon (CS) was accompanied by a shock to the tail (UCS) resulting in a conditioned gill withdrawal response (Carew et al. 1981). The efficacy of using simple neural circuits to study complex paradigms is exemplified in the *Aplysia* studies of classical conditioning, as it allows a well identified circuit to be easily manipulated. Since the discovery of the ability for *Aplysia* to be classically conditioned, more recent studies have developed a wide range of animal models to determine the underlying cellular and molecular networks that govern this form of associative learning and memory.

Human infants have the ability to be classically conditioned very early in life. One of the earliest examples of complex classical conditioning in infants involved exposing one-day old infants to a novel odor (CS) paired with tactile stimulation of stroking by the mother (UCS). This pairing produced a conditioned preference response of head tilting towards the conditioned odor 24 h later (Sullivan et al. 1991b). Conditioned eye-blink response in rabbits exemplifies a classical conditioning paradigm where rabbits blink in response to a tone previously paired with a puff of air to the eye (Schneiderman et al. 1962). Other models of classical conditioning include *Drosophila* (fruit flies). Fruit flies can be conditioned in an odor-taste paradigm where they learn to prefer an odor previously paired with a food reward of sugar (Gerber et al. 2013; Kim et al. 2007). Neonatal mice can learn to prefer an odor previously paired with a thermoneutral temperature and avoid odors paired with cold temperatures (Bollen et al. 2012).

Pairing of odor+shock in mice has shown long lasting changes in synaptic plasticity (Roth et al. 2013) as well as behavioural changes. There are many models of classical conditioning encompassing a wide range of species, this variety provides a range of tools for dissecting the structural and functional underpinnings of adaptive change in both invertebrates and vertebrates. Recent studies of classical conditioning in mammals have used this paradigm to assess the cellular and molecular correlates associated with learning and memory. For the purposes of this study **we employ the use of an early olfactory preference paradigm where neonatal rat pups are classically conditioned to prefer a typically aversive odor.**

1.5 Attachment Learning in Neonatal Rats

Maternal attachment is characterized as a critical learned association that is formed between a caregiver and infant in the early stages of life, especially with altricial animals, and is centered on the requirement for support and survival. This learned association persists in humans and across many species and is heightened during a sensitive period when the ability to form this attachment is critical.

Attachment learning is easily modeled in the neonatal rat. They rely extensively on their sense of smell to locate the dam as their eyes and ears are not yet fully developed (Raineki et al. 2010). This naturally occurring attachment is critical for their survival. Neonatal rats must learn to form an association to maternal odors and produce approach behaviors towards the dam, as she provides their source of food and protection from predators and protection from their environment. Associations of these olfactory cues are formed during a critical period, post-natal days (PD) 1-10. Both pleasant and aversive stimuli can produce approach behaviours in neonatal

rat pups, highlighting a unique resistance to disruption. This is evidenced by persistence during abusive conditions where a rat pup will learn to approach maternal odors when paired with shock (Camp and Rudy 1988; Sullivan et al. 2000a). Near the end of the sensitive period (PD10), the rat pup's sensory input begins to expand beyond tactile and olfactory cues. They also become exposed to odors outside the nest as their motor skills develop (Raineki et al. 2010). The major reliance of neonatal rats on olfactory cues in the sensitive period provides a useful model to study learning and memory early in life.

1.6 Olfactory Preference Learning

The central nervous system (CNS) is immature in rat pups and connections with higher order structures are not yet developed. As a result, they use different circuitry than the more complex adult rat to form new memories. The locus coeruleus (LC) and the OB have been shown to be critical for heightened odor learning in neonatal rats during the sensitive period whereas the hippocampus and frontal cortex are still developing (Moriceau and Sullivan 2005; Raineki et al. 2010; Sullivan 2003). The LC provides the sole source of norepinephrine (NE) for the OB (McLean and Shipley 1991). Release of NE, and subsequent activation of β-adrenoceptors (β-AR), is necessary and sufficient when paired with novel odor to produce neonatal odor preference learning (Harley et al. 2006; Sullivan et al. 1991a). Removing pups from the nest and pairing stroking (using a soft paintbrush) with novel odor exposure (peppermint) for a single 10 min training session produces learning and an odor preference lasting 24 h that is shown by approach behavior towards peppermint odor. This reward, mimicking a maternal interaction (grooming), produces increased NE released from the LC into the OB (Sullivan et al. 1991a).

Odor preference is also found when stroking is replaced with isoproterenol (iso), a β-AR agonist (Langdon et al. 1997; Sullivan et al. 1991a; Sullivan et al. 1989).

1.7 Advantages of Olfactory Preference Learning Model

Early odor preference learning is a valuable model when studying the neurobiology of mammalian learning due to its relatively simple circuitry. It is also advantageous that changes in synaptic plasticity as a result of olfactory learning occur within the olfactory bulb itself (Sullivan and Leon 1986; Sullivan et al. 1989; Yuan et al. 2002), allowing a specific locus of learning of which we can take advantage. When a novel odor is paired with tactile stimulation (odor+stroke), rat pups show a preference for that odor accompanied by an increase in 2-deoxyglucose (DG) uptake in glomeruli of the OB (Sullivan and Leon 1986). Specifically, the increases in 2-DG uptake in the glomerular region of the OB occurs as a result of learning. This increase is not seen in animals where learning was blocked using propranolol (NE antagonist), or when odor was presented alone (Sullivan et al. 1991a). Early olfactory preference learning also produces increases in CREB phosphorylation in the OB after odor+stroke pairing (McLean et al. 1999) and increased ON depolarization of mitral cells (Yuan et al. 2000). The advantages of the olfactory preference paradigm allow us to manipulate a simple circuit in an attempt to gain insight into the mechanisms that drive this form of learning.

1.8 Neuromodulators in Learning and Memory

1.8.1 Neurotransmission in the Olfactory Bulb

Olfactory learning is associated with changes in synaptic transmission between neurons in the OB. Changes in synaptic plasticity are seen within the bulb itself and are an integral advantage when using this system as a learning model. One of the most unique aspects of the OB is that olfactory processing occurs without relaying to the thalamus (Kay and Sherman 2007). The OB receives neuromodulatory input from multiple regions encompassing noradrenergic, serotonergic, cholinergic, dopaminergic, glutamatergic and GABAergic transmission into the OB (Fletcher and Chen 2010). The role of these modulators for the purposes of this thesis is focused primarily on early preference learning.

1.8.2 Norepinephrine

The OB receives extensive noradrenergic input, in fact, at least 40% of LC noradrenergic neurons project to the OB (Shipley et al. 1985). A role for NE in maternal infant attachment has been suggested where associative learning between neonatal rat and the dam is essential for survival of the pup and dependent on NE input to olfactory structures. When LC input to the OB is blocked, acquisition of new memories is inhibited (Sullivan et al. 1989). The majority of noradrenergic LC input is in the GCL (McLean et al. 1989), as both mitral cells and granule cells express NE receptors (Devore and Linster 2012). Such a substantial noradrenergic innervation during olfactory processing suggests NE plays a crucial role in its function. Most critically, for early odor preference learning, LC-NE input (Sullivan et al. 1989) or the activation of

noradrenergic receptors (see next section) can serve the role of the unconditioned stimulus in this form of classical conditioning. In adult rats, the noradrenergic system has been implicated in several types of information processing including learning, and NE has been described as a neuromodulator in this process (Devore and Linster 2012).

Adrenoceptors, the target receptors of NE, are ubiquitously expressed in the OB (Woo and Leon 1995). Evidence supports a role for both α- and β-ARs in mitral cell mediated activity. It has been suggested that α-1 receptors increase excitability of mitral cells, enhancing its ability to detect and discriminate between weak odors and weak ON input (Hayar et al. 2001). Neonatal olfactory preference models also provide evidence for noradrenergic mediated β-AR activation where activation of β-ARs is both necessary and sufficient to produce learning (Sullivan et al. 2000b). Adrenoceptor agonists phenylephrine (α1-AR agonist) and dobutamine (β1-AR agonist) have been shown to induce learning in a neonatal odor preference learning model (Harley et al. 2006). Intrabulbar infusions of clonidine, an α_2-AR agonist, when paired with odor has also shown to induce learning (Shakhawat et al. 2012). These agonists also displayed an inverted U-curve dose response consistent with previous findings using isoproterenol (Langdon et al. 1997; Sullivan et al. 1989). This suggests that noradrenergic activity mediated by the LC is critical in learning and its associated changes in synaptic plasticity in the rat pup olfactory bulb.

1.8.3 Serotonin

Serotonin (5-HT), a neuromodulator in olfactory processing, has inputs in all layers of the OB with the most dense projections into the GL (McLean and Shipley 1987b). An *in situ* hybridization study revealed 5-HT receptors are located primarily in mitral cells and tufted cells

of the OB (McLean et al. 1995). A role for 5-HT in the OB has been associated with the acquisition of olfactory preference memories, exemplified by selective depletion of 5-HT input to the OB producing deficits in acquisition of olfactory preference memories in neonatal rat pups (McLean et al. 1996; McLean et al. 1993) without affecting cellular development (McLean and Darby-King 1994). When 5-HT is depleted in the rat OB, increased β-AR activation can overcome this deficit in memory acquisition (Langdon et al. 1997). It has therefore been proposed that NE via β-AR activation and supported by 5-HT is normally required for stroking-induced classical conditioning of olfactory preference. Immunohistochemical analysis of β1-AR and 5-HT receptor co-localization reveal both receptors are present on OB mitral cells. Serotonin depletion did not appear to directly induce cAMP signaling, but β-AR mediated upregulation of cAMP expression was impaired (Yuan et al. 2003b).

1.8.4 Acetylcholine

The OB receives cholinergic projections from the basal forebrain (Wilson et al. 2004). Cholinergic input is primarily in the GL and GCL of the OB (Kasa et al. 1995). Cholinergic receptors, both nicotinic and muscarinic, have been thought to play a role in learning and memory. Nicotinic receptors are critical in olfactory discrimination and regulation of mitral cell olfactory receptive fields. Increased cholinergic receptor activation using neostigmine enhanced the mitral cell's ability to discriminate between similar odors in adult rats (Chaudhury et al. 2009). Evidence for muscarinic receptor involvement was shown when muscarinic receptors were blocked with scopolamine and performance on a short-term odor-discrimination memory task was impaired (Devore et al. 2012).

1.8.5 Dopamine

Dopamine (DA) neurons are located primarily in the GL of the rat OB and are regulated by ON input in both the developing and adult rat (McLean and Shipley 1988; Wilson et al. 2004). Localization of dopamine-1 (D1) receptors was found in the internal GCL, the IPL and the EPL. Dopamine-2 (D2) receptors were found in the ONL, GL and EPL (Levey et al. 1993; Wilson et al. 2004). Distributions of D2 receptors were also found in olfactory axon terminals and in the glomerular neuropil including the dendrites of mitral and tufted cells and periglomerular cells (Gutierrez-Mecinas et al. 2005). In a study looking at OB changes in response to olfactory preference learning in neonatal rat pups it was found that there was a significant increase of 400% above baseline of DA in the OB during odor stroke pairing (Coopersmith et al. 1991). Weldon and colleagues (1991) showed that subcutaneous injections of the D1 receptor antagonist (±)-SKF 83566 before or after odor+stroking conditioning produces decreased approach behaviours towards the odor 24 h after conditioning. Learning was restored when apomorphine (dopamine receptor agonist) was given immediately after training (Weldon et al. 1991). This suggests a critical supportive role for DA in neonatal olfactory learning.

1.8.6 Glutamate

Glutamate is the major excitatory neurotransmitter in the brain. In the mammalian OB, the first site of synaptic contact in olfactory processing is from the glutamatergic ON onto individual glomeruli. ON input can activate inhibitory neurons in glomeruli or mitral/tufted cell dendrites. When excited by ON input, glutamate is released by the dendrites of mitral cells in a local dendrodendritic circuit and excites granule cells (Didier et al. 2001). The release of glutamate causes a release of GABA from granule cell dendrites back onto mitral cells forming

the dendrodendritic circuit discussed previously. An odor preference food discrimination task provides evidence that response to a conditioned odor in mice produces increased glutamate response from the output cells of the OB, mitral and tufted cells, an increased GABA response from granule cells and periglomerular cells and an increased NE response from the LC (Brennan et al. 1998).

1.8.7 NMDA/AMPA

Excitatory glutamate release activates the glutamate receptors N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Both NMDA and AMPA receptors have been implicated in synaptic plasticity as mediators in long-term memory. Transgenic mice studies where the NMDA receptor 2B (NR2B) is overexpressed show enhanced memory in these mice on a novel object recognition task (enhanced memory lasting 3 days), contextual and cued fear-conditioning task (enhanced memory lasting 10 days), fear-extinction task (faster fear extinction to contextual, 4 days, and cued, 3 days) and on the water-maze (Tang et al. 1999). AMPA receptors are equally important in learning and memory as NMDA receptors require AMPA receptor facilitated depolarization to become activated (Rao and Finkbeiner 2007). AMPA receptors are also the main mediators of glutamate effects when memory is expressed. Olfactory preference training leads to increases in AMPA receptors at the time of the 24 h olfactory preference memory. This increase is no longer seen at 48 h post-training, consistent with decreased memory retention at 48 h (Cui et al. 2011). Blocking NMDAR activation has been shown to reduce ON evoked mitral cell firing that typically accompanies odor preference learning (Lethbridge et al. 2012). NMDARs and L-type calcium channels (LTCCs) have been suggested to play a co-operative and sequential role in the changes

that initiate early odor preference learning (Jerome et al. 2012). LTCC function was shown to be required for 24 h odor preference learning (iso+odor) and activating LTCC function was sufficient to rescue isoproterenol induced learning in the presence of the NMDA receptor antagonist D-APV (Jerome et al. 2012).

1.8.8 GABA

The reciprocal dendrodendritic synapse of mitral cells involves, as described, activation of feedback inhibitory GABA input from the granule cells, and there are also glomerular GABAergic neurons that modulate directly, in a feedforward manner, the response of mitral cells. GABAergic control of mitral cell activation is believed to play a role in olfactory learning in neonatal rat pups. With bulbar infusions, muscimol, a $GABA_A$ receptor agonist, prevented odor-shock learning while bicuculline, a $GABA_A$ receptor antagonist, paired with odor could induce associative odor learning without shock. This suggests that GABAergic inhibition of mitral cells is an important component of olfactory preference learning (Okutani et al. 1999).

1.9 Stages of Memory Duration

The process of learning new information about the world and representing it as a change in behaviour can be characterized as the acquisition of a new memory. Memories can last for minutes, days, months or years. The practice of conferring resistance to disruption (encoding a long-term memory), involves a process of consolidation. Stored memories can later be retrieved, often in the presence of a cue that was previously present when the memory was formed. Our lab has recently shown that odor preference memory in neonatal rat pups can last for short- ,

intermediate- or long-term durations. Short-term memory was characterised as lasting for up to 3 h and was a protein-translation and transcription independent process. Intermediate-term memory was characterised being evident at 5 h and dependent on protein translation, but not transcription (Grimes et al. 2011). Translation dependent intermediate-term memory was also shown in the pond snail (Parvez et al. 2005). Long-term memory was defined as 24 h memory and was both protein transcription and translation dependent (Grimes et al. 2012; Grimes et al. 2011). Short-term memory could occur when protein kinase A (PKA)-dependent long-term memory was blocked. Short-term memory, however, was absent when PKA activation triggered long-term memory (Grimes et al, 2012), suggesting short-term memory depends on an alternate signalling cascade than the canonical CREB pathway (see next section).

Early odor preference learning involving the CS-UCS pairing of odor+stroking or odor+β-AR activation was previously shown to produce a 24 h memory (Christie-Fougere et al. 2009; Cui et al. 2011; Grimes et al. 2012; Grimes et al. 2011; Harley et al. 2006; Langdon et al. 1997; Sullivan et al. 2000b; Sullivan et al. 1989; Yuan et al. 2000), which required activation of the cAMP/PKA signaling cascade leading to an increase in CREB phosphorylation 10 min after training (McLean et al. 1999). Long-term memory has typically been defined as memory lasting 24 h or more. However the rat pup model shows that long-term memories themselves can have varying durations. A single training trial produces behavioural memory that is not seen at 48 h, but is seen at 24 h, while multiple trials can lead to memory lasting many days (Fontaine et al. 2013). What remains unclear are the mechanisms by which we can obtain these varying long-term memory durations. What events differentiate a memory lasting 24 h from a memory lasting beyond 24 h (extended long-term memory)?

1.10 Cyclic-AMP Response Element Binding Protein (CREB)

CREB, CREM and ATF-1 are collectively referred to as the CREB family of transcription factors. CREB family members are components of the superfamily of bZIP transcription factors. Characteristics of bZIP transcription factors include a c-terminal for DNA binding and a leucine zipper for facilitation of dimerization (Mayr and Montminy 2001). Phosphorylation of CREB by PKA occurs at a centrally located kinase-inducible domain consisting of 60 amino acids (Mayr and Montminy 2001). CREB forms a dimer with a cAMP-responsive element (CRE), recruiting CREB-binding protein (CBP) and basal transcription machinery required to carry out gene transcription (Silva et al. 1998). CREB responds to a variety of stimuli involved in a vast array of functions within the nervous system including development and plasticity (Lonze and Ginty 2002).

1.11 cAMP/PKA/CREB Pathway

CREB governs cAMP and Ca^{2+} mediated gene transcription. Activation of CREB is induced by phosphorylation at the regulatory site Serine-133 (Ser-133), facilitated by PKA (Gonzalez and Montminy 1989; Kobrinsky et al. 2011). cAMP is a second messenger involved in a variety of biological functions (Sutherland and Rall 1958). Regulation of this second messenger is controlled by two enzymes, adenylyl cyclases and phosphodiesterases (Sassone-Corsi 2012). Synthesis of cAMP from ATP is catalyzed by adenylyl cyclases (Gancedo 2013). Phosphodiesterases control the amount of available cAMP by initiating the degradation of cAMP to AMP (Gancedo 2013; Sassone-Corsi 2012). A target of cAMP activation is PKA. PKA functions to phosphorylate proteins, one of particular interest in learning and memory being

CREB (pCREB). Phosphorylation of CREB at a specific site, Ser-133, allows CREB to become activated and bind to CREs on the promoter region of target genes. This complex is then able to interact with the transcriptional coactivator CBP, a crucial step in the initiation of gene transcription required for long-term memory formation (Kida 2012).

Initiation of the cAMP/PKA/CREB pathway has been implicated in long-term memory formation as a critical cascade required for the transcription and translation dependent components of consolidation. In olfactory preference learning CREB has been shown to be significantly phosphorylated 10 min after odor+stroking pairing in the mitral cells of the OB (McLean et al. 1999). In 5-HT depleted animals where memory is rescued with a normally super-optimal dose of isoproterenol (6 mg/kg), pCREB is also increased 10 min after conditioning (Yuan et al. 2000). This suggests that CREB phosphorylation 10 min after conditioning is required for 24 h memory, exemplified by the fact that CREB phosphorylation is not increased under non-learning conditions (Yuan et al. 2000). Expression of mutant CREB, via HSV viral vector, abolished early odor preference learning. Memory was rescued when a superoptimal dose of a β-AR was used (4 mg/kg). However, when CREB is overexpressed a suboptimal dose of isoproterenol is sufficient to induce learning (Yuan et al. 2003a). CREB's role in synaptic plasticity stems from its ability to alter protein expression in ways that support functional change and the cAMP/PKA/CREB pathway has long been considered to be an integral pathway in the consolidation and maintenance of long-term memories.

Other mammalian models of long-term memory and/or synaptic plasticity provide evidence for biphasic increases in CREB phosphorylation after conditioning treatments. Immunohistochemistry studies of CREB phosphorylation after hippocampal long-term potentiation (LTP) induction reveal a biphasic pattern of pCREB expression with a short-lasting

peak at 30 min post induction followed by a longer lasting peak at 2 h post induction (Schulz et al. 1999). Contextual-fear conditioning (context+shock), typically producing a memory greater than 24 h, also produces biphasic pCREB expression with an early phase between 0 and 30 min post-conditioning followed by a second phase between 3 and 6 h post-conditioning (Stanciu et al. 2001).

Examining changes in the expression pattern of pCREB after conditioning leading to different memory durations is one focus of this thesis. It is thought that differing expression profiles of CREB phosphorylation post-conditioning may provide insight into potential mechanisms by which memory is extended. It is hypothesized that pCREB expression patterns will differ with memory models of different durations.

1.12 Histone Involvement in Learning and Memory

Chromatin remodelling (chromosomal DNA and protein) through interactions with histones (H2A, H2B, H3 and H4) controls the activity of gene transcription. DNA is wrapped around a core of proteins that comprises histone octamers collectively referred to as nucleosomes. Transcriptional activity is regulated by post-translational modifications of histones. Specifically, transcription is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) and the interplay between them (Hebbes et al. 1988). During acetylation, negatively charged acetyl groups are added onto histone proteins by HATs, changing the conformation of chromatin rendering it more accessible for gene transcription (Brownell and Allis 1996). Acetyl groups are removed from histone proteins by HDACs (Vecsey et al. 2007), coiling the DNA into a compact conformation making it inaccessible for transcription factors to

bind and subsequently suppressing transcriptional activity (de Ruijter et al. 2003). Changes in histone acetylation are driven by changes in neuronal activity

Promoting histone acetylation by preventing deacetylation via HDAC inhibitors has been of interest as a potential mechanism for the promotion of memory extension that would act downstream of CREB phosphorylation. A role for HDAC inhibition in long-term memory formation was first shown in a contextual fear conditioning study where histone acetylation of the histone H3 was significantly increased in the hippocampus of fear conditioned rats (Levenson et al. 2004). Stimulation of the ERK pathway also produced increased acetyl H3 as did administration of trychostatin A (TSA), an HDAC inhibitor (Levenson et al. 2004). CBP is engaged by pCREB to recruit transcriptional machinery for the transcription of new genes. CBP acts as a HAT in the acetylation of histones (Korzus et al. 2004), a critical role for CBP in long-term memory formation (Vecsey et al. 2007). The cAMP/PKA/CREB pathway is tightly coupled as each step is critical for the function of the pathway. It has been shown that CREB phosphorylation is rate limited by PKA translocation (Hagiwara et al. 1993) and as such histone acetylation has been looked at as a potential enhancer for long-term memory formation by modulating CREB mediated transcription. A study by Vecsey and colleagues studied the relationship between the CBP:CREB interaction and the effects of histone acetylation (Vecsey et al. 2007). They showed that the CBP:CREB interaction is required for consolidation of contextual fear memories and furthermore that gene transcription and memory is enhanced when animals are given the HDAC inhibitor TSA (Vecsey et al. 2007). The critical relationship between CREB and histone acetylation provides another avenue by which memory duration may be extended.

1.13 Pharmacological Models of Olfactory Preference Learning

1.13.1 24 h Isoproterenol

The natural early odor preference learning model of odor paired with stroking can be modified by using pharmacological agents to mimic the effects of stroking. Isoproterenol, a β-AR agonist, produces β-AR activation that parallels that produced by stroking, resulting in a reliable 24 h olfactory preference memory (Christie-Fougere et al. 2009; Cui et al. 2011; Grimes et al. 2012; Grimes et al. 2011; Harley et al. 2006; Langdon et al. 1997; Sullivan et al. 2000b; Sullivan et al. 1989; Yuan et al. 2000). Isoproterenol exhibits an inverted u-dose response curve where a low and high dose (1 mg/kg or 4 mg/kg) blocks learning and an optimal dose of 2 mg/kg is sufficient to produce 24 h memory (Sullivan et al. 1989). As previous studies have reliably produced 24 h memory using odor paired with 2 mg/kg isoproterenol, for the purposes of this study, we used this previously established olfactory memory model of odor+2mg/kg isoproterenol as our long-term 24 h memory model.

