THE MOLECULAR SIGNALING OF THE UNCONDITIONED
STIMULUS IN RAT PUP

LONG-TERM
ODOR PREFERENCE MEMORY: SUPPORT FOR
PARALLEL, NOT SERIAL, MEMORY MODELS

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The molecular signaling of the unconditioned stimulus in rat pup long-term odor preference memory: Support for parallel, not serial, memory models

by

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Abstract

The formation of memory was originally thought to occur through learning causing the formation of early short-term memory (STM), which transitions into long-term memory (LTM) through the process of consolidation. Work in *Aplysia* found that memory consists of three distinct phases that are defined by their unique dependence on protein synthesis. Through altering serotonin exposure, experimenters could selectively generate a translation-independent STM, a translation-dependent intermediate-term memory (ITM), and a transcription- and translation-dependent LTM. Here we specifically examine the memory mechanisms of LTM using neonatal odor preference learning, which is a well-defined, associative mammalian learning and memory model. Within this model a single pairing of the conditioned stimulus (CS) of novel odor exposure with the unconditioned stimulus (US) of the activation of bulbar β-adrenoceptors within rat pups causes the formation of a preference for the conditioned odor that lasts for 24 h. Previous analysis of molecular signaling found that odor preference learning increased bulbar cAMP levels and the phosphorylation of CREB. Manipulating the US of β-adrenoceptor activation causes the alteration of cAMP signaling resulting in the formation or impairment of neonatal odor preference memory such that there is an optimal level of activation, either too little or excessive activation of the US does not produce learning.

In the present thesis, we set out to identify the underlying molecular mechanisms that produce 24 h neonatal odor preference memory with specific focus on the US of cAMP signaling. In earlier work we had shown, using translational and transcriptional protein synthesis inhibitors, that as in *Aplysia*, neonatal odor preference memory
includes a translation-independent STM lasting till 3 h after learning, a translation-dependent ITM occurring 5 h after learning, and a transcription- and translation-dependent LTM occurring 24 h after learning. Altering the US through decreasing β-adrenoceptor activation causes the selective generation of ITM, demonstrating the three characteristics of invertebrate memory in mammalian memory (see Appendix). Neonatal odor preference learning causes the activation of bulbar PKA 10 min after training (Chapter 2) and the activation of bulbar ERK immediately after training (Chapter 3). The direct activation of bulbar PKA acts as a sufficient US to selectively generate ITM and LTM and extend odor preference memory to 72 h (Chapter 2). The activation of Epac is also a sufficient US for the generation of long-term neonatal odor preference memory (Chapter 3), demonstrating that cAMP can work through the PKA or Epac/ERK pathway to generate longer protein synthesis-dependent phases of neonatal odor preference memory. Inhibition of either PKA (Chapter 2) or ERK (Chapter 3) prevented the LTM of rat pup odor preference learning with a normal US, suggesting both pathways contribute synergistically to LTM. LTM can be rescued from the inhibition of either bulbar PKA (Chapter 2) or ERK (Chapter 3) through increasing β-adrenoceptor activation to a level which normally would not produce learning. Thus higher levels of activation of either pathway alone are sufficient to produce LTM. However, the simultaneous inhibition of both kinases inhibits LTM at any level of β-adrenoceptor activation tested. PKA (Chapter 2) and Epac (Chapter 3) activation, when given alone, as an intracellular US do not produce STM. Thus ITM and LTM occur independently of STM. In addition a high level of β-adrenoceptor activation in the presence of either a PKA (Chapter 2) or ERK (Chapter 3) antagonist does not lead to ITM although LTM is
now supported. Thus, while PKA and Epac/ERK synergistically generate normal LTM through odor preference learning, the distinct memory phases of STM, ITM and LTM can occur without the prior occurrence of the temporally earlier memory. Here we have demonstrated for the first time that PKA and/or Epac activation can act directly as an US for a long-term mammalian memory. In addition, we find that memory is likely composed of parallel intracellular processes which are quasi-independent rather than occurring as a set of serial, interdependent “consolidating” processes.
Acknowledgements

I wish to thank my supervisor Dr. John McLean, who has provided me the opportunity to be in the neuroscience department of BioMedical Sciences at the Faculty of Medicine, Memorial University of Newfoundland. He has given me guidance and support through the many frustrations and successes of the experimental research while working in his lab during my PhD. The opportunities and support he has granted for teaching during my PhD. has also opened my eyes to another passion I did not know existed.

I wish to thank my co-supervisor Dr. Carolyn Harley, whose intelligence and creativity has been an asset and inspiration for the experimental design and scientific writing. Her uncanny ability to see different angles and stories from the results is truly amazing and exciting to experience.

I wish to thank Andrea Darby-King, who has provided her expertise and assistance in the lab during my PhD. Her ability to teach and assist in experimental techniques is an asset to the laboratory research environment.