1.13.2 4-Day Isoproterenol+Cilomilast

Since the early studies of memory in Aplysia, cAMP has been linked to a role in learning and memory. In olfactory preference learning, cAMP appears to be critical in the acquisition and maintenance of memory (Cui et al. 2007). As previously described, stroking of neonatal rat pups evokes an increase in norepinephrine coupled to increased cAMP production. Norepinephrine induced β-AR activation in a single trial is sufficient to produce a 24 h memory, however, this only occurs when activation of β-ARs is at an optimal level since, as described, either suboptimal

or above optimal activation is inefficient at producing learning (Sullivan et al. 1989). Stroking and isoproterenol (UCS) activates β-ARs in the mitral cells of the OB, and norepinephrine induced β-AR activation leads to an increase in the cAMP signal (McLean et al. 2005; Yuan et al. 2003b) in those neurons. Elevated levels of cAMP increase PKA activity leading to increased PKA translocation and CREB phosphorylation, the critical component already described of the intracellular signaling pathways involved in long-term memory formation. To test the causal role of cAMP in inducing odor preference learning, McLean et al (2005) used the phosphodiesterase IV (PDE4) inhibitor cilomilast (Cilo) to enhance the cAMP signal by slowing its inactivation. Under those conditions the previously ineffective, suboptimal, 1 mg dose of isoproterenol was effective, and 24 h memory was seen. Unexpectedly, the use of the PDE4 inhibitor with 1 mg/kg Iso also extended memory to 4 days. This suggested that some parameter of the cAMP signal was enhanced and it became effective at recruiting longer lasting memory. Adding the PDE4 inhibitor to a normally effective learning dose of isoproterenol did not extend memory, but rather prevented any memory expression, consistent with the inverted dose response profile of isoproterenol.

Effective learning is associated with an oscillatory pattern of cAMP that is increased immediately after olfactory preference training and followed by a decrease 5 min later with another increase at 10 min post-conditioning, a time when CREB is maximally phosphorylated (Cui et al. 2007). A similar pulsatile pattern was seen in natural olfactory preference learning (odor+stroking). The oscillatory pattern of cAMP characteristic of learning was observed with 1 mg/kg isoproterenol paired with cilomilast, but not in the absence of cilomilast (McLean et al. 2009). In the present experiments I take advantage of the prior observation that 1 mg/kg

isoproterenol + 3 mg/kg cilomilast paired with odor produces a 4-Day olfactory preference memory and use this as one extended (4 day) long-term memory model.

1.13.3 5-Day Iso+Cilo+NaB

As previously described, β-AR activation coupled with phosphodiesterase inhibition is hypothesized to extend memory by enhancing phosphorylation of CREB. Phosphorylation of CREB promotes transcription of new targeted gene products, a requirement for the establishment of long-term memory. Vecsey and colleagues explored the role of HDACs in memory as a potential mechanism for memory enhancement (Vecsey et al. 2007). Their findings show that in wild-type mice CREB phosphorylation initiates gene transcription, a process that is enhanced in the presence of an HDAC inhibitor when the CREB:CBP interaction is intact. This in turn enhances downstream gene expression. In the absence of CREB, in CREB knock-out mice, CBP is not recruited and HDAC inhibitors have no effect on enhancing transcription due to the lack of recruitment of required transcription machinery. The same is true when mice express mutant CBP (Vecsey et al. 2007). This suggests a critical role not only for CREB:CBP interaction, but also for histone acetylation in CREB mediated gene expression.

Inhibition of HDACs via selective class I (HDAC1, HDAC2, HDAC3, and HDAC8) HDAC inhibitors enhances long-term object location memory, suggesting class I HDACs are involved in learning and memory (Hawk et al. 2011; McQuown et al. 2011). Specifically, when HDAC3 is blocked in knock-out mice, long-term memory for object location is enhanced and extended from 24 h to 7 days. This memory extension is also exhibited when mice are given RGFP136 [N-(6-(2-amino-4-fluorophenylamino)-6-oxohexyl)-4-methylbenzamide], a selective

HDAC3 inhibitor (McQuown et al. 2011). A recent study looking at the effects of sodium butyrate in cocaine-associated contextual memory showed enhancement of memory consolidation and impaired extinction (Itzhak et al. 2013). With recent studies supporting a role for HDAC inhibition as a modulator of CREB mediated gene transcription and possible target for memory extension, we combined isoproterenol (1mg/kg), cilomilast (3 mg/kg) and the HDAC inhibitor sodium butyrate (NaB; 1.2 g/kg) in an attempt to further extend olfactory preference memory. Findings in our lab revealed memory extension lasting 5 days (McLean Lab, unpublished data; Appendix B). For the purposes of this study, iso+cilo+NaB was used as a second (5-Day) extended long-term memory model.

1.14 CREB target genes

The signaling cascade initiated by NE and β-AR activation engages the cAMP/PKA/CREB pathway, catalyzing transcription of new genes and production of proteins, requirements for consolidation of long-term memory (McLean and Harley 2004). Pharmacologically targeting this pathway with a focus on assessment of the phosphorylation of CREB is a major component of the present study. Targeting both cAMP, using phophodiesterase inhibitors (McLean et al. 2005), and PKA, using Sp-cAMPs (Grimes et al. 2012), extend memory beyond the 24 h memory typically seen with the natural odor preference model of odor+stroking. Targeting the events after CREB is phosphorylated has also shown promise for memory extension, in particular targeting histone acetylation using histone deacetylation inhibitors (Vecsey et al. 2007), and thus increasing the window for transcription. These findings suggest the underpinnings of longer memory duration are likely to relate to the phosphorylation of CREB and the subsequent transcription of its downstream genes.

CREB target genes have been a recent focus of our lab in an attempt to gain insight into how memory is supported by increased or novel protein translation. CREB activation has been linked to a number of target genes implicated in long-term learning and memory. Any gene that has a CRE element has the potential to be CREB related. The list of plasticity-related genes with CRE elements is extensive, including but not limited to the following: brain-derived neurotrophic factor (*BDNF*), early growth response element 1 (*Egr-1*), CCAAT enhancer-binding protein (*C/EBP*$_{\beta}$), *c-fos*, and *jun* (Alberini 2009; Vecsey et al. 2007). Other genes of interest include the NR4A family of nuclear receptors (Vecsey et al. 2007). It is these plasticity-related genes modulated by CREB that are of initial interest in the present study as potential downstream regulators of CREB mediated extended long-term memory.

1.14.1 NR4A

The NR4A family of transcription factors are a set of orphan nuclear receptors known to be involved in a multitude of biological processes including learning and memory (Bridi and Abel 2013). This subfamily consists of three members, *Nr4a1*, *Nr4a2*, and *Nr4a3*. NR4A nuclear receptors are immediate-early genes that respond to changes in the external environment, and , are believed to be a product of the CREB mediated transcriptional response involved in the consolidation of long-term memories. The transcriptional activity of NR4A proteins is regulated by post-translation modifications, namely histone acetylation. *Nr4a* transcription is directly controlled by the cAMP/PKA/CREB pathway, as members of the NR4A family are CRE containing genes (Barneda-Zahonero et al. 2012; Bridi and Abel 2013; Hawk and Abel 2011; Hawk et al. 2012; Volakakis et al. 2010). In the absence of *Nr4a* expression in mutant mice, hippocampal LTP is impaired and cannot be rescued by an HDAC inhibitor, suggesting Nr4a

mediates changes in synaptic plasticity and ultimately memory through its modulation by histone acetylation (Bridi and Abel 2013). In Nr4a deficient mice (NR4ADN), downstream genetic targets including *BDNF* and *Fosl2* are also down-regulated (Barneda-Zahonero et al. 2012; Bridi and Abel 2013). *Nr4a1* and *Nr4a2* may play different roles in memory as shown by evidence that *Nr4a1* is involved in long-term object location and object recognition whereas *Nr4a2* is only involved in object location memory (McNulty et al. 2012).

As previously mentioned, the CREB:CBP interaction is critical in the consolidation of long-term memories. When the CREB:CBP interaction is intact and memory consolidation can occur, *Nr4a1* and *Nr4a2* gene expression was increased. When this interaction was impaired, so too was expression of these genes (Vecsey et al. 2007). A study by Hawk and colleagues revealed that all three members of the NR4A family were increased in response to contextual fear training. When NR4A activity was blocked, LTP was impaired but short-term memory was intact. This suggested that NR4A gene expression plays a role in consolidation and long-term memory. It was also found that when memory was enhanced using the HDAC inhibitor TSA, so too was NR4A gene expression. These memory enhancing effects were attenuated when NR4A gene expression was inhibited (Hawk et al. 2012).

In the present study, all three members of the NR4A family were assessed to determine if they are differentially expressed in olfactory preference models of different durations as part of my effort to examine their possible roles in extending memory duration.

1.14.2 CCAAT/Enhancer Binding Protein

CCAAT/Enhancer binding proteins (C/EBPs) belong to the bZIP class of transcription factors whose family members consist of C/EBP$_{\alpha}$-C/EBP$_{\zeta}$. C/EBP subtypes differ in structure and function, playing a role in several cellular responses (Ramji and Foka 2002). A role for C/EBP in long-term synaptic plasticity was first reported in *Aplysia* (ApC/EBP) as a potential immediate-early gene involved in the consolidation of long-term facilitation (LTF). Here it was found that ApC/EBP expression was induced by both serotonin (5-HT), (proposed to parallel the role of NE as a UCS in mammals (Brunelli et al. 1976)) , and cAMP during consolidation. LTF was impaired when ApC/EBP was inhibited. There is a CRE-element in the 5'-region of ApC/EBP, suggesting it may become transcriptionally activated by CREB proteins (Alberini et al. 1994). C/EBPs have since been examined in the mammalian brain where a similar story has emerged for a role in hippocampal LTP. In mammals C/EBP$_{\beta}$ is the most constitutively expressed C/EBP in the mouse hippocampus and expression is increased in response to cAMP and Ca^{2+} signaling (Yukawa et al. 1998). Interestingly, electrophoretic mobility shift assays (EMSA) also revealed that C/EBP$_{\beta}$ is a potential CREB target as evidenced by CREB binding to a small region of the C/EBP$_{\beta}$ gene containing two CRE sites (Yukawa et al. 1998), consistent with C/EBP$_{\beta}$ as a potential downstream CREB regulated immediate-early gene (Alberini 1999; Alberini 2009). In more recent studies, C/EBP$_{\beta}$ has been expressed 9-28 h following learning in the same hippocampal neurons that showed increased CREB expression immediately after learning (Taubenfeld et al. 2001a). There is also elevated C/EBP$_{\beta}$ expression in the gustatory cortex 18 h after novel taste learning, an increase in expression that is attenuated during behavioural interference (Merhav et al. 2006). The inhibitory avoidance study in rats highlights the relationship between CREB and C/EBP. CREB becomes phosphorylated after inhibitory

avoidance training and remains elevated for nearly 24 hours. C/EBP$_\beta$ mRNA levels are increased in both control and trained animals, however, inhibitory avoidance training increased C/EBP$_\beta$ mRNA and protein levels above controls at 9 h and 20 h post-conditioning. This delay in C/EBP expression after CREB phosphorylation suggests C/EBPs are induced in response to activation of the cAMP/PKA/CREB pathway. Inhibiting CREB phosphorylation by fornix lesions also inhibited the previously seen increases in C/EBP$_\beta$ expression. Support for CREB regulation of C/EBP was also shown by anatomical co-localization of CREB and C/EBP in hippocampal slices (Taubenfeld et al. 2001b).

C/EBPs have also been linked to long-term memory gene expression and chromatin remodeling. A study in *Aplysia* by Guan and colleagues suggests C/EBP activation as a mechanism responsible for the differentiation between short-term and long-term memories. They propose that 5-HT induces expression of C/EBP through CREB phosphorylation and CREB:CBP interaction and subsequent binding of CREB1a to C/EBP. This was shown by increased C/EBP 15 min to 2 h after 5-HT activation of CREB by PKA phosphorylation. They then suggested that C/EBP induction depended on histone acetylation, as evidenced by 5-HT inducing H3 and H4 acetylation at the C/EBP promoter region (Guan et al. 2002).

Evidence for C/EBP as a downstream CREB IEG and as possibly mediating effects of histone acetylation suggests a potential role of C/EBP in memory duration. C/EBP$_\beta$ was thus also examined as a possible modulator of memory extension in our olfactory preference learning models of different durations.

1.14.3 Jun and Fos

AP-1 is an early-response transcription factor comprised of dimers from the Jun, Fos, and ATF families. Playing a role in gene transcription regulation, members of the AP-1 family have been implicated in the same long-term changes in synaptic plasticity as CREB target genes (Alberini 2009). Proteins from the Fos and Jun families are bZIP transcription factors (Glover and Harrison 1995) that bind to several DNA recognition sites including CRE binding sites (Kerppola and Curran 1991), and form heterodimers with CREB (Hai and Curran 1991).

Several learning paradigms provide evidence for Fos and Jun involvement in long-term changes in synaptic plasticity and memory. In an operant conditioning task requiring lever pressing for food in response to a light cue, q-PCR revealed increases in both *c-fos* and *c-jun* mRNA 15 minutes after conditioning (Rapanelli et al. 2009). In an olfactory preference learning task a significant increase in *fos* immunoreactivity was seen in periglomerular cells of the midlateral OB 45 min after conditioning, suggesting a role in olfactory learning (Johnson et al. 1995). Countryman and colleagues provide supporting evidence for CREB mediated *c-fos* transcription in a socially transmitted food preference task (Countryman et al. 2005). Both pCREB and *c-fos* immunoreactivity were increased in the dorsal hippocampus after training (Countryman et al. 2005). Evidence supports a potential role for *c-Fos* and *c-Jun* involvement in learning and memory. In the present study mRNA levels of both genes were assessed in two olfactory preference learning models of different durations (24 h and 5-Day) in an attempt to determine if their role in long-term memory is related to memory extension.

1.14.4 Brain-derived neurotrophic factor (BDNF)

BDNF is a CRE containing CREB target gene and member of the neurotrophin family (Kesslak et al. 1998). BDNF plays a role in cell survival and in the changes in synaptic plasticity that accompany learning and memory. BDNF is stored in glutamatergic synapses and released in response to LTP induced stimulation (Hartmann et al. 2001).

There are several lines of evidence supporting a role for BDNF in learning and memory. When BDNF mRNA expression is inhibited in the hippocampus of adult mice, significant impairments in novel object recognition and spatial learning are observed. Surprisingly, no behavioural deficits are observed on fear-conditioning tasks, however, reduced extinction is reported (Heldt et al. 2007). BDNF is activated in response to CREB phosphorylation and expression is impaired when CREB dependent learning is disrupted (Finkbeiner et al. 1997). A recent study by Hawk and colleagues provide evidence for BDNF gene expression as a downstream target in memory consolidation (Hawk et al. 2012). When memory is enhanced using HDAC inhibitors, and NR4A expression is increased, downstream targets *Bdnf* and *Fosl2* are also increased. When NR4A expression is knocked-down, so too is expression of *Bdnf* and *Fosl2* (Hawk et al. 2012). Mammalian learning and memory studies suggest BDNF plays a role in long-term memory. Here we explore its role as a potential marker for memory extension using our memory models of differing durations.

1.14.5 Egr-1/zif268

Egr-1 (also known as *zif268*) is an immediate early gene implicated in the maintenance of long-term changes in synaptic plasticity. Egr-1 contains two CRE sites within its promoter region (Wei et al. 2012), which CREB binding can target to activate Egr-1 gene expression (Zhang et al. 2005). Previous studies have shown that Egr-1 is activated in response to behavioural learning and memory training, and is related to the maintenance of late phase hippocampal LTP (Bozon et al. 2002; Jones et al. 2001). In a study using mutant *zif268* mice both wild-type and mutants performed the same on short-term learning and memory tasks and also exhibited multi-hour LTP, indicating *zif268* is not required for the induction of LTP or for behavioural performances on certain memory tasks. There was, however, a deficit in the maintenance of LTP which was maintained in wild-type mice for 48 h but was at baseline levels after 24 h in mutant mice. Similar results were found on the behaviour performance tasks (Jones et al. 2001). The evidence for Egr-1 as a factor in the maintenance of long-term memories rather than the acquisition of memory, suggests it is a potential candidate for memory extension in olfactory preference learning. As a CREB target gene we examined its role in memory extension through analysis of mRNA levels under behavioural paradigms of both long-term (24 h) and extended long-term (5-Day) memory.

1.14.6 Objectives

The purpose of this thesis is to investigate potential mechanisms that could contribute to memory extension, thus gaining insight into its molecular correlates. The experiments in this study attempt to assess several lines of evidence previously linked to learning and memory with the intention of investigating specific roles in the maintenance and extension of memory duration

in early olfactory preference learning. First, it is hypothesized that phosphorylated CREB, having a well-known and critical role in the consolidation of long-term memories, may temporally vary in its expression for long-term memories of shorter and longer duration, and, thus, vary the pattern of activation for downstream targets that support memory extension. Second, a role for histone acetylation in long-term memory duration was also assessed as several lines of evidence suggest chromatin remodelling may extend the window for transcription, another possible mechanism for memory extension. Lastly, we looked at level of recruitment of the downstream targets of CREB specifically as potential mediators of memory extension between the long-term (24 h) and extended long-term (5-Day) memory models.

We first sought to determine whether the expression profile of CREB phosphorylation differed across our three memory models of differing durations (Appendix C). It is hypothesized that if CREB phosphorylation is a requirement for the wave of transcriptional responses that initiates the generation of new gene products and ultimately long-term memory formation, then the expression profiles of pCREB will vary at different time intervals post-conditioning for different memory durations.

We next sought to determine if histone acetylation contributes to changes in memory duration. Having shown that the HDAC inhibitor sodium butyrate, in combination with β-AR activation and phosphodiesterase inhibition, extends memory beyond 24 h, then the expression profile of histone acetylation across a time course post-conditioning is predicted to vary across our three memory models.

Finally, we pursued downstream targets of both CREB and histone acetylation by looking at the expression of CREB-related genes post-conditioning. It was hypothesized that, even

though CREB phosphorylation was found to be similarly bi-phasically increased in both our long-term (24 h) and extended long-term (5-Day) memory models, the downstream recruitment patterns of gene expression might differ.

CHAPTER 2 – METHODS

2.1 Animals

Sprague Dawley (Charles River, Saint-Constant, Quebec, Canada) rat pups of both sexes were used in this study. The day of birth was considered PD 0. Litters were culled to 12 rat pups on PD 1. All animals used in this study were PD 6. No more than one animal of each sex, per litter, was assigned to each training condition. Animals were housed in temperature-controlled rooms (20-25°C) on reverse 12 h light/dark cycles with *ad libitum* access to food and water. All experimental procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland following the guidelines set by the Canadian Council on Animal Care.

2.2 Drugs

Subcutaneous injections (s.c.) of vehicle or drug (50μl) were administered to PD6 rat pups before exposure to the peppermint odor. Combinations of three pharmacological agents, when paired with peppermint odor, were used to obtain experimental learning groups with long-term memories of different durations (24 h, 4-Day and 5-Day). The β-adrenoceptor agonist, isoproterenol, (Sigma-Aldrich Co., St. Louis, MO, USA), was given 40 min before odor exposure. Concentrations of 1mg/kg or 2mg/kg isoproterenol were prepared on day of use in saline. The PDE4 inhibitor, cilomilast (Selleckchem, Houston, TX, USA), was given 30 min before odor exposure. A concentration of 3 mg/kg cilomilast was prepared in advance with aliquots stored at -80°C (Appendix D), thawed on day of use and the concentration adjusted to the mean weight of pups with 5% dimethyl sulfoxide (DMSO, EMD Chemicals Inc., Germany).

The histone deacetylase inhibitor, NaB (Sigma-Aldrich Co., St. Louis, MO, USA), was given 30 min before odor exposure. A concentration of 1.2 g/kg NaB was prepared fresh on day of use in saline. Control animals were given saline in lieu of isoproterenol or NaB and 5% DMSO in lieu of cilomilast before odor exposure.

2.3 Olfactory Preference Training

A single 10 min training session was performed on PD 6 rat pups in temperature controlled (28°C) behaviour rooms. Rat pups were given three separate s.c. injections prior to training as previously described. Pups were returned to the dam between injections until 10 min before odor exposure when pups were removed from the dam and placed in isolation (Appendix E). Training consisted of placing the pup on peppermint scented bedding for 10 min. Peppermint bedding was prepared by adding 0.3 ml of peppermint extract (G. E. Barbour Inc.) to 500 ml of regular unscented bedding (PWI Brand Pro-chip, Hardwood Sawdust) and covered for 10 min. Scented bedding was then left uncovered in a fume hood for 10 min allowing any ethanol to evaporate. Pups were returned to the dam immediately after training until sacrificed.

2.4 Experiment 1

2.4.1 Experimental Groups for Western Blotting

Rat pups were randomly divided into five groups: saline control group, 2 mg/kg Iso (24 h memory), 1 mg/kg Iso + 3mg/kg Cilo (4-Day memory), 1mg/kg Iso + 3 mg/kg Cilo + 1.2 g/kg NaB (5-Day memory), and 1.2 g/kg NaB non-learning group. Rat pups were trained using olfactory preference learning as previously described (*2.3*).

2.4.2 Tissue Collection

Tissue was collected at 6 different time points (0 min, 10 min, 30 min, 1 h, 2 h, and 4 h) after odor conditioning. Animals were decapitated and olfactory bulbs were quickly removed and flash frozen on dry ice. Tissue was stored in 1.5 ml centrifuge tubes at -80°C.

2.4.3 Sample Preparation

Lysis buffer [100 µl, 50mM Tris-HCL, 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN,USA), phosSTOP cocktail (Roche Diagnostics, Indianapolis, IN,USA), 1mM EDTA, 1mM PMSF; Appendix F] was added to a pair of olfactory bulbs from an individual animal in lysis tubes (Micro tube 0.5ml, SARSTEDT AG & Co., Germany) containing beads (1.4 mm Zirconium oxide beads, Precellys 24) and homogenized (Precellys 24) for 20 sec at 5500 rpm. Samples were placed immediately on ice for 15 min then centrifuged for 20 min at 13300 rpm at 4°C. Supernatant was transferred to 1.5 ml centrifuge tubes for protein determination.

2.4.4 Protein Determination

Duplicates of bovine serum albumin (BSA, 2.0 mg/ml, Pierce BCA Protein Assay Kit, Thermo Scientific) and water were used as standards for protein determination (1/10). Samples were loaded into a 96-well plate (25µl/well) and 200 µl bicinchoninic acid (BCA, 1 ml BCA Protein Assay Reagent B: 10 ml BCA Protein Assay Reagent A, Pierce BCA Protein Assay Kit, Thermo Scientific) was added to each well. Plates were incubated at 37°C for 30 min. Next, plates were read using Microplate Reader (Bio-Rad Model 3550) at 540nm. Readings were taken relative to a standard curve [standards of BSA and water prepared in serial dilution (0 µg, 5 µg, 10 µg, 15 µg, 20 µg, 25 µg, 30 µg, and 40 µg)] and analysed to determine the volume required to load 50 µg of protein per sample.

2.4.5 Gel Electrophoresis

Samples were prepared using a calculated volume of protein lysate to obtain a concentration of 50 µg/20 µl. Lysate, 5X sample buffer (5 ml glycerol, 1 g sodium dodecyl sulfate, 3 ml 1.0 M Tris pH 6.8) + 0.5 M dithiothreitol (DTT; 4 µl), and water (total volume 20 µl) were mixed and boiled for 5 min. Samples were loaded into a 10-well 10% or 15% acrylamide gel and 4-6 µl of protein ladder (PageRuler Plus, Thermo Scientific) was used (Appendix F). The positive control for anti-phosphorylated CREB was forskolin treated NIH-3T3 cells and for anti-acetylated H3 was TSA treated NIH-3T3 cells. Gels ran at 110V (60mA) for 1.5 h until the sample buffer dye-band reached the bottom of the gels using GIBCO BRE Electrophoresis power supply.

2.4.6 Protein Transfer

Gels were separated from the glass plates and placed onto blotting paper. Polyvinylidene difluoride (PVDF) membrane (Immobilon-P Transfer Membrane, Millipore) was immersed in 100% methanol for 15 sec followed by water for 2 min and transfer buffer (700 ml dH_2O + 200 ml methanol + 100 ml 10X transfer buffer, Appendix F) for 5 min. PVDF membrane was placed on top of gels followed by a second piece of blotting paper (Chromatography Paper 3mm, Whatman). Blotting paper, gel and membrane were sandwiched by sponges (pre-soaked in transfer buffer) and placed in cassette grids. The sandwich was placed in a Bio-Rad transfer apparatus containing transfer buffer and transferred at 100 V for 60 min at $4^{o}C$ using the Bio-Rad power supply.

2.4.7 Antibody Application

PDVF membranes were removed from the cassettes and washed in TBST (3x5min),blocked in 5% milk + TBST (Appendix F) for 1 h, and immersed in primary antibody (in 5% milk + TBST) with gentle shaking overnight at $4^{o}C$. The following morning blots were washed in TBST (3x10min) and incubated in secondary antibody (goat anti-rabbit conjugated to horseradish peroxidase in 5% milk + TBST, Thermo Scientific) for 1.5 h at room temperature.

2.4.8 Antibodies

For experiments looking at CREB phosphorylation (rabbit polyclonal anti-phosphorylated CREB, 1/2000, Millipore, Billerica, MA, USA), β-Actin (purified rabbit anti-β-actin, 1/5000, Cedarlane, Burlington, ON, CANADA) was used as a loading control. Total

CREB (rabbit polyclonal anti-CREB, 1/3000, Millipore, Billerica, MA, USA) was collected but was not used as a loading control due to variability in CREB expression not explained by loading. Experiments looking at histone acetylation (rabbit monoclonal anti-acetyl H3, 1/000, Cell Signaling, Danvers, MA, USA) used total H3 (rabbit monoclonal anti-H3, 1/1000, Cell signaling, Danvers, MA, USA) as a loading control.

2.4.9 Enhanced Chemiluminescent Exposure

Membranes were washed (3x10 min TBST) and immersed in Enhanced Chemiluminescent (ECL, Thermo Scientific, Rockford, IL, USA) substrate for 5 min (1 ml SuperSignal West Pico Stable Peroxide Solution: 1 ml SuperSignal West Pico Luminol Enhance Solution) at room temperature. Blots were placed in a sheet of plastic page protector and excess ECL was gently removed by wiping the plastic protector with a kimwipe. Blots were placed in a film box (18x24 cm Blue 400 HC Systems, CURIX Screens AGFA) and exposed to film (18x24 cm, Kodak ClinicSelect Green) in a dark room using mini medical 90 developer (AFP Imaging Corp., Elmsford, NY, USA).

Blots were stripped as necessary (maximum of two times) and new antibody was applied. Stripping consisted of placing the blots in 8 ml of stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific, Rockford, IL, USA) and then placing them in a 37°C (anti-acetyl H3, anti-total H3) or 42°C (anti-CREB, anti-pCREB, anti-β-actin) water bath. Blots were rinsed 3x10 min in TBST and probed for the next primary antibody application or stored at 4°C (wrapped in saran wrap and stored in 4°C fridge).

2.4.10 Statistical analysis

Densitometry values for western blotting data were obtained using ImageJ (width: 188, height: 432, 25% zoom, http://imagej.nih.gov/ij/) where all samples were put into ratios of pCREB or acetyl H3 over β-actin and normalized to saline controls and represented as area means ± SEM. A one-way repeated-measures ANOVA was performed with Dunnett post-hoc tests. For all statistical tests $p<0.05$ was considered significant (InStat, http://www.graphpad.com/scientific-software/instat/). The western blotting experiments described in this study follow a randomized block experimental design. Randomized block designs use the same statistical analyses as a one-way repeated measures ANOVA therefore using InStat this was the function chosen (InStat, http://www.graphpad.com/scientific-software/instat/; Howell, 2002, p. 516).

2.5 Experiment 2

2.5.1 Real-time Polymerase Chain Reaction (q-PCR)

2.5.1.1 Tissue Collection

Rat pups were randomly divided into four groups: saline control group, 2 mg/kg Iso (24 h memory), 1 mg/kg Iso + 3 mg/kg Cilo + 1.2 g/kg NaB (5-Day memory), and 1.2 g/kg non-learning NaB group. Animals were trained using the olfactory preference training protocol previously described (*2.1.1*). Animals were sacrificed 10 min or 2 h post-conditioning in an RNA-free environment (RNase Away, Molecular BioProducts Inc, San Diego, CA, USA) and

both OBs were removed. Sacrifice times were chosen to correspond with significant increases in CREB phosphorylation as seen with Western blotting data. Olfactory bulbs were removed and flash frozen on dry ice then stored at -80°C.

2.5.1.2 RNA extraction

RNA extraction was performed using the RNeasy Micro Kit and followed the protocol outlined in the *RNeasy Micro Handbook* (Quiagen, Venlo, Limburg, Netherlands). Samples were transferred from -80°C (each sample contained one pair of OBs from one animal) to lysis tubes (Micro tube 0.5ml, SARSTEDT AG & Co., Germany) containing beads (1.4 mm zirconium oxide beads, Precellys 24. France). Buffer RLT + β-mercaptoethanol (β-ME, 350 µl) were added to each tube. Tissue was homogenized (TissueLyser, Precellys 24) for 20 sec at 5500 rpm. Samples sat at room temperature for 1 h. Lysate was transferred to a new 1.5 ml centrifuge tube and centrifuged at full speed (17000 x g) for 3 min (room temperature). Supernatant was collected and transferred to a new 1.5 ml centrifuge tube where 350 µl of 70% ethanol was added, mixed by pipetting then transferred immediately to RNeasy MinElute spin columns placed in 2 ml collection tubes and centrifuged for 15 s at 8000 x g (room temperature). Flow-through was discarded between steps. Buffer RW1 (350 µl) was added to the RNeasy MinElute spin column and centrifuged for 15 s at 8000 x g (room temperature) to wash the spin column membrane. DNase I stock solution (10 µl) was added to Buffer RDD (70 µl) and added directly to the spin column membrane. Samples were placed on the bench at room temperature for 15 min. To wash spin column membranes, 350 µl Buffer RW1 was added and centrifuged for 15 s at 8000 x g (room temperature). Buffer RPE + ethanol (500 µl) was added to the spin columns and centrifuged 15 s at 8000 x g (room temperature) followed by 500 µl 80% ethanol and then

centrifuged 2 min at 8000 x g (room temperature). RNeasy MinElute spin columns were transferred to a new 2 ml collection tube and centrifuged at full speed (17000 x g) for 5 min (room temperature) with open lids. Spin columns were transferred to new 1.5 ml collection tubes and centrifuged for 1 min at full speed (17000 x g, room temperature) to elute the RNA. Quality and quantity of RNA was assessed using NanoDrop (ThermoScientific, ON, Canada). RNA was stored at -80°C.

2.5.1.3 cDNA Synthesis

RNA samples were removed from -80°C and thawed on ice. A mixture of 5X VILO Reaction Mix (4 µl, Invitrogen) and 10X SuperScript Enzyme Mix (2 µl, Invitrogen) was added to each sample followed by 0.1 % diethylpyrocarbonate (DEPC)-treated water and 2.0 µg of RNA for a total volume of 20 µl. Samples were placed in Thermal Cycler (Bio-Rad) at 25°C for 10 min, 42°C for 90 min 85°C for 5 min and held at 4°C. cDNA was stored at -20°C.

2.5.1.4 Real-Time PCR amplification (q-PCR)

q-PCR was performed using 0.1 ml MicroAmp Fast Optical 96-well plates (Applied Biosystems, Burlington, ON, Canada). Samples were prepared in triplicate according to the TaqMan Fast Advanced Master Mix protocol (Applied Biosystems, Burlington, ON, Canada). PCR reaction mix consisted of TaqMan Fast Advanced Master Mix (10 µl), TaqMan Gene Expression Assay (1.0 µl) or Endogenous control gene assay (18S, 1.0 µl, external control), 2.0 µg cDNA sample (2.0 µl) and nuclease-free water (to a total volume of 20 µl per reaction). Non-template controls were also prepared for each gene (TaqMan Gene Expression Assay and

nuclease-free water). A total volume of 3 reactions was calculated and combined into a single 0.5 ml centrifuge tube allowing enough volume for triplicates (3 x 20 µl) with extra volume to account for pipetting errors. Reaction mix was vortexed and spun down and 20 µl was transferred into three separate wells. Plates were covered with Optical Adhesive Covers (Applied Biosystems) and spun down to eliminate any air bubbles in the bottom of the wells. Plates were then loaded into a ViiA 7 Real-Time PCR System (Invitrogen). Experimental set-up was set to the comparative CT (ΔΔCT) method using TaqMan Fast reagents to detect target sequence. Plate parameters for thermal-cycling profiles consisted of two hold stages (2 min at 50°C and 20 sec at 95°C) followed by a 40 cycle PCR stage consisting of a 1 sec denature stage at 95°C and a 20 sec annealing/extending stage at 60°C. All plates contained non-template controls for each gene of interest.

2.5.1.5 Statistical Analysis

Real-time PCR data were assessed using the comparative CT method (ΔCT) and represented as relative fold change + SEM (Appendix G). Threshold values for each gene were averaged across plates and set to a common threshold. Gene expression was analyzed using 2-tailed unpaired t-tests. For all statistical tests $p<0.05$ was considered significant.

CHAPTER 3 – RESULTS

3.1 CREB phosphorylation plays a role in long-term memory and memory extension

In order to identify the role of CREB in long-term memory, and how expression of phosphorylated CREB may change when memory is extended, we designed behavioural experiments using three memory models of different durations. To test the hypothesis that the expression pattern of CREB phosphorylation changes as memory is sustained (a possible mechanisms for memory extension), a time course western blotting analysis was performed. Densitometry values were recorded for the expression of phosphorylation of this transcription factor, and normalized to saline+odor controls relative to β-actin levels. CREB has long been considered a critical candidate in long-term memory and as such is a viable potential candidate as a mediating mechanism aiding in memory extension.

3.1.1 24 h long-term early odor preference training induces biphasic increases in CREB phosphorylation

Using an optimal US learning dose of 2 mg/kg isoproterenol for 24 h memory paired with peppermint scented odor, we found, through western blotting, significant changes in the levels of pCREB in the OB after training (Repeated measures ANOVA, $F_{(6, 48)} = 2.55$, $p < .05$) compared to saline+odor controls (Fig. 1A). We show, for the first time in olfactory preference learning, a biphasic pattern of pCREB. Post-hoc Dunnett multiple comparisons tests revealed significant

increases in the levels of pCREB at both 10 min ($p < .01$) and 4 h ($p < .05$) post conditioning, with a near significant ($q = 2.64$) increase in pCREB at 2 h post conditioning (The Dunnett multiple comparisons test yielding a q value greater than 2.66 is normally considered significant). Biphasic pCREB has been observed in fear conditioning models, but has not been reported with 24 h olfactory preference training (Yuan et al. 2000). We had assumed the biphasic pCREB in fear conditioning was related to the long lasting and robust aversion memory produced by shock as a US. However a similar biphasic pattern was seen here with a 24 h appetitive memory suggesting the biphasic profile may be unrelated to memory duration per se.

3.1.2 CREB phosphorylation in the 4-Day olfactory memory training paradigm is unchanged by training

Animals trained using an olfactory learning paradigm (1 mg/kg Iso + 3 mg/kg Cilo US) previously shown to produce an extended long-term memory lasting up to 4-Days (McLean et al. 2005) yielded no significant changes in pCREB levels compared to saline controls (Fig. 1B) across all 6 time points (Repeated measures ANOVA $p = .298$). These findings were unexpected as CREB is a known immediate early gene that has been characteristically activated in a range of memory models including early olfactory preference learning

3.1.3 CREB phosphorylation in the 5-Day olfactory memory paradigm displays a biphasic pattern of post training increases

In an attempt to gain further insight into the role of pCREB in extended long-term memory we looked at pCREB in a 5-Day memory model (1 mg/kg Iso + 3 mg/kg Cilo + 1.2 g/kg

NaB US, Fig. 1C). Western blotting time course revealed significant changes in pCREB (Repeated measures ANOVA, $p < .0001$, $F_{(6/96)} = 5.47$), with significant increases in the level of pCREB at 10 min ($p < .01$), 30 min ($p < .05$) and 2 h ($p < .01$) post-conditioning (Dunnett multiple comparisons test). These findings parallel the pattern of phasic increases in phosphorylated CREB seen after 24 hr long-term memory training (Fig. 1A).

Changes in the pattern of CREB phosphorylation revealed similar patterns of phasic increases in both 24 h and 5-Day extended long-term memory models. This suggests that pCREB is a potential mediator in both 24 h and 5-Day memory, however, we have yet to distinguish its role in differentiating these two memory durations.

3.2 Histone acetylation may contribute to memory extension

Histone acetylation occurs downstream of CREB phosphorylation and has been linked to long-term memory as it plays a key role in extending the duration of the active transcriptional window through conformational changes in chromatin (Vecsey et al. 2007). To investigate whether histone acetylation plays a role in memory extension beyond 24 h in olfactory preference learning we designed a similar set of experiments where we looked at histone acetylation, specifically acetyl H3, in the same three memory models with the same time points (0 min, 10 min, 30 min, 1 h, 2 h, and 4 h) as discussed previously, with the addition of a non-learning control group.

3.2.1 24 h memory training is not associated with changes in histone acetylation

In an attempt to gain further insight into the potential mechanism governing memory extension and having shown a biphasic increase in pCREB 10 min and 2 h post 24 hr olfactory memory training, I examined histone acetylation using the same 24 h animals as used for pCREB analysis. No significant changes in histone acetylation were found (Fig. 2A), as evidence by no significant changes in histone acetylation in a western blotting time course analysis ($p= .135$, $F_{(6,18)}= 1.907$) for the same animals as used previously for pCREB.

3.2.2 4-Day memory training is not associated with changes in histone acetylation

Our previous findings indicate no changes in pCREB expression as a result of training leading to 4-Day memory. This is consistent with the present analysis whereby no changes were observed in the levels of histone acetylation in neonates trained using the same paradigm ($p= .2970$, $F_{(6,18)}= 1.324$) compared to saline controls (Fig. 2B). What remains unclear is why no such changes have been found when it would be expected that a 4-Day memory is a transcription and translation dependent process.

3.2.3 5-Day memory reveals robust increases in histone acetylation immediately after learning

Animals trained using our 5-Day model revealed a robust increase in histone acetylation (Repeated measures ANOVA $p< .0001$, $F_{(6,24)}= 10.269$). Post-hoc Dunnett multiple comparisons tests showed significant increases in acetyl H3 at both 0 min ($p< .01$) and 10 min ($p< .01$) post-conditioning compared to saline+odor controls (Fig. 2C). The data show a robust increase in

acetyl H3 immediately after training with a gradual decrease to that seen with non-learning (saline) levels by 4 h post-conditioning.

3.2.4 Sodium butyrate produces rapid increase in histone acetylation

Our 5-Day memory model involves giving the animals a 1.2 g/kg dose of NaB, a histone deacetylase inhibitor. The actions of this drug prevent the deacetylation of histones resulting in an increase in histone acetylation. In order to determine if the robust increase in acetyl H3 immediately after training is a result of odor preference or due to drug effects we next looked at non-learning animals given solely 1.2 g/kg NaB. A similar pattern of increased acetyl H3 was evident (Repeated measures ANOVA, $p < .0001$, $F_{(6,18)} = 13.806$), with a significant increase starting at 0 min ($p < .01$) and remaining elevated 10 min ($p < .01$) and 30 min ($p < .01$) after training and returning to baseline by 4 h (Fig. 2D). Taken together these data suggest acetyl H3 increases may be contributing to extended memory duration since NaB both extends memory and increases acetyl H3. Our unpublished data suggest no preference memory is generated by pairing odor and NaB alone (McLean et al, 2011), thus in the absence of an associative memory there is no effect of enhancing chromatin remodelling.

3.3 Downstream CREB target gene expression differences provide insight into potential mechanisms of memory extension

Western blotting data revealed that both CREB phosphorylation and histone acetylation are recruited in a 5 day olfactory preference training model, while only CREB phosphorylation is recruited in a 24 h model. However, we have yet to identify a downstream mechanism that

differentiates between these two models. With the novel finding of biphasic pCREB in both the 24 h model and the 5-Day model we next designed a set of real-time PCR experiments that looked at downstream CREB target genes at two times that parallel the previously reported rises in pCREB, 10 min and 2 h post-conditioning.

3.3.1 NR4a family of nuclear receptors

The Nr4a family of orphan nuclear receptors has been linked to learning and memory through increased histone acetylation and long-term memory (Hawk et al. 2012; Vecsey et al. 2007). We investigated the role of all three family members of the NR4A orphan nuclear receptors, as downstream CREB target genes, as possible sources of differences between long-term memory (24 h) and extended long-term memory (5-Day). We examined gene expression using q-PCR in saline non-learning control group, a 24 h memory group (2 mg/kg Iso), a 5-Day memory group (1 mg/kg Iso+3 mg/kg Cilo+1.2 g/kg NaB- ICN) and a NaB only (1.2 g/kg dose) control group (Fig. 3-10).

At 10 min following training, there were no significant differences in relative fold change in the Nr4a1 gene in non-learning NaB (0.86 $\pm$ .13) and 5-Day ICN (1.19 $\pm$ 0.21) groups compared to saline+odor controls (1 $\pm$ 0.09; Fig. 9A). Animals in the Isoproterenol group yielded a near significant difference in fold change (1.35 $\pm$ .12) relative to controls ($t_{(6)} = 2.47$, $p = .05$; Fig. 3A). Interestingly, at 2 h post-conditioning *Nr4a1* gene expression did not significantly differ between Isoproterenol (1.26 $\pm$ 0.27) and saline+odor controls (1 $\pm$ 0.07; Fig. 3B). Given the sample size is relatively small, increased *Nr4a1* expression in the 24 h learning group may be a potential target gene involved in 24 h long-term memory that differentiates itself from extended

long-term memory. This idea is further supported by the finding that Nr4a1 fold change is significantly increased in ICN animals 2 h post-conditioning (2.37 ± 0.28) compared to controls (1 ± .10, $t_{(6)}$= 4.56, p= .00; Fig. 3B). This increase in gene expression was not due to NaB only effects as non-learning NaB only animals (1.73 ± 0.39) did not show significant changes in relative gene expression.

We next looked at the second subtype of the Nr4a family of nuclear receptors, *Nr4a2*. An increase in relative fold change was found in the 24 h Isoproterenol (2.01 ± 0.27) group compared to saline+odor controls (1 ± .07; $t_{(6)}$= 3.67, p= .01; Fig. 4A) 10 min after learning. Increase in fold change was also found in 5-Day ICN (1.39 ± 0.14) animals relative to saline+odor controls (1 ± 0.07; $t_{(6)}$= 2.57, p= 0.04; Fig. 4A). Non-learning NaB only animals (0.77 ± 0.11) did not show this increase 10 min post-conditioning. *Nr4a2* expression did not differ in 24 hr Isoproterenol (0.97 ± 0.07), NaB non-learning (0.94 ± 0.10) or 5-Day ICN (0.86 ± 0.07) groups 2 h after learning compared to controls (1 ± 0.11; 4B). This suggests that *Nr4a2* may be a product of the initial phase of pCREB expression and is potentially involved in acquiring or consolidating the memory.

The third member of the Nr4a orphan nuclear receptors is *Nr4a3*. q-PCR data shows no significant differences in relative fold change 10 min post-conditioning in 24 h Isoproterenol (1.26 ± 0.06), NaB non-learning (0.79 ± 0.06) or 5-Day ICN (1.17 ± 0.24) animals compared to controls (1 ± 0.12; Fig. 5A). Also, no significant changes were found at 2 h post-conditioning in 24 h Isoproterenol (0.97 ± 0.07), NaB non-learning (0.94 ± 0.10) and 5-Day ICN (0.86 ± 0.07) animals compared to saline non-learning controls (Fig. 5B).

3.3.2 CCAAT-enhancer binding protein (C/EBPβ)

A significant increase in fold change was found for the gene *C/EBP*$_{\beta}$ (Fig. 6A). At 10 min post-conditioning both 24 h Isoproterenol ($p < .0001$) and the 5-Day ICN ($p = .01$) learning groups showed significant increases in relative fold change expression compared to saline+odor controls. No such changes were found in the NaB non-learning groups ($p = .4049$) at 10 min post-conditioning. No significant changes in relative fold change were found at 2 h post-conditioning in Isoproterenol ($p = .53$), ICN ($p = .31$) or NaB non-learning ($p = .87$) groups (Fig. 6B). This suggests a possible role for *C/EBP*$_{\beta}$ as a product of the first wave of transcription responses initiated by pCREB.

3.3.3 Jun

There were no significant differences in relative fold change of the *Jun* gene in 24 h learning animals at 10 min (0.84 $\pm$ 0.20; Fig. 7A) or 2 h (0.88 $\pm$ 0.21; Fig. 7B) post-conditioning compared to saline+odor controls (10 min 1 $\pm$ 0.12; 2 h 1 $\pm$ 0.16). Also, no significant changes were found in 5-Day ICN animals at 10 min (1.38 $\pm$ 0.27) or 2 h (0.95 $\pm$ 0.19) compared to controls. Nor were there any difference in the NaB only groups at 10 min (1.02 $\pm$ 0.17) or 2 h (0.87 $\pm$ 0.17).

3.3.4 Fos

No significant changes in *Fos* expression were found in 24 h Isoproterenol at 10 min (1.48 $\pm$ 0.32; Fig. 8A) or 2 h (1.33 $\pm$ 0.06; Fig. 8B) post-conditioning compared to controls (10 min, 1 $\pm$ 0.11; 2 h, 1 $\pm$ 0.17). Also, no changes were found at 10 min in both 5-Day ICN (1.00 $\pm$

0.15) and NaB (0.86 ± 0.12) or at 2 h in both 5-Day ICN (1.72 ± 0.36) and NaB (1.56 ± 0.28) groups compared to controls.

3.3.5 Egr1

No differences in relative fold change expression were found at 10 min post-conditioning in Isoproterenol (0.92 ± 0.13), ICN (0.97 ± 0.15) or NaB (1.03 ± 0.20) groups compared to saline controls (1 ± 0.18; Fig. 9A). At 2 h post-conditioning there were also no changes in *Egr1* expression in both Isoproterenol (1.09 ± 0.13) and NaB (1.40 ± 0.41) compared to controls (1 ± 0.21; Fig. 9B). ICN animals did show an increase in fold changes at 2 h post-conditioning (1.89 ± 0.23) compared to controls ($t_{(6)}$= 2.85, p= 0.03; Fig. 9B).

3.3.6 BDNF

q-PCR results yielded no significant changes in *BDNF* expression levels at 10 min post-conditioning in Isoproterenol (0.92 ± 0.15), NaB (1.01 ± 0.12) or ICN (0.93 ± 0.06) groups compared to controls (1 ± 0.17; Fig. 10A). The same holds true at 2 h in Isoproterenol (0.53 ± 0.07), NaB (0.45 ± 0.12) and ICN (0.46 ± 0.06) groups compared to controls (1 ± 0.47; Fig. 10B).

CHAPTER 4 – DISCUSSION

4.1 Summary of major findings

The purpose of these experiments was to gain insight into potential mechanisms responsible for extending the duration of memory. Specifically, we sought to determine the key molecular players involved in forming long lasting long-term memories compared to long-term memories lasting only a day. We first tested the role of CREB and its generated pattern of phosphorylation following training as a potential mediator for governing memory duration. We examined the expression of CREB phosphorylation with western blotting using 3 models of olfactory preference learning that have been shown to yield odor preferences for different durations (24 h, 4-Day, or 5-Day). Training induced significant biphasic increases in CREB phosphorylation in two of the long-term olfactory preference models, 24 h and 5-Day. Our 4-Day memory model generated no detectable changes in CREB phosphorylation after conditioning, suggesting memory duration in this model is governed by alternate factors. Next, we tested the role of histone acetylation in memory extension as it has been implicated in promoting transcription, a requirement for long-term memory consolidation, and it is dependent on the CREB: CBP interaction (Vecsey et al. 2007). We showed that the HDAC inhibitor NaB, when paired with the 1 mg/kg isoproterenol and 3 mg/kg cilomilast, induced robust increases in the acetylation of H3 immediately after learning. This increase in acetyl H3 was paralleled in non-learning conditions where NaB was present, suggesting sodium butyrate increases acetylation of H3 in extended memory conditions but also in non-learning conditions, suggesting sodium butyrate activates H3 but this, in itself, is insufficient to account for long-term memory.

Next, we tested the hypothesis of certain genes downstream of CREB might be differentially expressed in 24 h versus extended memory for the model in which pCREB increased following training. Corresponding with the increases found in CREB phosphorylation at 10 min and 2 h, increases in CREB target genes following olfactory preference training were assessed. Using q-PCR we found differential gene expression for the CREB target genes *Nr4a1* and *Egr1* between long-term (24 h) and extended long-term (5-Day) olfactory preference learning. Both genes were significantly increased 2 h after training in the extended memory paradigm, but not in the 24 h memory paradigm. In addition, we observed two other CREB target genes, *Nr4a2* and C/EBP_{β}, to be increased 10 min after training with both 24 h and 5-Day memory training. The differences in gene expression following odor preference training could play a role in the initiation of potential structural and cellular changes that accompany learning. Insights into these changes will be discussed.

4.2 Expression pattern of pCREB across long-term olfactory preference learning models

We know that memories can be formed under a variety of conditions. In the case of classical conditioning, when a CS and UCS are paired, a learned association between these two stimuli can be formed as shown by the CS eliciting a specified CR. The duration of this memory varies under a multitude of conditions including saliency to the animal. The scenario of an animal that retains a memory for 24 h versus an animal that retains that same memory for 4 or 5 days, generates the question of what governs the ability of this memory to be sustained for longer periods of time when that memory has the same intrinsic and meaningful value to the animal.

With the aim of gaining insight into the cellular mechanisms responsible for governing the duration of memory, a logical point of departure was to look at CREB, which can arguably be referred to as a universal memory molecule (Silva et al. 1998). Phosphorylation of this transcription factor is best known to be mediated by PKA translocation across the nuclear membrane in response to cAMP signaling, and is rate limited by this action (Hagiwara et al. 1993). Phosphorylation of CREB can also be achieved by a multitude of stimuli including activation via the MEK/ERK pathway (Davis et al. 2000). In neurons, CREB is activated in response to stimuli that produce changes in synaptic strength and is responsible for regulating gene transcription associated with the consolidation of long-term memory (Silva et al. 1998, Review). Previous findings in our lab provide evidence that pharmacologically targeting and manipulating different aspects of the cAMP/PKA pathway, including β-AR activation , phosphodiesterase inhibition, and HDAC inhibition are successful in producing long-term and extended long-term olfactory preference learning (Christie-Fougere et al. 2009; Cui et al. 2011; Grimes et al. 2012; Grimes et al. 2011; Harley et al. 2006; Langdon et al. 1997; McLean et al. 2005; Sullivan et al. 2000b; Sullivan et al. 1989; Yuan et al. 2000).We employed the use of three pharmacological models shown to produce 24 h odor preference or longer to assess the role of the phosphorylation of CREB as it pertains to memory duration.

CREB phosphorylation is enhanced in a biphasic pattern after olfactory preference training as shown here in both 24 h and 5-Day odor conditioning models. This is a novel finding for olfactory preference learning. Yuan and colleagues (2000) previously reported a single increase in CREB phosphorylation 10 min after early odor preference training of odor paired with 2 mg/kg isoproterenol to produce 24 h preference learning. They did not find any subsequent increases in pCREB expression at 1 h or 2 h (Yuan et al. 2000). Closer examination

of the graphical representation of the optical density value for pCREB in that study suggests there is a possible trend towards an increase in pCREB at 2 h. It is possible that an increase in sample size (here n=17/group for the 5 Day memory group versus n=8 group in Yuan et al 2000) would reveal similar findings to those reported here. In the present study, the reported initial peak in pCREB at 10 min post-conditioning provides supportive evidence for its requirement as an early transcription factor involved in early olfactory preference learning, and is consistent with the previously reported increase in pCREB after 24 h odor preference training using isoproterenol (McLean et al. 1999; Yuan et al. 2000).

Hawk and Abel (2011) propose involvement of the cAMP/PKA/CREB pathway as the initiator of two waves of transcriptional responses required for memory consolidation, with evidence suggesting the second wave is initiated by the gene products of the CREB mediated first wave (Hawk and Abel 2011). My findings in the present study of biphasic pCREB expression support the view for two waves of gene expression with the common requirement being that they are CREB mediated. These results suggest an initial peak occurs 10 min after conditioning with the second peak occurring at 2 h post-conditioning. It is possible that there are subsequent peaks in pCREB occurring at later times than those examined here. Although this is a novel finding in olfactory preference learning, biphasic pCREB has been reported during hippocampal LTP. Schulz and colleagues (1999) demonstrate direct involvement of biphasic CREB during hippocampal LTP *in vivo*, where the initial peak in pCREB was shown to correspond with early phase LTP while the late secondary peak corresponded with late phase LTP and was implicated in the maintenance of late phase LTP. Contextual fear-conditioning memory models also provide supportive evidence for biphasic pCREB post-conditioning in mice with evidence of an early peak in pCREB between 0 min and 30 min post-conditioning followed

by a second peak lasting between 3 h and 6 h post-conditioning (Stanciu et al. 2001). Biphasic pCREB has also been reported in forced swim studies, fear conditioning and spatial water maze tasks (Bilang-Bleuel et al. 2002; Martel et al. 2007; Tomobe et al. 2007). The difference in the timing of when these biphasic peaks of pCREB occur may be a result of the type of learning and the structures recruited. Taken together with what has been observed previously, the present results suggest that protein synthesis-dependent long-term memory may characteristically be associated with two waves of gene transcription.

If two waves of pCREB are required for the consolidation of long-term memory, our findings a single peak in pCREB in long-term (24 h) and biphasic pCREB in extended long-term (5-Day) memory do not support biphasic pCREB expression itself as being the mediator of memory extension. Previous findings in our lab reveal that when translation is blocked via intrabulbar infusions of anisomycin immediately after training 24 h memory is impaired. When protein translation is blocked 1 h or 3 h post-conditioning, 24 h odor preference learning is unaffected (Grimes et al. 2011). This suggests that the proteins required for long-term memory formation have been translated prior to 1 h after conditioning and that the second peak in pCREB may in fact not be critical for 24 h long-term memory. The finding of no increases in any of the 8 CREB target genes (*Nr4a1*, *Nr4a2*, *Nr4a3*, *C/EBP$_\beta$*, *Jun*, *Fos*, *Bdnf*, and *Egr-1*) examined in this study 2 h after 24 h memory conditioning is consistent with the suggestion that CREB-driven transcription may not occur at 2 h post-conditioning in the 24 h memory model, despite increased pCREB. Blocking translation/transcription at this interval for the extended odor preference training would be useful in determining the involvement of this second wave of pCREB in our extended memory model. If the second wave of pCREB is required for extended long-term memory then this would suggest the second wave of pCREB is a distinguishing characteristic

between long-term and extended long-term memory. This suggestion is supported by the finding of two CREB related genes being upregulated at 2 h post-training in the extended memory model.

Variability in gene expression with comparable levels of CREB phosphorylation has been reported previously [see for example (West et al. 2001) using BDNF as a reporter gene]. It is also possible that CREB modulated targets not examined here may have been altered by the second wave of pCREB activation in the 24 h memory model and could prime the circuitry for longer-lasting memory depending on later events such as spaced training. Finally a quantitative assay might be more sensitive to differences in pCREB levels at 2 h between models.

Of notable interest here is the level of pCREB at 4 h in the 24 h and 5-Day odor preference models. A significant increase in pCREB at 4 h seen in the 24 h odor preference memory paradigm was not observed in our 5-Day model. In fact, it appears in our 5-Day model that pCREB levels have returned to baseline by 4 h. Although not performed here, an extension beyond 4 h on the time points examined under western blotting may reveal other points of differential pCREB expression between these two models. However, if the second phase of pCREB is not critical for long-term consolidation, it is possible that any subsequent peak in pCREB may be arbitrary for the maintenance of long-term memory. Blocking transcription after 1 h post-conditioning in this model is necessary to confirm the role of the second peak in pCREB for 5-Day memory.

4.3 4-Day long-term memory does not show evidence of pCREB enhancement

Interestingly, not all of the long-term memory models examined in this thesis showed increased CREB phosphorylation after training. No evident changes in CREB phosphorylation were found in our 4-Day memory model. This suggests the possibility of an alternate means for the consolidation of long-term memory that may not be dependent on CREB signaling.

What might be an alternate pathway for long-term memory? Possibly it is an extension of the mechanisms responsible for short-term olfactory preference memory (< 3 h). The exact mechanism by which short-term odor preference learning is formed is not yet known. It has been suggested that the Ca^{2+}/calmodulin pathway has a role (Grimes et al. 2012). This pathway produces increases in Ca^{2+} influx through NMDA receptors in response to glutamatergic release resulting in CaMKIV activation and ultimately CREB phosphorylation at Ser-133 (Deisseroth et al. 1998). In the study by Grimes and colleagues (2012), application of Rp-cAMPs, a PKA inhibitor, produced no effect on short-term memory while disrupting intermediate (5 h) and long-term (24 h) memory. They also used an intrabulbar PKA activator paired with odor to create intermediate and long-term odor preference memory without creating shorter term odor preference memory. Thus, a non PKA-dependent intracellular signalling system can engage preference learning circuits. In the case of 4-Day odor preference memory, where the combination of isoproterenol (β-AR agonist) and cilomilast (phosphodiesterase inhibitor) paired with odor drives the prolonged memory, an alternative to the cAMP/PKA/CREB pathway may be activated. Clearly the strict dosage requirements for isoproterenol in this paradigm (1 mg/kg but not 2 mg/kg is required) suggests a different profile of cAMP signalling. Although cilomilast normalizes the pulsatile cAMP pattern, the level of cAMP cannot be so high as to pass the

hyperbolic threshold. Epac is an agonist activated by cAMP that might mediate associative learning in the 4 Day model and not generate normal pCREB activation profiles.

Taken together, the level of pCREB, post-olfactory preference training, suggests it is not solely responsible for the initiation and maintenance of long-term memories. In fact, a parallel profile of biphasic pCREB in both long-term and extended long-term memory suggests CREB may possibly play a role in priming the brain for another memory molecule to enhance the duration of memory. We proposed a possible role of histone acetylation in memory maintenance.

4.4 NaB initiates histone 3 acetylation

Having shown CREB phosphorylation is biphasic in both long-term and extended long-term (5-Day) models, it is perhaps histone acetylation and the promotion of the transcriptional window that leads to enhancement of memory duration. We next sought to determine the role of histone acetylation in the olfactory preference memory model. Vecsey and colleagues (2007) provide evidence when given HDAC inhibition to act as a memory enhancer immediately after contextual fear conditioning. Their findings suggest that the HDAC inhibitor TSA promotes contextual fear conditioning through acetylation of histones and subsequent promotion of CREB-mediated gene transcription. Further support is shown by evidence of memory impairment in the absence of a successful CREB: CBP interaction. When this interaction is disrupted, learning cannot be rescued by TSA due to the lack of recruitment of necessary transcriptional machinery (Vecsey et al. 2007).

Previous findings in our lab show that administration of the HDAC inhibitor NaB alone and paired with odor does not produce significant odor preference learning (McLean et al. 2011).

In contrast, when NaB is given in combination with β-AR activation and phosphodiesterase inhibition, odor preference memory is enhanced, with odor preferences observed at least 5-Days after the conditioning trial. We show through western blotting that the HDAC inhibitor NaB, when given 40 min before conditioning, increases histone acetylation immediately after training in both learning (extended long-term, 5-Day) and non-learning (NaB+odor) conditions.

I demonstrated that NaB effectively crosses the blood brain barrier and is active to acetylate H3 in the OB of neonatal rats following systemic injection of the drug. This confirms that when given by itself, the observation of a no learning effect is not due to a lack of NaB entering the brain. NaB aids in extending olfactory preference memory to last at least 5-Days, as shown by the effectiveness of the combination of iso+cilo to produce 4-Day memory while the addition of NaB extends that memory to 5-Days. Increased histone acetylation via NaB administration suggests that the parallel acetylation patterns in both 5-Day learning groups that received NaB and NaB non-learning controls indicates that HDAC inhibition is only effective in initiating and extending memory formation when pCREB is increased and the OB's transcription machinery is recruited. This suggests that enhanced acetyl H3 is contributing to memory extension, but it is not sufficient to enhance memory by itself.

The relationship between histone acetylation and the promotion of gene transcription is by remodelling chromatin via the addition and removal of acetyl groups on lysine residues from histone tails increasing the accessibility of DNA to transcription factors (Brownell and Allis 1996). This interaction is initiated by HATs to promote a transcriptionally active conformation and HDACs which render chromatin to a transcriptionally inactive conformation. HAT activity is not limited to histone targets, but also includes non-histone proteins such as CREB (Imhof et al. 1997). It is possible that promotion of acetyl H3 in both learning and non-learning groups

shown here may lie in the ability of CBP to act as a HAT and acetylate both histone proteins and CREB itself. Lu and colleagues (2003) showed that CBP can directly activate CREB via 3 lysines affecting its transactivation domain. They postulated that an increase in CREB acetylation could lead to increases in the interaction of CREB binding with CRE elements and successive interactions with CBP (Lu et al. 2003). This could be a possible role for HDAC inhibition in memory extension of olfactory preference memory.

Histone 3 acetylation pattern found in the non-learning group and paralleled in our extended long-term model does not rule out the possibility of HDAC involvement in memory extension. With evidence that histone acetylation is only effective in enhancing memory and gene transcription when CREB is activated and bound to CRE followed by recruitment of CBP and the CREB:CBP interaction (initiated by β-AR activation and promoted by phosphodiesterase inhibition), in the case of NaB by itself, cAMP levels are not pharmacologically enhanced and as such promotion of CREB phosphorylation is absent. In the case of our 5-Day model, we have pharmacologically primed the OB to promote CREB phosphorylation as we saw with our pCREB western blotting data, and have created an environment where CREB:CBP interaction is promoted and histone acetylation is effective. Without the transcription factor, transcription is not initiated. Histone acetylation occurs independently of CREB phosphorylation, therefore increasing the time window for transcription in the absence of increased pCREB is ineffective as recruitment of basal transcription machinery is absent. This is consistent with the increases in CREB transcribed genes in the 2 h window for the 5 Day model, which are not seen in the 24 h group.

Exploration of HDAC inhibitors as a potential treatment for memory impairments associated with neurological diseases has generated recent success. For example, a mouse model

of Huntington's disease (HD) with pronounced cognitive impairments has shown promise for rescue of associated memory deficits when given the HDAC inhibitor TSA (Giralt et al. 2012). Giralt and colleagues (2012) show that long-term object recognition and spatial memory in heterozygous HD knock-in mutant mice ($Hdh^{Q7/Q111}$) is impaired. They show associated reduced CBP function in these animals as well as reduced CBP in human patients. Interestingly, pCREB levels were not affected by TSA. This suggests that memory enhancement is occurring downstream of CREB phosphorylation. In addition, they found reduced acetyl H3 levels in mice at 8 months of age, suggesting a reduction in CBP may be affecting histone acetylation resulting in the associated cognitive deficits. When given TSA, short-term memory (15min) is unaffected, but 24 h long-term object recognition was rescued. This suggests that reduced CBP HAT activity might contribute to the long-term memory deficits in HD mice. In addition, they found a significant reduction in CREB target gene expression, including a ~25% reduction in *Nr4a2* expression. Consistent with these findings, our extended long-term olfactory preference model yielding memory extension of 5-Days may be a result of increased CBP HAT activity and resulting increased acetyl H3 activity by NaB. Recruitment of CBP and the associated basal transcription machinery can affect transcription of downstream genes.

A recent paper looking at 24 h aversive odor conditioning using shock in 11 day old rat pups also reported that TSA, like NaB here, extended 1 day memory to a multiday memory (Wang et al. 2012). In their experiments both H3 and H4 were increased in the first 10 min after training. They noted that these were earlier times for a drug-induced increase in histone acetylation than had been reported previously, but they are consistent with the time line in the present study. Histone acetylases were increased in both mitral cells and granule cells when examined immunohistochemically. An unusual finding in their study was that TSA paired with

odor 30 min after the odor was presented could induce an odor aversion memory 24 h later itself. This is not yet understood mechanistically. No aversion was seen with pre-training administration of TSA paired with odor, which is similar to the lack of conditioning observed in our appetitive paradigm with NaB (John McLean private communication).

4.5 Differential gene expression suggests CREB regulated genes are implicated in the promotion of memory duration

The absence of phosphorylated CREB after memory conditioning negatively affects downstream CREB mediated gene expression (Vecsey et al. 2007). The pharmacological agents used in our olfactory preference models target specific components of the cAMP/PKA signalling cascade: isoproterenol activates β-adrenoceptors on mitral cells (Yuan et al., 2003), phosphodiesterase inhibitors prevent the breakdown of cAMP and increase cAMP stores (Cui et. al, 2007), and HDAC inhibitors prevent the deacetylation of histones. These pharmacological targets encompass a specific goal, that being to promote the phosphorylation of CREB and ultimately gene transcription. A logical progression of the investigation into the mechanism responsible for memory extension lies in the generation of new gene product as a result of this response. I identified two genes, *Nr4a1* and *Egr-1*, which are differentially expressed between our long-term (24 h) and extended long-term (5-Day) odor preference models. I have also identified CREB related genes that are expressed in both long-term and extended long-term learning models, *Nr4a2*and *C/EBP*$_{\beta}$, suggesting they may play similar roles in mediating long-term memory formation. Differential gene expression suggests regulation of gene transcription

during learning is required for establishing the molecular memory trace. A discussion on the potential role these genes may play in memory extension follows.

4.6 NR4A gene expression is differentially expressed across long-term and extended long-term olfactory preference learning

CREB target genes belonging to the NR4A family of orphan nuclear receptors have been implicated in memory and the associated changes in synaptic plasticity as a result of learning (Bridi and Abel 2013). NR4A orphan nuclear receptors are CRE containing genes whose transcription is directly controlled by the cAMP/PKA/CREB signaling pathway (Barneda-Zahonero et al. 2012; Bridi and Abel 2013; Hawk and Abel 2011; Hawk et al. 2012; Volakakis et al. 2010). I identified *Nr4a1* as differentially expressed between 24 h and 5-Day odor preference models. A significant increase in *Nr4a1* expression was observed at 2 h post-conditioning in our 5-Day extended long-term memory model but not in our 24 h long-term model. My findings fit well with those reported by Vecsey and colleagues (2007), that show contextual-fear conditioning is enhanced following administration of the HDAC inhibitor TSA directly in the hippocampus, resulting in an increase in *Nr4a1* gene expression 2 h post-conditioning (Vecsey et al. 2007). This increase was abolished in the absence of CREB activation and subsequent binding with CRE to form the CREB: CBP interaction. I chose to look at gene expression at the time points that correlated with our increased pCREB expression profiles. As such, consistent with the reports by Vecsey and colleagues (2007), it can be suggested that pCREB mediates *Nr4a1* gene expression under the conditions of enhanced memory duration, specifically when HDACs are inhibited.

As previously mentioned, *Nr4a1* and its family members are CREB target genes. I found no such increase in *Nr4a1* gene expression in our long-term 24 h memory model despite parallel CREB phosphorylation profiles while *Nr4a1* gene expression was increased in the 5-Day memory model. This suggests that induction of *Nr4a1* gene transcription is initiated by recruitment of another factor after the initial peak in pCREB levels. With evidence of *Nr4a1* expression being enhanced in the presence of an HDAC inhibitor (Hawk et al. 2012; Vecsey et al. 2007), I postulate that NaB is aiding in the enhanced expression of this gene. However, expression of this gene was not significantly increased in NaB non-learning controls, suggesting *Nr4a1* gene expression is not enhanced solely by systemic administration of an HDAC inhibitor but requires prior associative learning conditions as in the Vecsey study and in the present study.

In addition to enhanced *Nr4a1* gene expression, I also find increased *Nr4a2* expression 10 min post-conditioning in both the 24 h and 5-Day models. *Nr4a1* and *Nr4a2* might play different roles in learning and memory (McNulty et al. 2012). *Nr4a1* has previously been implicated in long-term object location and object recognition memory while *Nr4a2* has only been implicated in long-term object location memory (McNulty et al. 2012). It may be the case in olfactory preference learning that *Nr4a1* and *Nr4a2* contribute to different aspects of long-term odor preference learning. It can be suggested that *Nr4a2* plays a unified role in olfactory preference memory as its expression appears to be a product of the necessary first wave of pCREB while *Nr4a1* expression appears to be a product of the second wave and only occurred in the case where memory is extended to 5-Days. It would be informative to block *Nr4a1* to determine if it is causal in extending long-term memory. *Nr4a3* was not significantly expressed above controls at either time point examined in 24 h or 5-Day memory. *Nr4a1* gene expression

is found to be enhanced after TSA administration in $Hdh^{Q7/Q11}$ mutant mice. This enhanced expression was correlated with rescued memory deficits (Giralt et al. 2012).

In contrast to Hawk and Abel (2011) who suggest *Nr4a1* to be implicated in the first of two waves of transcription required for long-term memory, I find *Nr4a1* expression to coincide with our second peak of pCREB, suggesting it may be a result of the second wave of transcription. Having found no such increase 2 h after 24 h memory training is consistent with previous findings suggesting all proteins required for 24 h long-term memory formation have been translated by this time after conditioning (Grimes et al. 2011). Members of the NR4A subfamily of orphan nuclear receptors have been implicated as immediate early genes whose transcriptional activity is regulated by transcriptional modification (Hawk and Abel 2011; Zhao and Bruemmer 2010). Histone acetylation plays an integral role in the promotion of gene transcription in long-term memory. Acetylation and deacetylation of histone and non-histone proteins can initiate cellular and molecular changes that occur aside from changes in DNA sequences, and permit lasting changes in gene activity. Histone acetylation and deacetylation are epigenetic modifications and as such, it can be suggested that these transcriptional alterations may be promoting phenotypic changes to neurons and contributing to the changes in synaptic plasticity associated with learning and memory.

4.7 C/EBPβ is significantly enhanced in the OB after long-term and extended long-term odor preference training

C/EBP$_{\beta}$ is the C/EBP family member with the greatest expression in the mouse hippocampus (Yukawa et al. 1998). First implicated in learning and memory in *Aplysia* studies

(Alberini et al. 1994), a similar role has emerged in mammalian studies of hippocampal LTP (Taubenfeld et al. 2001a). I found C/EBP_{β} expression is significantly increased 10 min after training in both 24 h and 5-Day odor-preference models. Expression is not enhanced at 2 h post-conditioning . Although not differentially expressed between our long-term and extended long-term models, C/EBP_{β}'s role in long-term memory may have important implications downstream of CREB. C/EBP_{β} expression is enhanced by cAMP signaling and Ca^{2+} activation in mouse cultured hippocampal neurons by increasing the transcription factors' DNA binding capabilities (Yukawa et al. 1998). Guan and colleagues (2002) reveal potential insight into the role of C/EBPs in long-term memory. They show, in *Aplysia* that 5-HT can induce downstream C/EBP expression by activation of $CREB_1$ whose role involves recruiting CBP for histone acetylation. In contrast, FMRFa, an inhibitory transmitter, displaces $CREB_1$ by activating $CREB_2$ along with HDAC5 recruitment promoting deacetylation of histones. They show that in long-term facilitation, inhibitory inputs dominate and the repression of transcription is promoted by $CREB_2$ (Guan et al. 2002). In the case of our olfactory preference learning models, I pharmacologically target the pathway leading to CREB activation. It is possible that these manipulations aid to overcome the dominating inhibitory inputs and transcription repressors by promoting the CREB: CBP interaction, resulting in the increased C/EBP_{β} expression seen here. In the *Aplysia* study, they suggest C/EBP plays a role in the switch from short-term to long-term facilitation. Such a role would coincide with enhanced expression in both 24 h and 5-Day memory as both are long-term and transcription dependent.

4.8 Jun, Fos and Bdnf expression are not enhanced after olfactory preference learning

No detectable increase in expression of *Jun*, *Fos*, or *Bdnf* was seen between long-term and extended long-term models and their respective controls. Although implicated in learning and memory as regulators of transcription, the two time points examined here (10 min and 2 h) may not correspond with the timing of expression of these genes after conditioning. An expansion of the times examined using q-PCR may be more inclusive of other CREB regulated genes than those found expressed here. In addition, when samples were collected for q-PCR, analysis was performed on whole OBs. It is possible that increases in gene expression solely in a small area or layer of the OB may not be detectable above controls. The previous report of increased cfos with odor preference learning in periglomerular cells suggests immunohistochemical staining or *in situ* hybridization for *Jun*, *Fos*, and *Bdnf* may be required for insight into expression level variation as well as localization of these products.

4.9 Egr-1 expression is enhanced in extended long-term olfactory preference learning

Egr-1 is an immediate early gene that has been considered to be a facilitator involved in the maintenance of memory duration (Bozon et al. 2002; Jones et al. 2001). When *Egr-1* (zif268) is mutated and no longer active in the hippocampus of mice, performance on spatial and non-spatial learning tasks show long-term memory retention is impaired, while short-term memory remains intact (Jones et al. 2001). It was also the case that *Egr-1* was required for the 24 h and 48 h expression of late phase hippocampal LTP. Consistent with a selective role in longer term

memories, I found *Egr-1* expression was significantly increased in our extended (5-Day) odor preference model 2 h after odor exposure. *Egr-1* expression was enhanced neither at 10 min nor 2 h in our 24 h long-term memory model, nor at 10 min in our 5-Day model. Expression was not increased in NaB treated non-learning controls, again suggesting that systemic NaB does not affect *Egr-1* gene expression in the absence of learning.

What remains unidentified is what might be aiding in the expression of *Egr-1* in addition to its modulation via CREB. This remains elusive given our findings that both 24 h and 5-Day memory yield biphasic pCREB at the same time points yet we find differential gene expression. Our 5-Day model receives, in addition to the iso, phosphodiesterase inhibition and HDAC inhibition. This suggests that *Egr-1* expression is promoted in conditions that enhance cAMP patterns as well as the transcriptional time frame. *Egr-1* gene expression in the 5 Day memory model suggests that it is involved in initiating potential downstream targets, independent from those implicated in 24 h memory, and possibly related to structural changes in the neurons.

Results of immunohistochemistry performed on neonatal rats trained for odor preference learning by pairing odor+stroking producing a 24 h memory, show that AMPA receptor signaling is increased 24 h after training, and is no longer enhanced at 48 h when odor preference is no longer exhibited (Cui et al. 2011). Although it has not yet been characterized, AMPA receptor staining at 48 h and later would be of interest in our extended model. Neuron activity regulated pentraxin (Narp), a synaptic protein implicated in the aggregation of AMPA receptors (O'Brien et al. 2002) was increased in the dentate gyrus of rats along with *Egr-1* (Soule et al. 2008). In addition, Arc, PSD-95 and α-CaMKII proteins were also upregulated (Soule et al. 2008). Interestingly, PSD-95 plays a role in the stabilization of AMPA receptors (Yudowski et

al. 2013), and is a possible downstream target of *Egr-1* and potential link to cellular changes associated with learning and memory and in particular the extension of memory duration.

4.10 Conclusions

These experiments have shown that both CREB phosphorylation and histone acetylation play a role in the cellular changes associated with the promotion of long-term memory formation and that downstream CREB mediated genes provide insight into the possible mechanisms responsible for governing memory duration. Long-term learning is associated with significant biphasic pCREB expression 10 min and 2 h after conditioning. Both long-term and extended long-term olfactory preference learning demonstrate this expression profile of pCREB, collectively re-affirming a role for pCREB in early odor preference learning. Together, these results suggest the mechanism responsible for facilitating memory duration does not collectively arise alone from phosphorylation of the memory molecule CREB. Rather, these findings suggest CREB phosphorylation is implicated in long-term memory formation but does not definitively distinguish its duration. This study focused on the timing of when CREB is phosphorylated. It is possible that the amount of phosphorylated CREB is a defining factor to differentiate long-term from extended long-term memory. Next, we suggested a role for histone acetylation in extended long-term olfactory preference learning. A robust increase in acetylation of H3 suggests that although NaB alone is sufficient to promote a significant increase in acetyl H3, it is not sufficient in promoting memory duration. It can be suggested that histone acetylation, by increasing the duration of transcriptional activity, aids in promoting memory extension under conditions that promote CREB phosphorylation. Lastly, CREB target genes *Nr4a1* and *Egr-1* are expressed solely after extended long-term memory training. Together, these results suggest that the

mechanism responsible for promoting memory extension is influenced by CREB phosphorylation and histone acetylation coupled with the aim of enhancing gene transcription. The exact mechanism by which memory duration is extended has yet to be determined. However, insight into the function of integral components of long-term memory formation provides support for the cAMP/PKA pathway and its downstream targets as mediators in the event.

Questions could be raised that I did not know if the animals I used for biochemistry (westerns, q-PCR) actually behaved as expected. This is a legitimate concern that occurs with any biochemical study where it is not possible to test the behaviour of the animals (pups must be sacrificed before a behavioural test can be practically performed). In order to undertake all the biochemical studies in the timeframe of a master's thesis and in consultation with my supervisors, I decided to assume that the animals used in these studies would be behaving in the manner found in other studies using the same techniques as referenced in this thesis. Thus, although I did not know with certainty that pups, for example, in the one day memory group (2 mg/kg isoproterenol paired with odor during training) behaved as expected, there is good reason to believe they would behave as learning pups based on numerous previous studies from my supervisor's lab and from others (Christie-Fougere et al. 2009; Cui et al. 2011; Grimes et al. 2012; Grimes et al. 2011; Harley et al. 2006; Langdon et al. 1997; Sullivan et al. 2000b; Sullivan et al. 1989; Yuan et al. 2000). I took a similar approach with the 4 day memory (McLean et al. 2005) and 5 day memory (Appendix B).

Understanding the cellular signaling cascade responsible for governing how and why we are able to form memories that last for different periods of time is important for addressing how to maintain these memories under compromised cognitive conditions. Finding the gateway for

memory duration is critical for combating neurological diseases leading to cognitive impairments in learning and memory such as Alzheimer's disease. The role of the memory mediators studied here, including CREB phosphorylation, histone acetylation and its downstream genetic targets are essential for understanding the functionality of this pathway.

4.11 Future directions

Further research with the goal of understanding the molecular correlates implicated in the extension of memory duration should be directed at the requirement for biphasic pCREB in extended long-term memory models, specifically in determining the purpose of the second wave of pCREB at 2 h post-conditioning. In addition, conditional knock-out or down regulation by siRNA of *Nr4a1* and *Egr-1* gene expression should be examined with respect to what aspects of extended long-term memory are impaired. Immunohistochemical analysis for localization of CREB target genes, *Nr4a1*, *Nr4a2*, *Egr-1*, and C/EBP_{β} would be of importance in determining where these genes are expressed. Having found an increase in mRNA of the CREB target genes discussed, it would be necessary to next verify that the protein is also upregulated. Future studies need to be directed at targeting specific components of CREB mediated signaling to distinguish what is critically involved in long-term memory formation and what is required for the extension of these memories beyond a 24 h time point. The genes examined in this thesis are only 8 of several CREB target genes and as such there may be other contributing genes to long-term memory not yet examined here. There is also the possibility of other transcription factors

implicated in 4-Day memory not examined here. A similar approach using western blotting and real-time PCR may provide insight into this 4-day memory model.

Reference List

1. Adam, Y. and Mizrahi, A. 2010. Circuit formation and maintenance--perspectives from the mammalian olfactory bulb. *Curr. Opin. Neurobiol.* **20**: 134-140.

2. Alberini, C.M. 1999. Genes to remember. *J. Exp. Biol.* **202**: 2887-2891.

3. Alberini, C.M. 2009. Transcription factors in long-term memory and synaptic plasticity. *Physiol Rev.* **89**: 121-145.

4. Alberini, C.M., Ghirardi, M., Metz, R., and Kandel, E.R. 1994. C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in Aplysia. *Cell* **76**: 1099-1114.

5. Aroniadou-Anderjaska, V., Ennis, M., and Shipley, M.T. 1999. Dendrodendritic recurrent excitation in mitral cells of the rat olfactory bulb. *J. Neurophysiol.* **82**: 489-494.

6. Bakalyar, H.A. and Reed, R.R. 1991. The second messenger cascade in olfactory receptor neurons. *Curr. Opin. Neurobiol.* **1**: 204-208.

7. Barneda-Zahonero, B., Servitja, J.M., Badiola, N., Minano-Molina, A.J., Fado, R., Saura, C.A., and Rodriguez-Alvarez, J. 2012. Nurr1 protein is required for N-methyl-D-aspartic acid (NMDA) receptor-mediated neuronal survival. *J. Biol. Chem.* **287**: 11351-11362.

8. Bilang-Bleuel, A., Rech, J., De, C.S., Holsboer, F., and Reul, J.M. 2002. Forced swimming evokes a biphasic response in CREB phosphorylation in extrahypothalamic limbic and neocortical brain structures in the rat. *Eur. J. Neurosci.* **15**: 1048-1060.

9. Boekhoff, I. and Breer, H. 1990. Differential stimulation of second messenger pathways by distinct classes of odorants. *Neurochem. Int.* **17**: 553-557.

10. Bollen, B., Matrot, B., Ramanantsoa, N., Van den Bergh, O., D'Hooge, R., and Gallego, J. 2012. Olfactory classical conditioning in neonatal mouse pups using thermal stimuli. *Behav. Brain Res.* **229**: 250-256.

11. Bozon, B., Davis, S., and Laroche, S. 2002. Regulated transcription of the immediate-early gene Zif268: mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation. *Hippocampus* **12**: 570-577.

12. Bradley, J., Reisert, J., and Frings, S. 2005. Regulation of cyclic nucleotide-gated channels. *Curr. Opin. Neurobiol.* **15**: 343-349.

13. Breer, H., Boekhoff, I., and Tareilus, E. 1990. Rapid kinetics of second messenger formation in olfactory transduction. *Nature* **345**: 65-68.

14. Brennan, P.A., Schellinck, H.M., de la Riva, C., Kendrick, K.M., and Keverne, E.B. 1998. Changes in neurotransmitter release in the main olfactory bulb following an olfactory conditioning procedure in mice. *Neuroscience* **87**: 583-590.

15. Bridi, M.S. and Abel, T. 2013. The NR4A orphan nuclear receptors mediate transcription-dependent hippocampal synaptic plasticity. *Neurobiol. Learn. Mem.*

16. Brownell, J.E. and Allis, C.D. 1996. Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. *Curr. Opin. Genet. Dev.* **6**: 176-184.

17. Brunelli, M., Castellucci, V., and Kandel, E.R. 1976. Synaptic facilitation and behavioral sensitization in Aplysia: possible role of serotonin and cyclic AMP. *Science* **194**: 1178-1181.

18. Camp, L.L. and Rudy, J.W. 1988. Changes in the categorization of appetitive and aversive events during postnatal development of the rat. *Dev. Psychobiol.* **21**: 25-42.

19. Carew, T.J., Walters, E.T., and Kandel, E.R. 1981. Classical conditioning in a simple withdrawal reflex in Aplysia californica. *J. Neurosci.* **1**: 1426-1437.

20. Chaudhury, D., Escanilla, O., and Linster, C. 2009. Bulbar acetylcholine enhances neural and perceptual odor discrimination. *J. Neurosci.* **29**: 52-60.

21. Christie, J.M., Schoppa, N.E., and Westbrook, G.L. 2001. Tufted cell dendrodendritic inhibition in the olfactory bulb is dependent on NMDA receptor activity. *J. Neurophysiol.* **85**: 169-173.

22. Christie-Fougere, M.M., Darby-King, A., Harley, C.W., and McLean, J.H. 2009. Calcineurin inhibition eliminates the normal inverted U curve, enhances acquisition and prolongs memory in a mammalian 3'-5'-cyclic AMP-dependent learning paradigm. *Neuroscience* **158**: 1277-1283.

23. Coopersmith, R., Weihmuller, F.B., Kirstein, C.L., Marshall, J.F., and Leon, M. 1991. Extracellular dopamine increases in the neonatal olfactory bulb during odor preference training. *Brain Res.* **564**: 149-153.

24. Countryman, R.A., Orlowski, J.D., Brightwell, J.J., Oskowitz, A.Z., and Colombo, P.J. 2005. CREB phosphorylation and c-Fos expression in the hippocampus of rats during acquisition and recall of a socially transmitted food preference. *Hippocampus* **15**: 56-67.

25. Cui, W., Darby-King, A., Grimes, M.T., Howland, J.G., Wang, Y.T., McLean, J.H., and Harley, C.W. 2011. Odor preference learning and memory modify GluA1 phosphorylation and GluA1 distribution in the neonate rat olfactory bulb: testing the AMPA receptor hypothesis in an appetitive learning model. *Learn. Mem.* **18**: 283-291.

26. Cui, W., Smith, A., Darby-King, A., Harley, C.W., and McLean, J.H. 2007. A temporal-specific and transient cAMP increase characterizes odorant classical conditioning. *Learn. Mem.* **14**: 126-133.

27. Davis, S., Vanhoutte, P., Pages, C., Caboche, J., and Laroche, S. 2000. The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. *J. Neurosci.* **20**: 4563-4572.

28. de Ruijter, A.J., van Gennip, A.H., Caron, H.N., Kemp, S., and van Kuilenburg, A.B. 2003. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem. J.* **370**: 737-749.

29. Deisseroth, K., Heist, E.K., and Tsien, R.W. 1998. Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. *Nature* **392**: 198-202.

30. Devore, S. and Linster, C. 2012. Noradrenergic and cholinergic modulation of olfactory bulb sensory processing. *Front Behav. Neurosci.* **6**: 52.

31. Devore, S., Manella, L.C., and Linster, C. 2012. Blocking muscarinic receptors in the olfactory bulb impairs performance on an olfactory short-term memory task. *Front Behav. Neurosci.* **6**: 59.

32. Didier, A., Carleton, A., Bjaalie, J.G., Vincent, J.D., Ottersen, O.P., Storm-Mathisen, J., and Lledo, P.M. 2001. A dendrodendritic reciprocal synapse provides a recurrent excitatory connection in the olfactory bulb. *Proc. Natl. Acad. Sci. U. S. A* **98**: 6441-6446.

33. Finkbeiner, S., Tavazoie, S.F., Maloratsky, A., Jacobs, K.M., Harris, K.M., and Greenberg, M.E. 1997. CREB: a major mediator of neuronal neurotrophin responses. *Neuron* **19**: 1031-1047.

34. Fletcher, M.L. and Chen, W.R. 2010. Neural correlates of olfactory learning: Critical role of centrifugal neuromodulation. *Learn. Mem.* **17**: 561-570.

35. Fontaine, C.J., Harley, C.W., and Yuan, Q. 2013. Lateralized odor preference training in rat pups reveals an enhanced network response in anterior piriform cortex to olfactory input that parallels extended memory. *J. Neurosci.* **33**: 15126-15131.

36. Gancedo, J.M. 2013. Biological roles of cAMP: variations on a theme in the different kingdoms of life. *Biol. Rev. Camb. Philos. Soc.* **88**: 645-668.

37. Gerber, B., Biernacki, R., and Thum, J. 2013. Odor-taste learning assays in Drosophila larvae. *Cold Spring Harb. Protoc.* **2013**.

38. Giralt, A., Puigdellivol, M., Carreton, O., Paoletti, P., Valero, J., Parra-Damas, A., Saura, C.A., Alberch, J., and Gines, S. 2012. Long-term memory deficits in Huntington's

disease are associated with reduced CBP histone acetylase activity. *Hum. Mol. Genet.* **21**: 1203-1216.

39. Glover, J.N. and Harrison, S.C. 1995. Crystal structure of the heterodimeric bZIP transcription factor c-Fos-c-Jun bound to DNA. *Nature* **373**: 257-261.

40. Gonzalez, G.A. and Montminy, M.R. 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**: 675-680.

41. Grimes, M.T., Harley, C.W., Darby-King, A., and McLean, J.H. 2012. PKA increases in the olfactory bulb act as unconditioned stimuli and provide evidence for parallel memory systems: pairing odor with increased PKA creates intermediate- and long-term, but not short-term, memories. *Learn. Mem.* **19**: 107-115.

42. Grimes, M.T., Smith, M., Li, X., Darby-King, A., Harley, C.W., and McLean, J.H. 2011. Mammalian intermediate-term memory: new findings in neonate rat. *Neurobiol. Learn. Mem.* **95**: 385-391.

43. Guan, Z., Giustetto, M., Lomvardas, S., Kim, J.H., Miniaci, M.C., Schwartz, J.H., Thanos, D., and Kandel, E.R. 2002. Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. *Cell* **111**: 483-493.

44. Gutierrez-Mecinas, M., Crespo, C., Blasco-Ibanez, J.M., Gracia-Llanes, F.J., Marques-Mari, A.I., Nacher, J., Varea, E., and Martinez-Guijarro, F.J. 2005. Distribution of D2 dopamine receptor in the olfactory glomeruli of the rat olfactory bulb. *Eur. J. Neurosci.* **22**: 1357-1367.

45. Hagiwara, M., Brindle, P., Harootunian, A., Armstrong, R., Rivier, J., Vale, W., Tsien, R., and Montminy, M.R. 1993. Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. *Mol. Cell Biol.* **13**: 4852-4859.

46. Hai, T. and Curran, T. 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. U. S. A* **88**: 3720-3724.

47. Harley, C.W., Darby-King, A., McCann, J., and McLean, J.H. 2006. Beta1-adrenoceptor or alpha1-adrenoceptor activation initiates early odor preference learning in rat pups: support for the mitral cell/cAMP model of odor preference learning. *Learn. Mem.* **13**: 8-13.

48. Hartmann, M., Heumann, R., and Lessmann, V. 2001. Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. *EMBO J.* **20**: 5887-5897.

49. Hawk, J.D. and Abel, T. 2011. The role of NR4A transcription factors in memory formation. *Brain Res. Bull.* **85**: 21-29.

50. Hawk, J.D., Bookout, A.L., Poplawski, S.G., Bridi, M., Rao, A.J., Sulewski, M.E., Kroener, B.T., Manglesdorf, D.J., and Abel, T. 2012. NR4A nuclear receptors support memory enhancement by histone deacetylase inhibitors. *J. Clin. Invest* **122**: 3593-3602.

51. Hawk, J.D., Florian, C., and Abel, T. 2011. Post-training intrahippocampal inhibition of class I histone deacetylases enhances long-term object-location memory. *Learn. Mem.* **18**: 367-370.

52. Hayar, A., Heyward, P.M., Heinbockel, T., Shipley, M.T., and Ennis, M. 2001. Direct excitation of mitral cells via activation of alpha1-noradrenergic receptors in rat olfactory bulb slices. *J. Neurophysiol.* **86**: 2173-2182.

53. Hayar, A., Karnup, S., Ennis, M., and Shipley, M.T. 2004. External tufted cells: a major excitatory element that coordinates glomerular activity. *J. Neurosci.* **24**: 6676-6685.

54. Hebbes, T.R., Thorne, A.W., and Crane-Robinson, C. 1988. A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J.* **7**: 1395-1402.

55. Heldt, S.A., Stanek, L., Chhatwal, J.P., and Ressler, K.J. 2007. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. *Mol. Psychiatry* **12**: 656-670.

56. Howell, C., David. (2002). *Statistical methods for psychology.* 5th ed. Pacific Grove, CA: Duxbury/Thomson Learning.

57. Imhof, A., Yang, X.J., Ogryzko, V.V., Nakatani, Y., Wolffe, A.P., and Ge, H. 1997. Acetylation of general transcription factors by histone acetyltransferases. *Curr. Biol.* **7**: 689-692.

58. Itzhak, Y., Liddie, S., and Anderson, K.L. 2013. Sodium butyrate-induced histone acetylation strengthens the expression of cocaine-associated contextual memory. *Neurobiol. Learn. Mem.* **102**: 34-42.

59. Jerome, D., Hou, Q., and Yuan, Q. 2012. Interaction of NMDA receptors and L-type calcium channels during early odor preference learning in rats. *Eur. J. Neurosci.* **36**: 3134-3141.

60. Johnson, B.A., Woo, C.C., Duong, H., Nguyen, V., and Leon, M. 1995. A learned odor evokes an enhanced Fos-like glomerular response in the olfactory bulb of young rats. *Brain Res.* **699**: 192-200.

61. Johnson, M.C., Biju, K.C., Hoffman, J., and Fadool, D.A. 2013. Odor enrichment sculpts the abundance of olfactory bulb mitral cells. *Neurosci. Lett.* **541**: 173-178.

62. Jones, M.W., Errington, M.L., French, P.J., Fine, A., Bliss, T.V., Garel, S., Charnay, P., Bozon, B., Laroche, S., and Davis, S. 2001. A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat. Neurosci.* **4**: 289-296.

63. Kasa, P., Hlavati, I., Dobo, E., Wolff, A., Joo, F., and Wolff, J.R. 1995. Synaptic and non-synaptic cholinergic innervation of the various types of neurons in the main olfactory bulb of adult rat: immunocytochemistry of choline acetyltransferase. *Neuroscience* **67**: 667-677.

64. Kaupp, U.B. 2010. Olfactory signalling in vertebrates and insects: differences and commonalities. *Nat. Rev. Neurosci.* **11**: 188-200.

65. Kay, L.M. and Sherman, S.M. 2007. An argument for an olfactory thalamus. *Trends Neurosci.* **30**:47-53.

66. Kerppola, T.K. and Curran, T. 1991. Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: implications for transcription factor cooperativity. *Cell* **66**: 317-326.

67. Kesslak, J.P., So, V., Choi, J., Cotman, C.W., and Gomez-Pinilla, F. 1998. Learning upregulates brain-derived neurotrophic factor messenger ribonucleic acid: a mechanism to facilitate encoding and circuit maintenance? *Behav. Neurosci.* **112**: 1012-1019.

68. Kida, S. 2012. A Functional Role for CREB as a Positive Regulator of Memory Formation and LTP. *Exp. Neurobiol.* **21**: 136-140.

69. Kim, Y.C., Lee, H.G., and Han, K.A. 2007. Classical reward conditioning in Drosophila melanogaster. *Genes Brain Behav.* **6**: 201-207.

70. Kobrinsky, E., Duong, S.Q., Sheydina, A., and Soldatov, N.M. 2011. Microdomain organization and frequency-dependence of CREB-dependent transcriptional signaling in heart cells. *FASEB J.* **25**: 1544-1555.

71. Korzus, E., Rosenfeld, M.G., and Mayford, M. 2004. CBP histone acetyltransferase activity is a critical component of memory consolidation. *Neuron* **42**: 961-972.

72. Langdon, P.E., Harley, C.W., and McLean, J.H. 1997. Increased beta adrenoceptor activation overcomes conditioned olfactory learning deficits induced by serotonin depletion. *Brain Res. Dev. Brain Res.* **102**: 291-293.

73. Lethbridge, R., Hou, Q., Harley, C.W., and Yuan, Q. 2012. Olfactory bulb glomerular NMDA receptors mediate olfactory nerve potentiation and odor preference learning in the neonate rat. *PLoS. One.* **7**: e35024.

74. Levenson, J.M., O'Riordan, K.J., Brown, K.D., Trinh, M.A., Molfese, D.L., and Sweatt, J.D. 2004. Regulation of histone acetylation during memory formation in the hippocampus. *J. Biol. Chem.* **279**: 40545-40559.

75. Levey, A.I., Hersch, S.M., Rye, D.B., Sunahara, R.K., Niznik, H.B., Kitt, C.A., Price, D.L., Maggio, R., Brann, M.R., and Ciliax, B.J. 1993. Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. *Proc. Natl. Acad. Sci. U. S. A* **90**: 8861-8865.

76. Liman, E.R. and Buck, L.B. 1994. A second subunit of the olfactory cyclic nucleotide-gated channel confers high sensitivity to cAMP. *Neuron* **13**: 611-621.

77. Liu, S., Plachez, C., Shao, Z., Puche, A., and Shipley, M.T. 2013. Olfactory bulb short axon cell release of GABA and dopamine produces a temporally biphasic inhibition-excitation response in external tufted cells. *J. Neurosci.* **33**: 2916-2926.

78. Lledo, P.M., Saghatelyan, A., and Lemasson, M. 2004. Inhibitory interneurons in the olfactory bulb: from development to function. *Neuroscientist.* **10**: 292-303.

79. Lonze, B.E. and Ginty, D.D. 2002. Function and regulation of CREB family transcription factors in the nervous system. *Neuron* **35**: 605-623.

80. Lu, Q., Hutchins, A.E., Doyle, C.M., Lundblad, J.R., and Kwok, R.P. 2003. Acetylation of cAMP-responsive element-binding protein (CREB) by CREB-binding protein enhances CREB-dependent transcription. *J. Biol. Chem.* **278**: 15727-15734.

81. Macrides, F. and Schneider, S.P. 1982. Laminar organization of mitral and tufted cells in the main olfactory bulb of the adult hamster. *J. Comp Neurol.* **208**: 419-430.

82. Maher, B.J. and Westbrook, G.L. 2008. Co-transmission of dopamine and GABA in periglomerular cells. *J. Neurophysiol.* **99**: 1559-1564.

83. Martel, G., Blanchard, J., Mons, N., Gastambide, F., Micheau, J., and Guillou, J.L. 2007. Dynamic interplays between memory systems depend on practice: the hippocampus is not always the first to provide solution. *Neuroscience* **150**: 743-753.

84. Mayr, B. and Montminy, M. 2001. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat. Rev. Mol. Cell Biol.* **2**: 599-609.

85. McLean, J.H. and Darby-King, A. 1994. Lack of evidence for serotonergic effect on the cellular development of the olfactory bulb in the postnatal rat. *Brain Res. Bull.* **34**: 249-259.

86. McLean, J.H., Darby-King, A., and Harley, C.W. 2005. Potentiation and prolongation of long-term odor memory in neonate rats using a phosphodiesterase inhibitor. *Neuroscience* **135**: 329-334.

87. McLean, J.H., Darby-King, A., and Hodge, E. 1996. 5-HT2 receptor involvement in conditioned olfactory learning in the neonate rat pup. *Behav. Neurosci.* **110**: 1426-1434.

88. McLean, J.H., Darby-King, A., and Paterno, G.D. 1995. Localization of 5-HT2A receptor mRNA by in situ hybridization in the olfactory bulb of the postnatal rat. *J. Comp Neurol.* **353**: 371-378.

89. McLean, J.H., Darby-King, A., Sullivan, R.M., and King, S.R. 1993. Serotonergic influence on olfactory learning in the neonate rat. *Behav. Neural Biol.* **60**: 152-162.

90. McLean, J.H. and Harley, C.W. 2004. Olfactory learning in the rat pup: a model that may permit visualization of a mammalian memory trace. *Neuroreport* **15**: 1691-1697.

91. McLean, J.H., Harley, C.W., Darby-King, A., and Yuan, Q. 1999. pCREB in the neonate rat olfactory bulb is selectively and transiently increased by odor preference-conditioned training. *Learn. Mem.* **6**: 608-618.

92. McLean JH, Powell M, Arsenault S, Darby-King A, Harley CW (2011) The synergistic effect of combining a phosphodiesterase inhibitor and histone deacetylase inhibitor on memory. Soc for Neurosci. 41:98.13.

93. McLean, J.H. and Shipley, M.T. 1987a. Serotonergic afferents to the rat olfactory bulb: I. Origins and laminar specificity of serotonergic inputs in the adult rat. *J. Neurosci.* **7**: 3016-3028.

94. McLean, J.H. and Shipley, M.T. 1987b. Serotonergic afferents to the rat olfactory bulb: II. Changes in fiber distribution during development. *J. Neurosci.* **7**: 3029-3039.

95. McLean, J.H. and Shipley, M.T. 1988. Postmitotic, postmigrational expression of tyrosine hydroxylase in olfactory bulb dopaminergic neurons. *J. Neurosci.* **8**: 3658-3669.

96. McLean, J.H. and Shipley, M.T. 1991. Postnatal development of the noradrenergic projection from locus coeruleus to the olfactory bulb in the rat. *J. Comp Neurol.* **304**: 467-477.

97. McLean, J.H., Shipley, M.T., Nickell, W.T., Aston-Jones, G., and Reyher, C.K. 1989. Chemoanatomical organization of the noradrenergic input from locus coeruleus to the olfactory bulb of the adult rat. *J. Comp Neurol.* **285**: 339-349.

98. McLean, J.H., Smith, A., Rogers, S., Clarke, K., Darby-King, A., and Harley, C.W. 2009. A phosphodiesterase inhibitor, cilomilast, enhances cAMP activity to restore conditioned odor preference memory after serotonergic depletion in the neonate rat. *Neurobiol. Learn. Mem.* **92**: 63-69.

99. McNulty, S.E., Barrett, R.M., Vogel-Ciernia, A., Malvaez, M., Hernandez, N., Davatolhagh, M.F., Matheos, D.P., Schiffman, A., and Wood, M.A. 2012. Differential roles for Nr4a1 and Nr4a2 in object location vs. object recognition long-term memory. *Learn. Mem.* **19**: 588-592.

100. McQuown, S.C., Barrett, R.M., Matheos, D.P., Post, R.J., Rogge, G.A., Alenghat, T., Mullican, S.E., Jones, S., Rusche, J.R., Lazar, M.A., and Wood, M.A. 2011. HDAC3 is a critical negative regulator of long-term memory formation. *J. Neurosci.* **31**: 764-774.

101. Meisami, E. and Safari, L. 1981. A quantitative study of the effects of early unilateral olfactory deprivation on the number and distribution of mitral and tufted cells and of glomeruli in the rat olfactory bulb. *Brain Res.* **221**: 81-107.

102. Merhav, M., Kuulmann-Vander, S., Elkobi, A., Jacobson-Pick, S., Karni, A., and Rosenblum, K. 2006. Behavioral interference and C/EBPbeta expression in the insular-cortex reveal a prolonged time period for taste memory consolidation. *Learn. Mem.* **13**: 571-574.

1023. Moriceau, S. and Sullivan, R.M. 2005. Neurobiology of infant attachment. *Dev. Psychobiol.* **47**: 230-242.

104. Nakamura, H. and O'Leary, D.D. 1989. Inaccuracies in initial growth and arborization of chick retinotectal axons followed by course corrections and axon remodeling to develop topographic order. *J. Neurosci.* **9**: 3776-3795.

105. Nickell, W.T. and Shipley, M.T. 1988. Two anatomically specific classes of candidate cholinoceptive neurons in the rat olfactory bulb. *J. Neurosci.* **8**: 4482-4491.

106. O'Brien, R., Xu, D., Mi, R., Tang, X., Hopf, C., and Worley, P. 2002. Synaptically targeted narp plays an essential role in the aggregation of AMPA receptors at excitatory synapses in cultured spinal neurons. *J. Neurosci.* **22**: 4487-4498.

107. Okutani, F., Yagi, F., and Kaba, H. 1999. Gabaergic control of olfactory learning in young rats. *Neuroscience* **93**: 1297-1300.

108. Parvez, K., Stewart, O., Sangha, S., and Lukowiak, K. 2005. Boosting intermediate-term into long-term memory. *J. Exp. Biol.* **208**: 1525-1536.

109. Pinching, A.J. and Powell, T.P. 1971a. The neuron types of the glomerular layer of the olfactory bulb. *J. Cell Sci.* **9**: 305-345.

110. Pinching, A.J. and Powell, T.P. 1971b. The neuropil of the glomeruli of the olfactory bulb. *J. Cell Sci.* **9**: 347-377.

111. Pinching, A.J. and Powell, T.P. 1971c. The neuropil of the periglomerular region of the olfactory bulb. *J. Cell Sci.* **9**: 379-409.

112. Prasad, B.C. and Reed, R.R. 1999. Chemosensation: molecular mechanisms in worms and mammals. *Trends Genet.* **15**: 150-153.

113. Price, J.L. and Powell, T.P. 1970. The mitral and short axon cells of the olfactory bulb. *J. Cell Sci.* **7**: 631-651.

114. Puopolo, M. and Belluzzi, O. 1998. Functional heterogeneity of periglomerular cells in the rat olfactory bulb. *Eur. J. Neurosci.* **10**: 1073-1083.

115. Raineki, C., Pickenhagen, A., Roth, T.L., Babstock, D.M., McLean, J.H., Harley, C.W., Lucion, A.B., and Sullivan, R.M. 2010. The neurobiology of infant maternal odor learning. *Braz. J. Med. Biol. Res.* **43**: 914-919.

116. Ramji, D.P. and Foka, P. 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem. J.* **365**: 561-575.

117. Rao, V.R. and Finkbeiner, S. 2007. NMDA and AMPA receptors: old channels, new tricks. *Trends Neurosci.* **30**: 284-291.

118. Rapanelli, M., Frick, L.R., and Zanutto, B.S. 2009. Differential gene expression in the rat hippocampus during learning of an operant conditioning task. *Neuroscience* **163**: 1031-1038.

119. Reed, R.R. 1992. Signaling pathways in odorant detection. *Neuron* **8**: 205-209.

120. Reisert, J., Lai, J., Yau, K.W., and Bradley, J. 2005. Mechanism of the excitatory Cl- response in mouse olfactory receptor neurons. *Neuron* **45**: 553-561.

121. Reyher, C.K., Lubke, J., Larsen, W.J., Hendrix, G.M., Shipley, M.T., and Baumgarten, H.G. 1991. Olfactory bulb granule cell aggregates: morphological evidence for interperikaryal electrotonic coupling via gap junctions. *J. Neurosci.* **11**: 1485-1495.

122. Ribak, C.E., Vaughn, J.E., Saito, K., Barber, R., and Roberts, E. 1977. Glutamate decarboxylase localization in neurons of the olfactory bulb. *Brain Res.* **126**: 1-18.

123. Roth, T.L., Raineki, C., Salstein, L., Perry, R., Sullivan-Wilson, T.A., Sloan, A., Lalji, B., Hammock, E., Wilson, D.A., Levitt, P., Okutani, F., Kaba, H., and Sullivan, R.M. 2013. Neurobiology of secure infant attachment and attachment despite adversity: A mouse model. *Genes Brain Behav.*

124. Sassone-Corsi, P. 2012. The cyclic AMP pathway. *Cold Spring Harb. Perspect. Biol.* **4**.

125. Schneiderman, N., Fuentes, I., and Gormezano, I. 1962. Acquisition and extinction of the classically conditioned eyelid response in the albino rabbit. *Science* **136**: 650-652.

126. Schulz, S., Siemer, H., Krug, M., and Hollt, V. 1999. Direct evidence for biphasic cAMP responsive element-binding protein phosphorylation during long-term potentiation in the rat dentate gyrus in vivo. *J. Neurosci.* **19**: 5683-5692.

127. Shakhawat, A.M., Harley, C.W., and Yuan, Q. 2012. Olfactory bulb alpha2-adrenoceptor activation promotes rat pup odor-preference learning via a cAMP-independent mechanism. *Learn. Mem.* **19**: 499-502.

128. Shipley, M.T. and Ennis, M. 1996. Functional organization of olfactory system. *J. Neurobiol.* **30**: 123-176.

129. Shipley, M.T., Halloran, F.J., and de la Torre, J. 1985. Surprisingly rich projection from locus coeruleus to the olfactory bulb in the rat. *Brain Res.* **329**: 294-299.

130. Silva, A.J., Kogan, J.H., Frankland, P.W., and Kida, S. 1998. CREB and memory. *Annu. Rev. Neurosci.* **21**: 127-148.

131. Soule, J., Penke, Z., Kanhema, T., Alme, M.N., Laroche, S., and Bramham, C.R. 2008. Object-place recognition learning triggers rapid induction of plasticity-related immediate early genes and synaptic proteins in the rat dentate gyrus. *Neural Plast.* **2008**: 269097.

132. Stanciu, M., Radulovic, J., and Spiess, J. 2001. Phosphorylated cAMP response element binding protein in the mouse brain after fear conditioning: relationship to Fos production. *Brain Res. Mol. Brain Res.* **94**: 15-24.

133. Sullivan, R.M. 2003. Developing a sense of safety: the neurobiology of neonatal attachment. *Ann. N. Y. Acad. Sci.* **1008**: 122-131.

134. Sullivan, R.M., Landers, M., Yeaman, B., and Wilson, D.A. 2000a. Good memories of bad events in infancy. *Nature* **407**: 38-39.

135. Sullivan, R.M. and Leon, M. 1986. Early olfactory learning induces an enhanced olfactory bulb response in young rats. *Brain Res.* **392**: 278-282.

136. Sullivan, R.M., McGaugh, J.L., and Leon, M. 1991a. Norepinephrine-induced plasticity and one-trial olfactory learning in neonatal rats. *Brain Res. Dev. Brain Res.* **60**: 219-228.

137. Sullivan, R.M., Stackenwalt, G., Nasr, F., Lemon, C., and Wilson, D.A. 2000b. Association of an odor with activation of olfactory bulb noradrenergic beta-receptors or locus coeruleus stimulation is sufficient to produce learned approach responses to that odor in neonatal rats. *Behav. Neurosci.* **114**: 957-962.

138. Sullivan, R.M., Taborsky-Barba, S., Mendoza, R., Itano, A., Leon, M., Cotman, C.W., Payne, T.F., and Lott, I. 1991b. Olfactory classical conditioning in neonates. *Pediatrics* **87**: 511-518.

139. Sullivan, R.M., Wilson, D.A., and Leon, M. 1989. Norepinephrine and learning-induced plasticity in infant rat olfactory system. *J. Neurosci.* **9**: 3998-4006.

140. Sutherland, E.W. and Rall, T.W. 1958. Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J. Biol. Chem.* **232**: 1077-1091.

141. Tang, Y.P., Shimizu, E., Dube, G.R., Rampon, C., Kerchner, G.A., Zhuo, M., Liu, G., and Tsien, J.Z. 1999. Genetic enhancement of learning and memory in mice. *Nature* **401**: 63-69.

142. Taubenfeld, S.M., Milekic, M.H., Monti, B., and Alberini, C.M. 2001a. The consolidation of new but not reactivated memory requires hippocampal C/EBPbeta. *Nat. Neurosci.* **4**: 813-818.

143. Taubenfeld, S.M., Wiig, K.A., Monti, B., Dolan, B., Pollonini, G., and Alberini, C.M. 2001b. Fornix-dependent induction of hippocampal CCAAT enhancer-binding protein [beta] and [delta] Co-localizes with phosphorylated cAMP response element-binding protein and accompanies long-term memory consolidation. *J. Neurosci.* **21**: 84-91.

144. Tomobe, K., Okuma, Y., and Nomura, Y. 2007. Impairment of CREB phosphorylation in the hippocampal CA1 region of the senescence-accelerated mouse (SAM) P8. *Brain Res.* **1141**: 214-217.

145. Trifilieff, P., Herry, C., Vanhoutte, P., Caboche, J., Desmedt, A., Riedel, G., Mons, N., and Micheau, J. 2006. Foreground contextual fear memory consolidation requires two independent phases of hippocampal ERK/CREB activation. *Learn. Mem.* **13**: 349-358.

146. Vecsey, C.G., Hawk, J.D., Lattal, K.M., Stein, J.M., Fabian, S.A., Attner, M.A., Cabrera, S.M., McDonough, C.B., Brindle, P.K., Abel, T., and Wood, M.A. 2007. Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. *J. Neurosci.* **27**: 6128-6140.

147. Volakakis, N., Kadkhodaei, B., Joodmardi, E., Wallis, K., Panman, L., Silvaggi, J., Spiegelman, B.M., and Perlmann, T. 2010. NR4A orphan nuclear receptors as mediators of CREB-dependent neuroprotection. *Proc. Natl. Acad. Sci. U. S. A* **107**: 12317-12322.

148. Wachowiak, M. and Shipley, M.T. 2006. Coding and synaptic processing of sensory information in the glomerular layer of the olfactory bulb. *Semin. Cell Dev. Biol.* **17**: 411-423.

149. Wang, Y.J., Okutani, F., Murata, Y., Taniguchi, M., Namba, T., and Kaba, H. 2012. Histone acetylation in the olfactory bulb of young rats facilitates aversive olfactory learning and synaptic plasticity. *Neuroscience* **232C**: 21-31.

150. Wei, W., Coelho, C.M., Li, X., Marek, R., Yan, S., Anderson, S., Meyers, D., Mukherjee, C., Sbardella, G., Castellano, S., Milite, C., Rotili, D., Mai, A., Cole, P.A., Sah, P., Kobor, M.S., and Bredy, T.W. 2012. p300/CBP-associated factor selectively regulates the extinction of conditioned fear. *J. Neurosci.* **32**: 11930-11941.

151. Weldon, D.A., Travis, M.L., and Kennedy, D.A. 1991. Posttraining D1 receptor blockade impairs odor conditioning in neonatal rats. *Behav. Neurosci.* **105**: 450-458.

152. West, A.E., Chen, W.G., Dalva, M.B., Dolmetsch, R.E., Kornhauser, J.M., Shaywitz, A.J., Takasu, M.A., Tao, X., and Greenberg, M.E. 2001. Calcium regulation of neuronal gene expression. *Proc. Natl. Acad. Sci. U. S. A* **98**: 11024-11031.

153. Wilson, D.A., Fletcher, M.L., and Sullivan, R.M. 2004. Acetylcholine and olfactory perceptual learning. *Learn. Mem.* **11**: 28-34.

154. Woo, C.C. and Leon, M. 1995. Distribution and development of beta-adrenergic receptors in the rat olfactory bulb. *J. Comp Neurol.* **352**: 1-10.

155. Young, J.M. and Trask, B.J. 2002. The sense of smell: genomics of vertebrate odorant receptors. *Hum. Mol. Genet.* **11**: 1153-1160.

156. Yuan, Q., Harley, C.W., Bruce, J.C., Darby-King, A., and McLean, J.H. 2000. Isoproterenol increases CREB phosphorylation and olfactory nerve-evoked potentials in normal and 5-HT-depleted olfactory bulbs in rat pups only at doses that produce odor preference learning. *Learn. Mem.* **7**: 413-421.

157. Yuan, Q., Harley, C.W., Darby-King, A., Neve, R.L., and McLean, J.H. 2003a. Early odor preference learning in the rat: bidirectional effects of cAMP response element-binding protein (CREB) and mutant CREB support a causal role for phosphorylated CREB. *J. Neurosci.* **23**: 4760-4765.

158. Yuan, Q., Harley, C.W., and McLean, J.H. 2003b. Mitral cell beta1 and 5-HT2A receptor colocalization and cAMP coregulation: a new model of norepinephrine-induced learning in the olfactory bulb. *Learn. Mem.* **10**: 5-15.

159. Yuan, Q., Harley, C.W., McLean, J.H., and Knopfel, T. 2002. Optical imaging of odor preference memory in the rat olfactory bulb. *J. Neurophysiol.* **87**: 3156-3159.

160. Yudowski, G.A., Olsen, O., Adesnik, H., Marek, K.W., and Bredt, D.S. 2013. Acute inactivation of PSD-95 destabilizes AMPA receptors at hippocampal synapses. *PLoS. One.* **8**: e53965.

161. Yukawa, K., Tanaka, T., Tsuji, S., and Akira, S. 1998. Expressions of CCAAT/Enhancer-binding proteins beta and delta and their activities are intensified by cAMP signaling as well as Ca2+/calmodulin kinases activation in hippocampal neurons. *J. Biol. Chem.* **273**: 31345-31351.

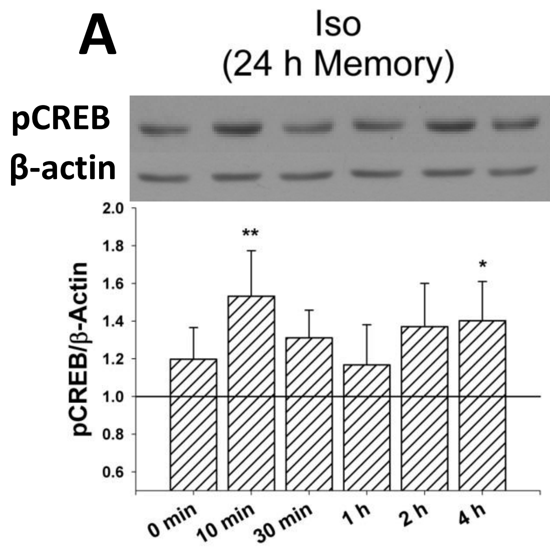
162. Zhang, X., Odom, D.T., Koo, S.H., Conkright, M.D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E., Kadam, S., Ecker, J.R., Emerson, B., Hogenesch, J.B., Unterman, T., Young, R.A., and Montminy, M. 2005. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proc. Natl. Acad. Sci. U. S. A* **102**: 4459-4464.

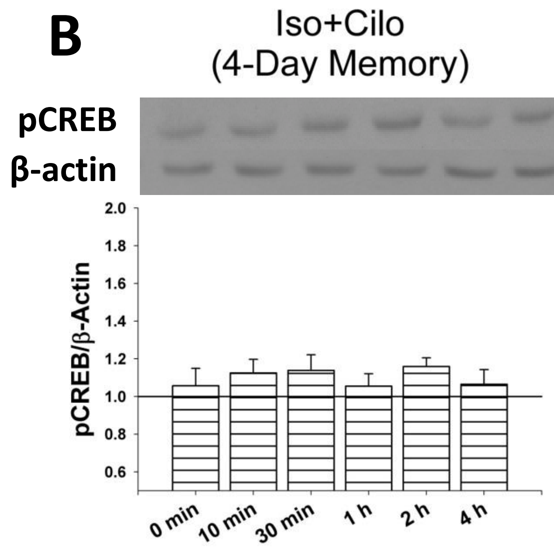
163. Zhao, Y. and Bruemmer, D. 2010. NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology. *Arterioscler. Thromb. Vasc. Biol.* **30**: 1535-1541.

GRAPHICAL RESULTS

Figure 1. Western blotting pCREB profile

Western of pCREB levels showing the average optical density of pCREB/β-actin (Mean±SEM) in the OB of PD 6 rat pups for 6 time points post-conditioning. All data are normalized to saline+odor (0 min post-conditioning) controls and are represented as a horizontal line. Representative densitometry bands for pCREB and β-Actin are at the top. ***A*** Time course analysis of pCREB/β-actin in rat pups trained using 24 h memory (2 mg/kg isoproterenol) paradigm reveals a biphasic increase in pCREB at 10 min ($p<.01$) and 2 h ($p<0.05$, N=9 pairs of OBs per condition; Bottom). ***B*** Time course analysis of pCREB/β-actin in rat pups trained using 4-Day memory (1 mg/kg isoproterenol + 3 mg/kg cilomilast) paradigm reveals no significant changes relative to saline+odor controls ($p= 0.298$, N=14 pairs of OBs per condition; Bottom). ***C*** Time course analysis of pCREB/β-actin in rat pups trained using 5-Day memory (1 mg/kg isoproterenol + 3 mg/kg cilomilast + 1.2 g/kg NaB) paradigm reveals significant increases at 10 min ($p<0.01$), 30 min ($p<0.05$) and 2 h ($p<0.05$) post-conditioning (N=17 pairs of OBs per condition; Bottom) compared to saline+odor controls.


A
Iso
(24 h Memory)
pCREB
β-actin
pCREB/β-Actin
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
**
*
0 min
10 min
30 min
1 h
2 h
4 h


B
Iso+Cilo
(4-Day Memory)
pCREB
β-actin
pCREB/β-Actin
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0 min
10 min
30 min
1 h
2 h
4 h

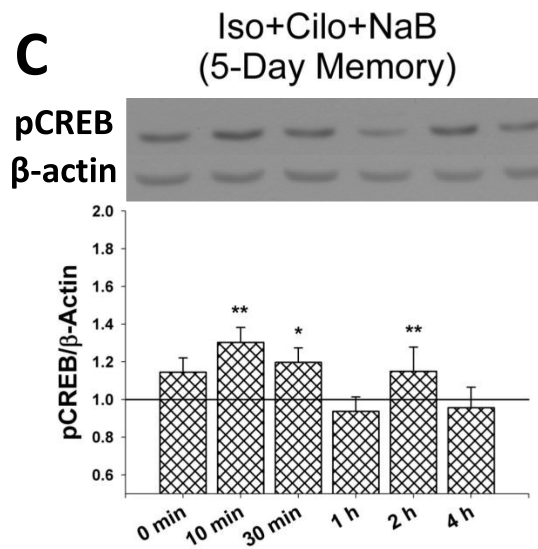
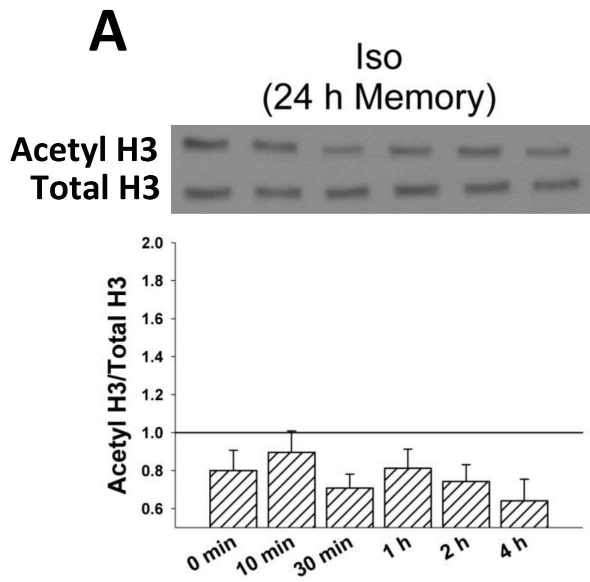
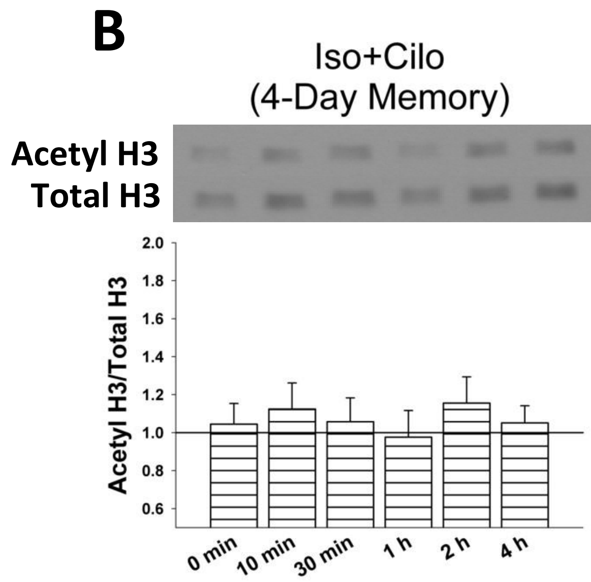

C
Iso+Cilo+NaB
(5-Day Memory)
pCREB
β-actin
pCREB/β-Actin
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
**
*
**
0 min
10 min
30 min
1 h
2 h
4 h

Figure 2. Western blotting Acetyl H3 profile

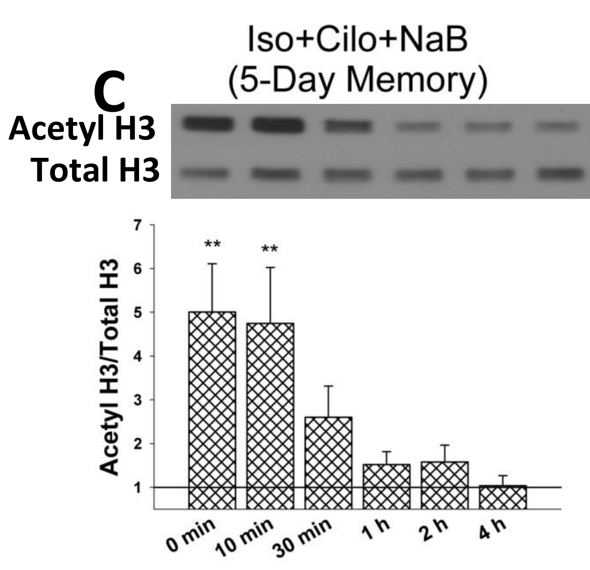
Westerns of acetyl H3 levels show the average optical density of Acetyl H3/ Total H3 (Mean±SEM) in the OB of PD 6 rat pups for 6 time points post-conditioning. All data are normalized to saline+odor controls (0 min post-conditioning) represented as a horizontal line. Representative densitometry bands for Acetyl H3 and Total H3 are at the top. ***A*** Time course analysis of Acetyl H3/ Total H3 in rat pups trained using 24 h memory (2 mg/kg iso) paradigm reveal no significant changes relative to controls ($p = 0.135$, N=4 pairs of OBs per condition; Bottom). ***B*** Time course analysis of Acetyl H3/ Total H3 in rat pups trained using 4-Day memory (1 mg/kg iso + 3 mg/kg cilomilast) paradigm show no significant changes relative to controls ($p = 0.297$, N=4 pairs of OBs per condition; Bottom). ***C*** Time course analysis of Acetyl H3/ Total H3 in rat pups trained using 5-Day memory (1 mg/kg iso + 3 mg/kg cilomilast + 1.2 g/kg NaB) paradigm reveal significant increases at 0 min ($p<0.01$) and 10 min ($p<0.01$) post-conditioning (N=4 pairs of OBs per condition; Bottom). ***D*** Time course analysis of Acetyl H3/ Total H3 in non-learning NaB controls (1.2 g/kg NaB) reveal significant increases in Acetyl H3 at 0 min ($p<0.01$), 10 min ($p<0.01$) and 30 min ($p<0.01$) post-conditioning (N=4 pairs of OBs per condition; Bottom) compared to saline+odor controls.



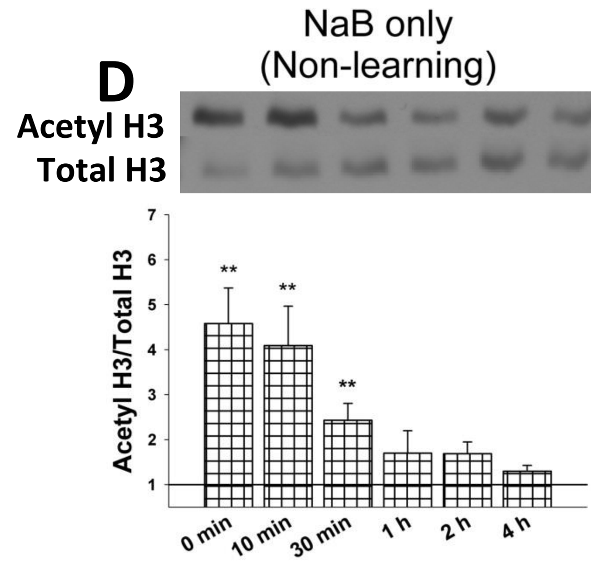
A
Iso
(24 h Memory)
Acetyl H3
Total H3
Acetyl H3/Total H3
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0 min
10 min
30 min
1 h
2 h
4 h



B
Iso+Cilo
(4-Day Memory)
Acetyl H3
Total H3
Acetyl H3/Total H3
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0 min
10 min
30 min
1 h
2 h
4 h



C
Iso+Cilo+NaB
(5-Day Memory)
Acetyl H3
Total H3
Acetyl H3/Total H3
7
6
5
4
3
2
1
**
**
0 min
10 min
30 min
1 h
2 h
4 h



D
NaB only
(Non-learning)
Acetyl H3
Total H3
Acetyl H3/Total H3
7
6
5
4
3
2
1
**
**
**
0 min
10 min
30 min
1 h
2 h
4 h

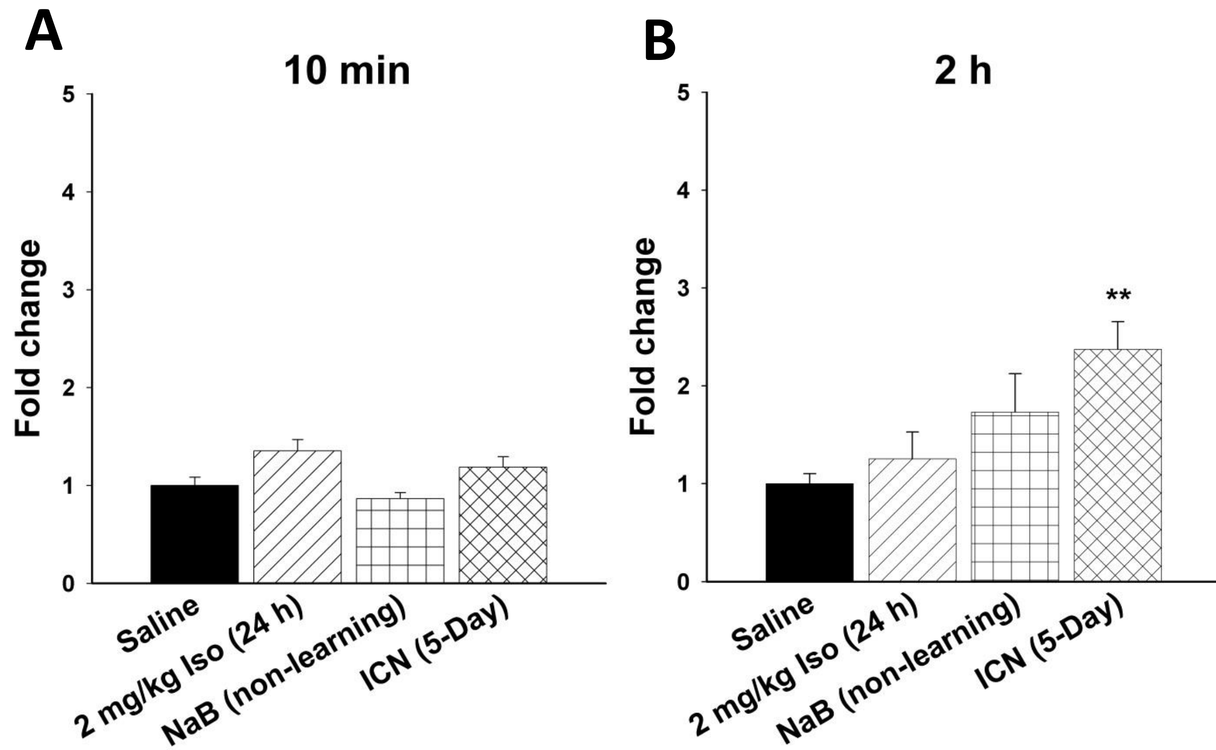


Figure 3. q-PCR relative fold change of Nr4a1 gene

q-PCR relative fold change of *Nr4a1* gene (Mean$\pm$SEM) in the OBs of PD6 rat pups (N=4 experiments). *Nr4a1* expression at 10 min yielded no significant changes in gene expression ($p>0.05$, ***A***). Significant increase in N*r4a1* gene expression was found in 5-Day memory group 2 hr post-conditioning ($p<0.01$, ***B***).

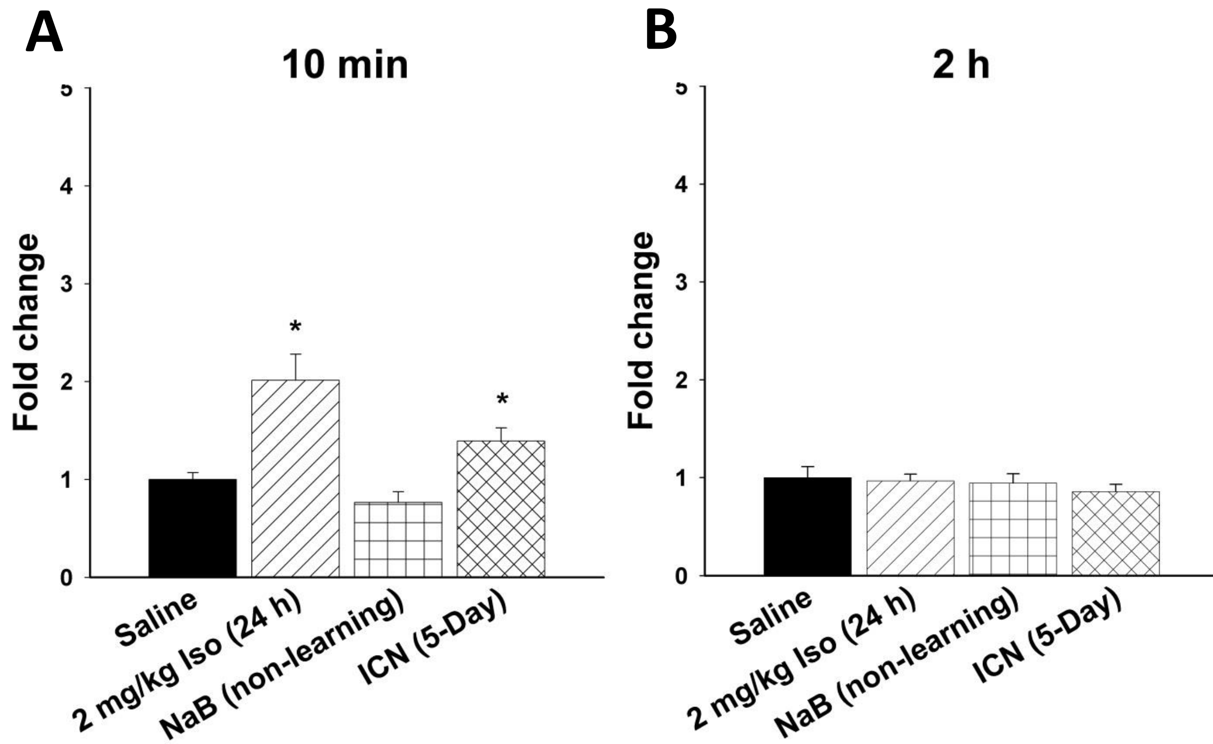


Figure 4. q-PCR relative fold change of Nr4a2 gene

q-PCR relative fold change of *Nr4a2* gene (Mean±SEM) in the OBs of PD6 rat pups (N=4 experiments). *Nr4a2* gene expression was significantly increased in both 24 hr (p=0.01) and 5-Day (p<0.05) learning groups 10 min post-conditioning (***A***). No significant increase in *Nr4a2* gene expression was found 2 hr post-conditioning (p>0.05, ***B***).

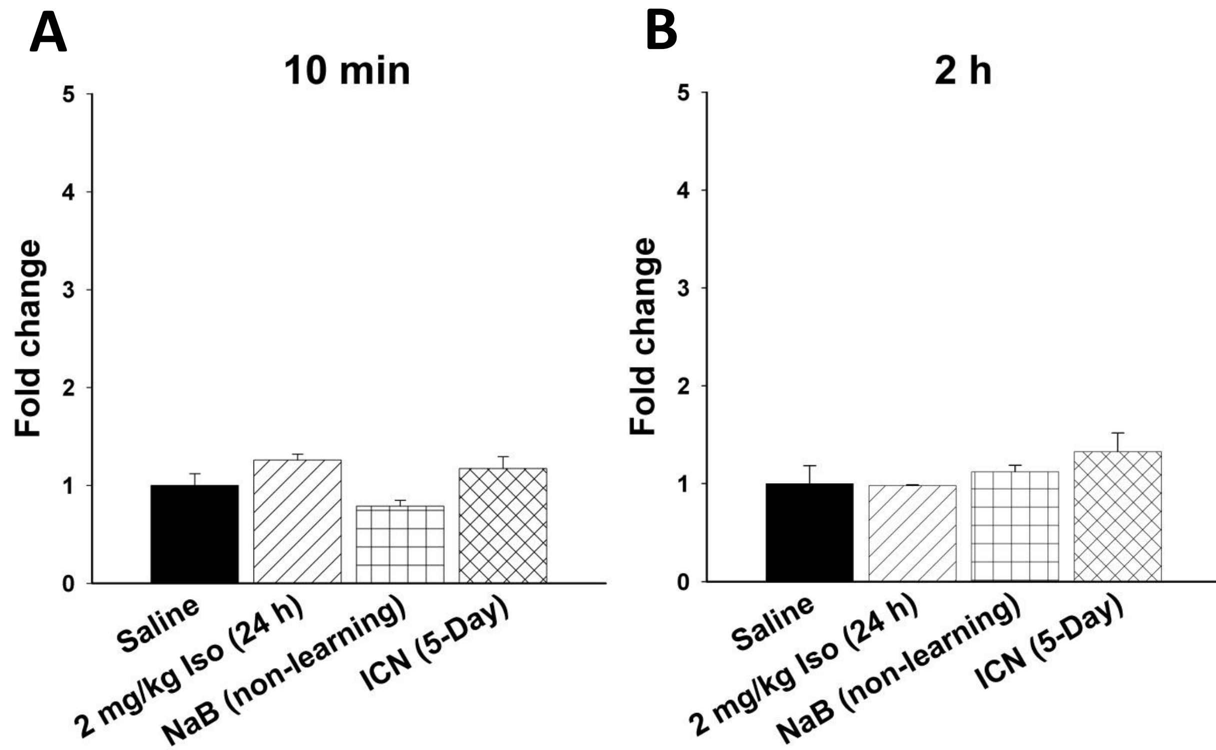


Figure 5. q-PCR relative fold change of Nr4a3 gene

q-PCR relative fold change of *Nr4a3* gene (Mean±SEM) in the OBs of PD6 rat pups (N=4 experiments). *Nr4a3* gene expression showed no significant increases in both 24 hr ($p>0.05$) and 5-Day ($p>0.05$) learning groups 10 min post-conditioning (***A***). No significant increase in *Nr4a3* gene expression was found 2 hr post-conditioning ($p>0.05$, ***B***).

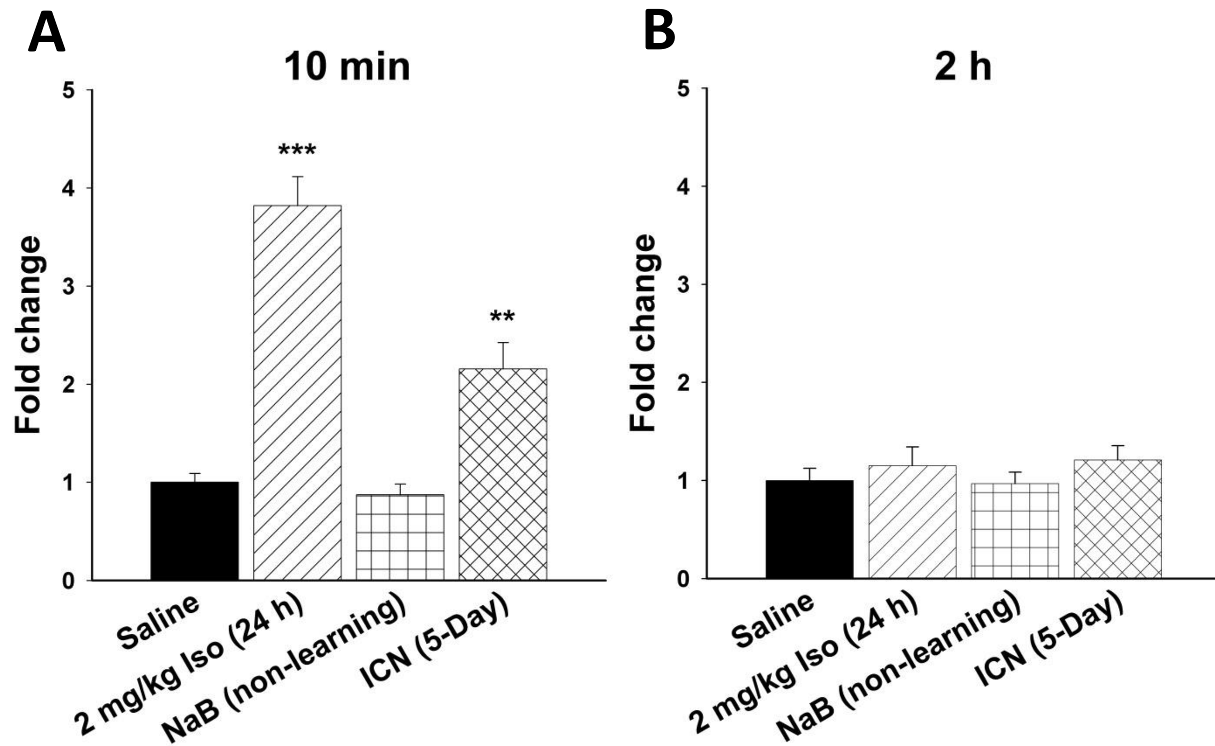


Figure 6. q-PCR relative fold change of C/EBPβ gene

q-PCR relative fold change of *C/EBP*$_{\beta}$ gene (Mean±SEM) in the OBs of PD6 rat pups (N=4 experiments). *C/EBP*$_{\beta}$ gene expression was significantly increased compared to controls in both 24 hr ($p<0.0001$) and 5-Day ($p<0.01$) learning groups 10 min post-conditioning (***A***). No significant increase in *C/EBP*$_{\beta}$ gene expression was found 2 hr post-conditioning ($p>0.05$, ***B***).

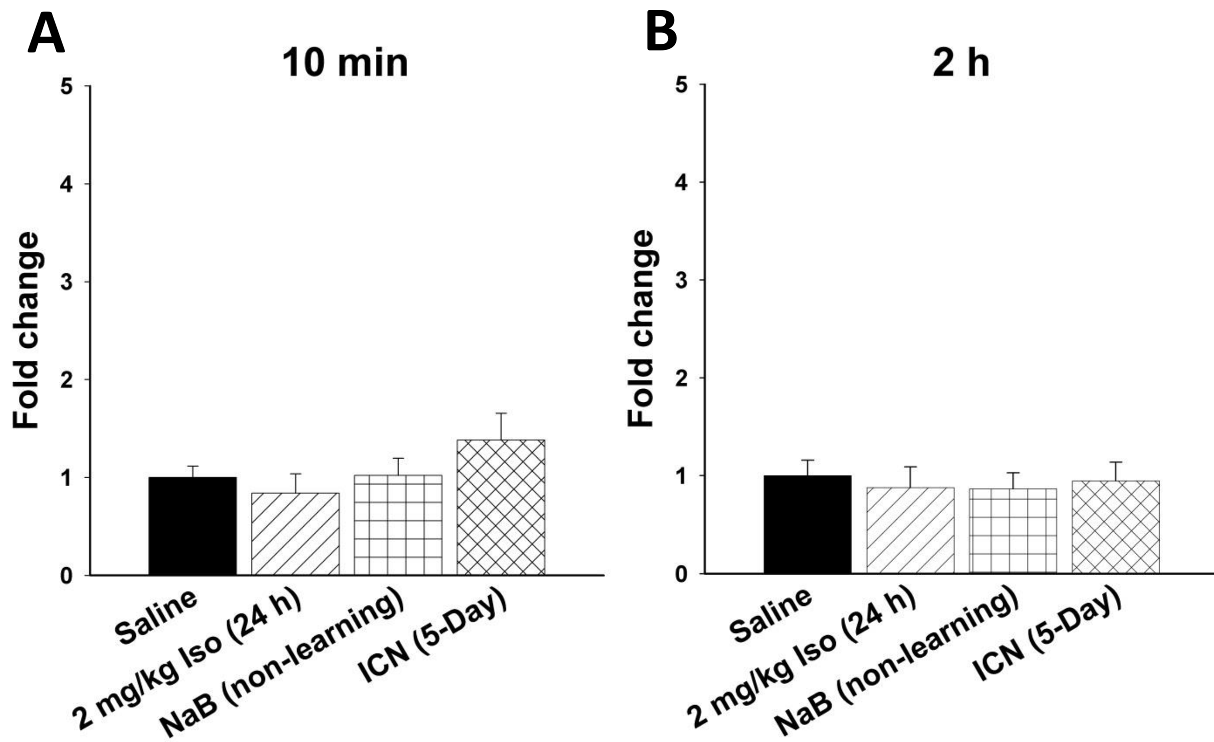


Figure 7. q-PCR relative fold change of Jun gene

q-PCR relative fold change of *Jun* gene (Mean±SEM) in the OBs of PD6 rat pups (N=4 experiments). *Jun* gene expression did not significantly differ from controls (p>0.05) 10 min post-conditioning (***A***). No significant increase in *Jun* gene expression was found 2 hr post-conditioning (p>0.05, ***B***).

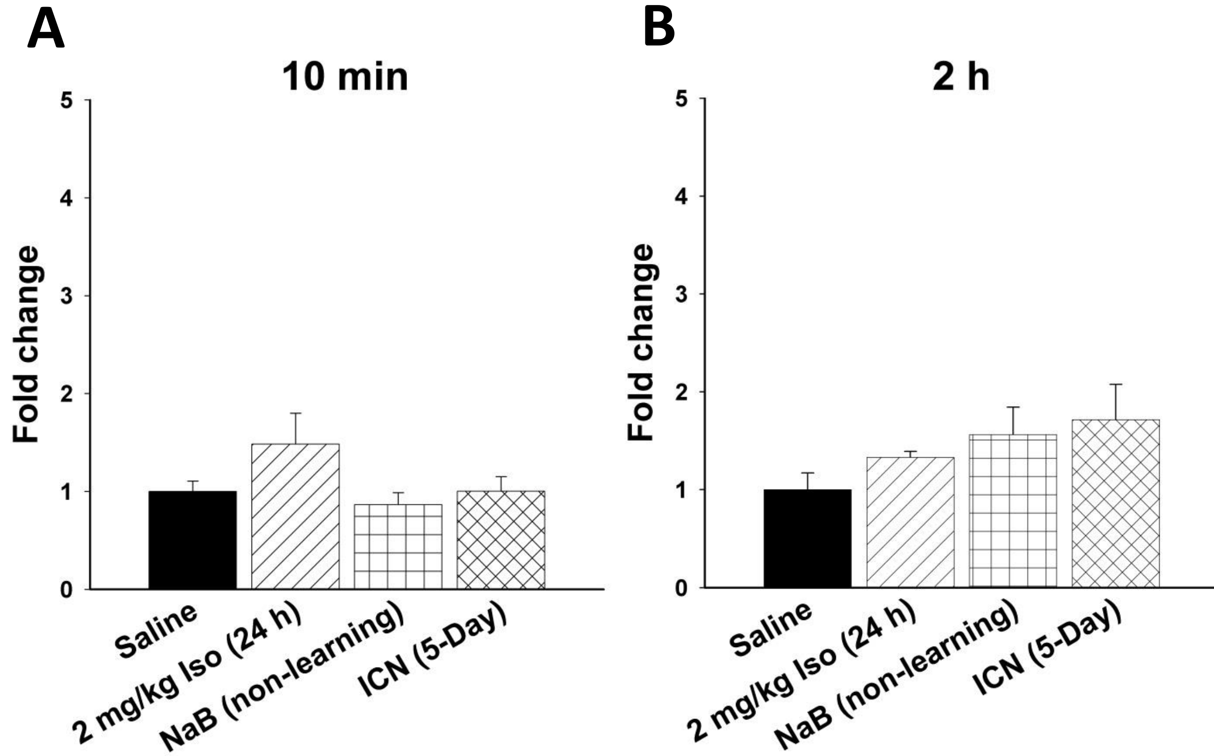


Figure 8. q-PCR relative fold change of Fos gene

q-PCR relative fold change of *Fos* gene (Mean$\pm$SEM) in the OBs of PD6 rat pups (N=4 experiments). *Fos* gene expression did not significantly differ from controls ($p>0.05$) 10 min post-conditioning (***A***). No significant increase in *Fos* gene expression was found 2 hr post-conditioning ($p>0.05$, ***B***).

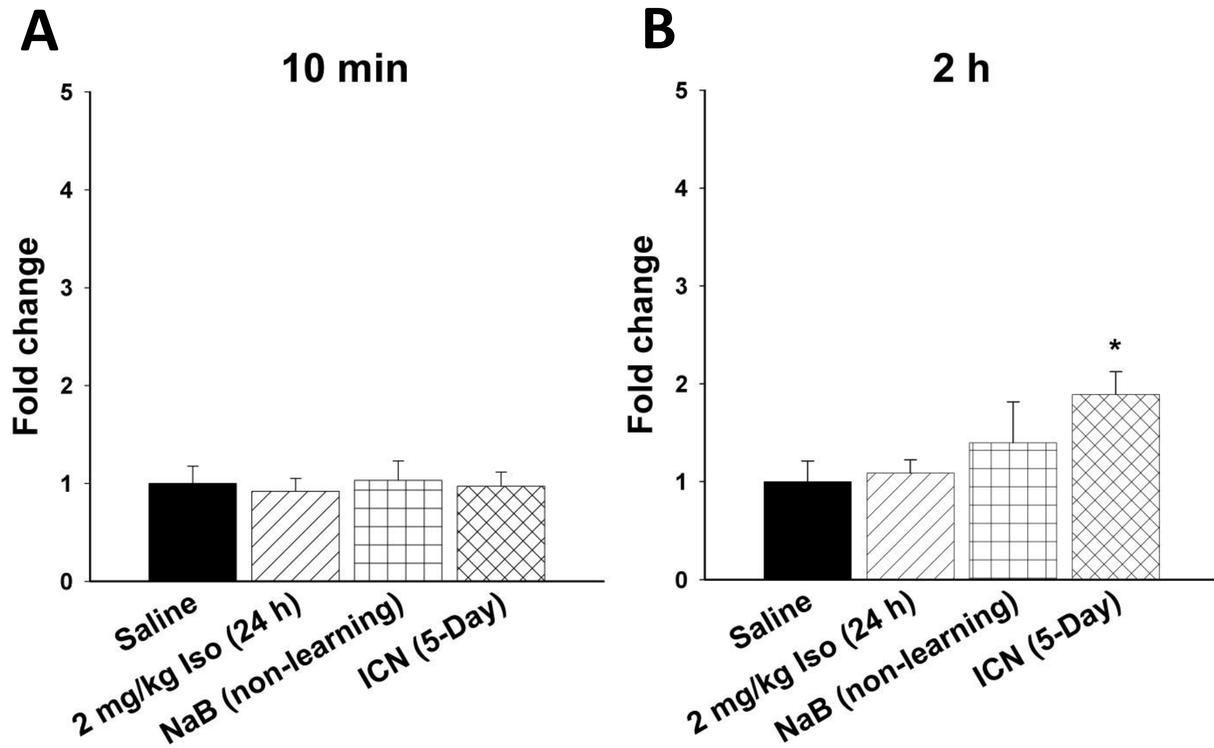


Figure 9. q-PCR relative fold change of Egr1 gene

q-PCR relative fold change of *Egr1* gene (Mean±SEM) in the OBs of PD6 rat pups (N=4 experiments). *Egr1* gene expression did not significantly differ from controls ($p>0.05$) 10 min post-conditioning (***A***). Significant increase in *Egr1* gene expression was found in 5-Day learning group 2 hr post-conditioning ($p<0.05$, ***B***).

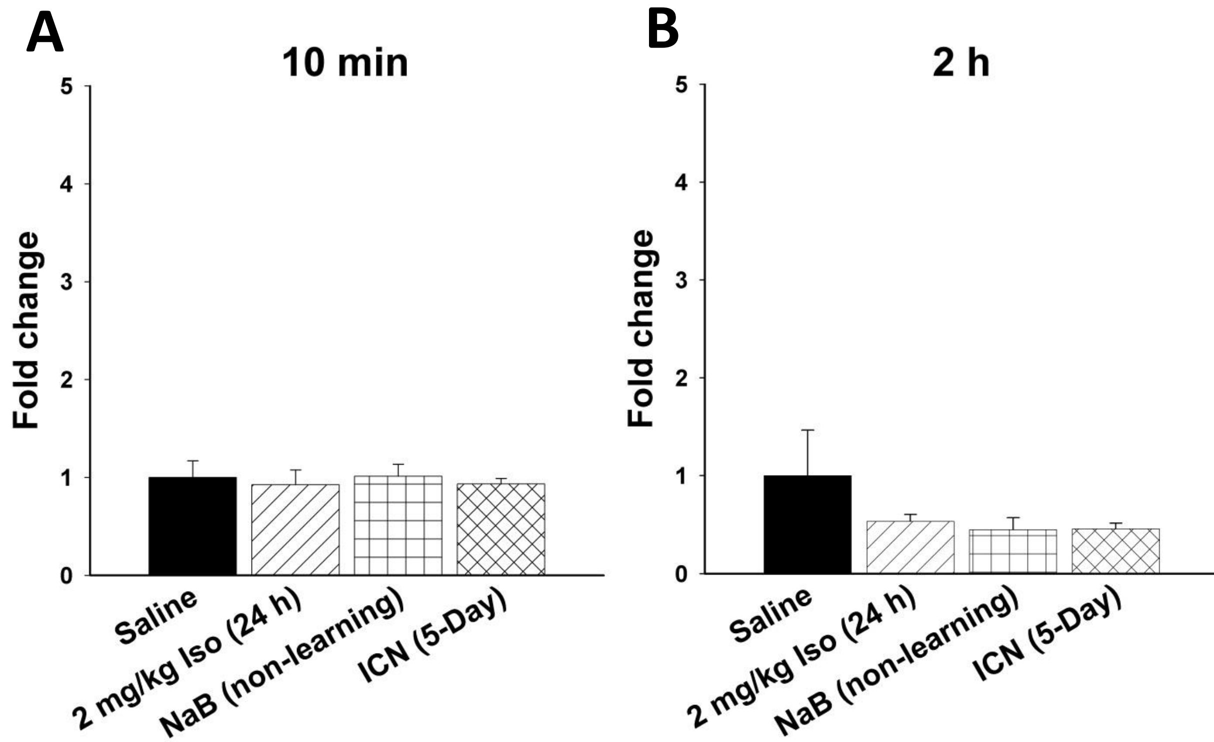


Figure 10. q-PCR relative fold change of BDNF gene

q-PCR relative fold change of *BDNF* gene (Mean±SEM) in the OBs of PD6 rat pups (N=4 experiments). *BDNF* gene expression did not significantly differ from controls (p>0.05) 10 min post-conditioning (***A***). No significant changes in *BDNF* gene expression was found 2 hr post-conditioning (p>0.05, ***B***).

APPENDIX A: Layers of the Olfactory Bulb

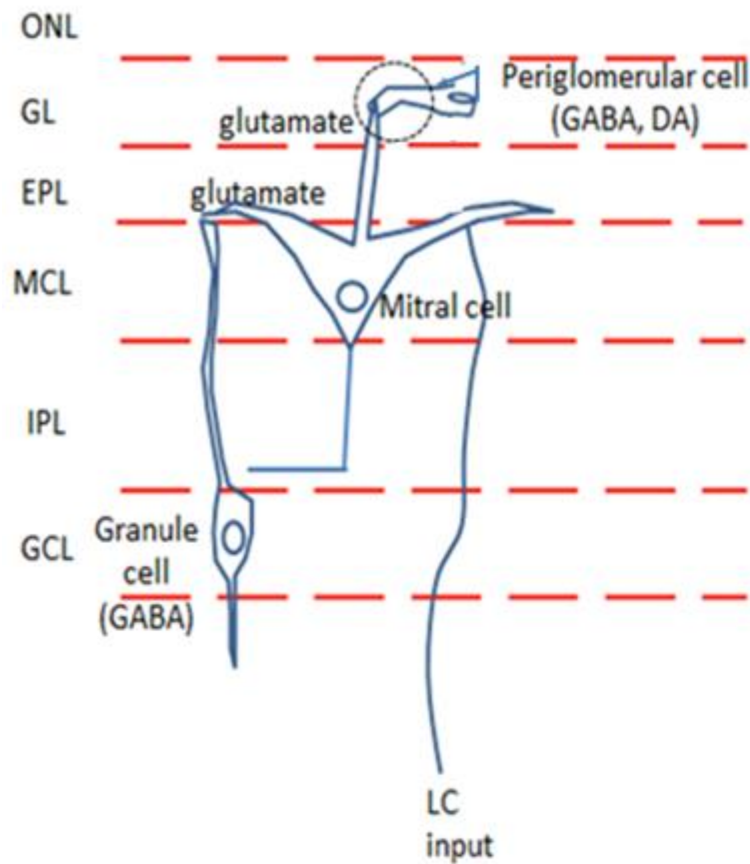


Figure 11. Diagram depicting the layers of the OB. The most superficial layer is the olfactory nerve layer (ONL). Deep to the ONL is the glomerular layer (GL) followed by the external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL) with the deepest layer being the granule cell layer (GCL).

APPENDIX B: 5-Day Odor Preference

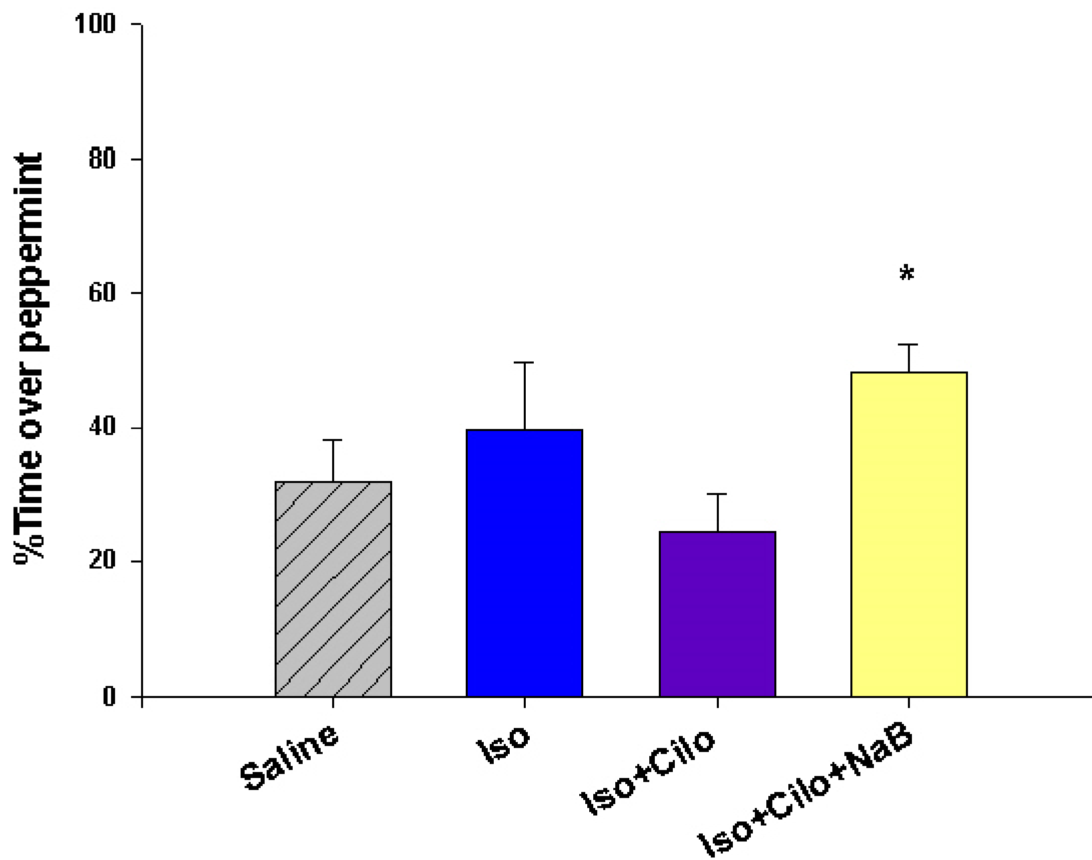


Figure 12. 5-Day Early Odor Preference. Percent of time spent over peppermint scented bedding during two-odor choice test 5 days after early odor preference training. Animals were trained on PD6 and tested on PD11. Preference for peppermint odor demonstrated by approach behaviour toward peppermint scented bedding. Significant odor preference 5 days after odor conditioning was observed in Iso+Cilo+NaB animals ($p<.05$, N=10) compared to Iso (N=6), Iso+Cilo (N=12) and saline (N=12) controls. (Results obtained from unpublished data from Dr. John McLean and Andrea Darby-King).

APPENDIX C: Role and function of pharmacological agents used for 24 h, 4-Day and 5-Day olfactory memory models

Drug	Function		Dosage	24 h memory	4-Day memory	5-Day memory
Isoproterenol (Iso)	β-adrenoceptor agonist	Increase cAMP	1 mg/kg or 2 mg/kg	2 mg/kg	1 mg/kg	1 mg/kg
Cilomilast (Cilo)	Phosphodiesterase inhibitor	Prevent breakdown of cAMP	3 mg/kg	X	3 mg/kg	3 mg/kg
Sodium butyrate (NaB)	HDAC inhibitor	Prevents deacetylation of histones	1.2 g/kg	X	X	1.2 g/kg

APPENDIX D: Cilomilast concentration adjustment sample equation

Aliquoting Cilomilast (5mg powder)

Want: Dilution of 1 mg/ml (5 mg/5000 µl)

$$X_1/50\ \mu l = 5\ mg/5000\ \mu l$$

$$\text{Solve for } X_1 = 0.05\ mg$$

Dosage of cilomilast = 3 mg/kg

$$3\ mg/1000\ g = 0.05\ mg/X_2$$

$$\text{Solve for } X_2 = 16.7\ g$$

A dilution of 1 mg/ml is appropriate for a 16.7 g animal receiving 3 mg/kg dosage of cilomilast.

- 5 ml total volume of 5% DMSO in saline

    50 µl 100% DMSO X 5 = 250 µl 100% DMSO

    950 µl Saline X 5 = 4750 µl Saline

Cilomilast dissolves best in 100% DMSO

Add 250 µl 100% DMSO then 750 µl Saline to tube (spin down before adding)

Vortex

Centrifuge

Repeat as necessary

Aliquot cilomilast into 350 µl volumes and stored at -80°C.

<u>Determining appropriate dilution of Cilomilast from aliquots on day of use</u>

If mean weight of the litter is less than 16.7 g you will need to dilute stored aliquot of Cilomilast.

<u>Sample calculation</u>:

M= 13.67 g

3 mg/1000 g = X/13.67 g

Solve for X = 0.041 mg

5 mg/5000 µl = 0.041 mg/X

Solve for X = 41 µl

Each pup receives 50 µl s.c. injection

41 µl stored Cilo + 9 µl 5% DMSO in Saline = 50 µl

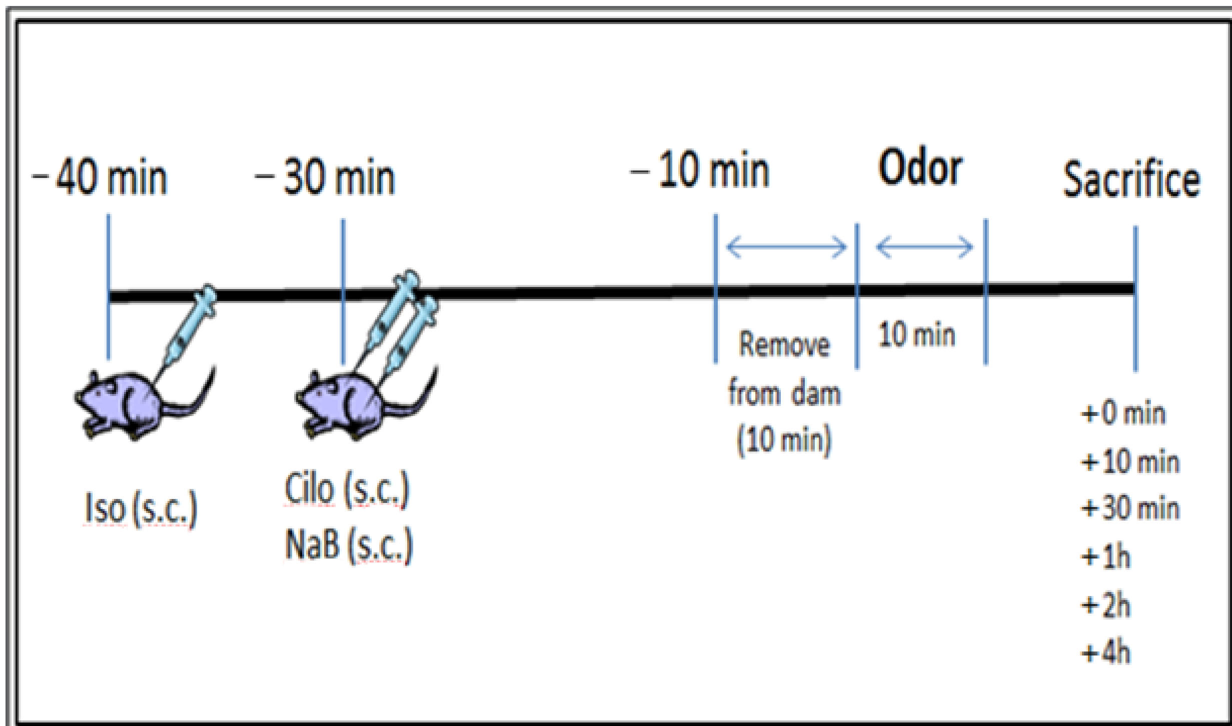
Calculation for 7 animals:

41 µl Cilo x 7 animals = 287 µl

9 µl 5% DMSO in Saline x 7 animals = 63 µl

287 µl Cilo Aliquot + 63 µl 5%DMSO in Saline = 350 µl (of 0.041 mg/50 µl)

APPENDIX E: Schematic representation of olfactory preference training protocol



APPENDIX F: Western Blotting Recipes

Lysis Buffer (RIPA) prepared on ice

Volumes	1.0ml	Final Conc.
dH_2O	370	
Tris-HCL, pH 7.4, 1.0M	50	50mM
NP-40	100	1%
Sodium Deoxycholate, 10%	25	0.25%
NaCL, 1.0M	150	150mM
10x protease inhibitor cocktail	100	1x
10x phosSTOP cocktail	100	1x
10mM EDTA pH8.0	100	1mM
200mM PMSF (in 100% isopropanol)	5	1mM
µl	1000	

Tris pH 6.8 (0.5 M) 50 ml

Tris Base	3.029g
dH_2O	40 ml

Adjust pH to 6.8

Tris pH 8.8 (1.5 M) 50 ml

Tris Base	9.086 g
dH_2O	40 ml

Adjust pH to 8.8

Running Buffer (10x) – Stock Solution

	For 500 ml	For 1L	Final concentration
Tris Base	14.14 g	30.3 g	250 mM
Glycine	72 g	144 g	1.92 mM
SDS	5.0 g	10 g	1.00%

pH should be 8.8 Use 1x on day of use.

Transfer Buffer (10x)

	For 500 ml	For 1L	Final concentration
Tris Base	15.14 g	30.3 g	250 mM
Glycine	72 g	144 g	1.92 mM

pH should be 8.3. Use 1X on day of use (700 ml dH_2O, 200 ml Methanol, 100 ml Transfer Buffer 10x).

TBS (10x)

	1 L	2 L	Final concentration
Tris Base	24.25 g	48.5 g	200 mM
NaCL	80 g	160.0 g	1.369 mM

Adjust pH to 7.6.

TBS/T (1x) – 1 L

TBS (1x)	1000 ml
10% Tween 20	10 ml

Sample Buffer (5x)

Glycerol	5 ml
SDS	1 g
Bromophenol Blue	25 mg
Tris 1.0 M, pH 6.8	3 ml

Adjust pH to 8.8 at 25°C.

On day of use

5x Sample Buffer	200 µl
Dithiothreitol (DTT)	15.4 mg

Warm 5x sample buffer to dissolve.

Blocking reagent (5% skim milk)

TBS/T	10 ml
Powdered skim milk	0.5 g

	4% stacking gel	10 %	15 %
Acrylamide/Bis-Acrylamide 30% 29:1	650 µl	3.3 ml	5.0 ml
dH_2O	3.3 ml	3.4 ml	1.7 ml
Tris, 1.5 M pH 8.8		2.5 ml	2.5 ml
Tris 0.5 M pH 6.8	630 µl		
SDS 20%	100 µl	50 µl	50 µl
APS 1.5%	332 µl	700 µl	700 µl
TEMED	8 µl	5 µl	5 µl

APPENDIX G: q-PCR Sample Calculation ΔCT Method

Control group	18S CT	Average	Nr4a1 CT	Average
Saline-10min Plate1	10.870		25.405	
	10.954		25.404	
	10.989	10.938	25.410	25.406
Saline-10min Plate2	11.009		25.982	
	11.119		25.986	
	11.133	11.087	25.971	25.980
Saline-10min Plate3	11.152		25.792	
	11.402		25.844	
	11.396	11.317	25.874	25.837
Saline-10min Plate4	10.468		25.480	
	10.522		25.484	
	10.599	10.530	25.494	25.486
Experimental group	**18S CT**	**Average**	**Nr4a1 CT**	**Average**
2mg Iso-10min Plate 1	10.906		25.225	
	10.717		25.171	
	10.708	10.777	25.130	25.175
2mg Iso-10min Plate 2	10.688		25.174	
	10.511		25.161	
	10.507	10.569	25.079	25.138
2mg Iso-10min Plate 3	11.307		25.351	
	11.204		25.234	
	11.068	11.193	25.227	25.271
2mgIso-10min Plate4	11.528		25.687	
	11.646		25.653	
	11.687	11.620	25.662	25.667

Control group	ΔCT	2^-ΔCT		
	Nra1 – 18S		**Mean**	**Standard Dev.**
Saline-10min Plate 1	14.469	4.4106E-05		
Saline-10min Plate 2	14.8936	3.28746E-05		
Saline-10min Plate 3	14.520	4.25642E-05		
Saline-10min Plate4	14.956	3.14554E-05	3.775E-05	6.505E-05
Experimental group	**ΔCT**	**2^-ΔCT**		
	Nra1 – 18S		**Mean**	**Standard Dev.**
2mg Iso-10min Plate 1	14.398	4.6309E-05		
2mg Iso-10min Plate 2	14.569	4.11333E-05		
2mg Iso-10min Plate 3	14.078	5.78363E-05		
2mgIso-10min Plate4	14.047	5.90788E-05	5.108E-05	8.781E-06

Control group	Fold Change		
	Sample mean/group mean	**Mean of group**	**Standard Dev. of group**
Saline-10min Plate 1	1.1683		
Saline-10min Plate 2	0.8708		
Saline-10min Plate 3	1.1275		
Saline-10min Plate4	0.8332	1	0.17233
Experimental group	**Fold Change**		
	Sample mean/group mean	**Mean of group**	**Standard Dev. of group**
2mg Iso-10min Plate 1	1.2267		
2mg Iso-10min Plate 2	1.08962		
2mg Iso-10min Plate 3	1.53208		
2mg Iso-10min Plate4	1.56499	1.35336	0.23261