I wish to thank Dr. Karen Mearow. Early in my PhD, I encountered a very challenging radioactive PKA activity assay that took a significant amount of time with no results. Feeling that I should give up the experiment, I approached Dr. Mearow for her expertise in molecular biology to revamp the assay protocol. Her advice allowed me to complete the assay successfully, leading to the overall success of the project.
Co-authorship Statement

I, Matthew T. Grimes, am the principal author of all the chapters and appendix that comprise this thesis. However, each chapter has been facilitated through assistance in writing and editing by my supervisors Drs. John McLean and Carolyn Harley, as well as assistance with experimental work from other students and co-workers. The specific contributions by these individuals for each experimental chapter are outlined below:

For Chapter 2, titled “Increased bulbar PKA activity is necessary and sufficient for neonate intermediate- and long-term odor preference memory, but not required for short-term memory”, I participated in the experimental design along with Drs. John McLean and Carolyn Harley, completed all of the experiments, and wrote the first draft of the manuscript. Carolyn W. Harley and John H. McLean helped me to improve the writing of the manuscript. Andrea Darby-King provided technical support.

For chapter 3, titled “The activation of the Epac/ERK pathway is required for specific phases of associative mammalian olfactory memory”, I participated in the experimental design along with Drs. John McLean and Carolyn Harley, completed all of the experiments, and wrote the first draft of the manuscript. Andrea Darby-King provided experimental support with the western blots by running the gel electrophoresis procedure and provided assistance with experimental design and technical support. Drs. Carolyn Harley and John McLean also helped me to improve the writing of the manuscript.
The manuscript in the appendix, titled "Mammalian intermediate-term memory: New findings in neonate rat", is co-authored by Melissa Smith, Xuqin Li, Andrea Darby-King, Carolyn W. Harley, and John H. McLean. As first author, I participated in the experimental design and wrote the first draft of the manuscript. Drs. Carolyn W. Harley and John H. McLean were involved in experimental design and helped me to improve the writing of the manuscript. The experimental work I contributed towards was the inhibiting of translation and exploring 5 h ITM (Fig. A.1C), inhibiting translation 1 h after learning and exploring 24 h LTM (Fig. A.2A), altering the US through varying doses of Iso and measuring 5 h ITM and 24 h LTM (Fig. A.4A/B). Melissa Smith contributed to the experimental work of inhibiting translation and exploring 1 h and 3 h STM and 24 h LTM (Fig. A.1A/B/D), and inhibiting translation 1 h and 3 h after learning and exploring 24 h LTM (Fig. A.2A/B). Xuqin Li contributed to the experimental work of inhibiting transcription and exploring 5 h ITM and 24 h LTM (Fig. A.3A/B). Andrea Darby-King was involved in experimental design and technical support and contributed to the experimental work of altering the US through varying doses of Iso and exploring 5 h ITM (Fig. A.4A) and 3 h STM (Fig. A.5). Dr. John H. McLean contributed to the experimental work of altering the US through varying doses of Iso and exploring 5 h ITM (Fig. A.4A) and 3 h STM (Fig. A.5). Dr. Carolyn W. Harley edited suggestions from myself and Dr. John H. McLean for the design of the summary figure (Fig. A.6).
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2-DG</td>
<td>2-deoxyglucose</td>
</tr>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebral spinal fluid</td>
</tr>
<tr>
<td>ACTI</td>
<td>actinomycin</td>
</tr>
<tr>
<td>AD</td>
<td>apical dendrite</td>
</tr>
<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid</td>
</tr>
<tr>
<td>ANI</td>
<td>anisomycin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>B-Raf</td>
<td>B-rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>C</td>
<td>catalytic subunit</td>
</tr>
<tr>
<td>Cal</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CaMK2</td>
<td>Ca2+/calmodulin dependent kinase 2</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CBS</td>
<td>cAMP binding site</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP responsive element</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic adenosine monophosphate responsive element binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>conditioned stimulus</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxy ribonucleic acid</td>
</tr>
<tr>
<td>Elk-1</td>
<td>Ets-like protein</td>
</tr>
</tbody>
</table>
E-LTP: early phase long-term potentiation
Epac: exchange protein activated by cAMP
epl: external plexiform layer
ERK: extracellular signal-regulated kinase
G: guanine nucleotide-binding proteins
GABA: gamma-amino butyric acid
GC: granule cell
gcl: granule cell layer
gl: glomerular layer
h: hour
HDAC: histone deacetylase
I-1: inhibitor-1
Iso: isoproterenol
ITM: intermediate-term memory
LD: lateral dendrite
LTD: long-term depression
LTF: long-term facilitation
LTM: long-term memory
LTP: long-term potentiation
L-LTP: late phase long-term potentiation
MC: mitral cell
mcl: mitral cell layer
MEK: mitogen-activated protein kinase kinase
min: minute
MOB: main olfactory bulb
mRNA: messenger ribonucleic acid
MSK: mitogen and stress-activated kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PG</td>
<td>periglomerular</td>
</tr>
<tr>
<td>PICK1</td>
<td>protein interacting with c-kinase 1</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKI</td>
<td>protein kinase A inhibitor cocktail</td>
</tr>
<tr>
<td>PND</td>
<td>post-natal day</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>R</td>
<td>regulatory subunit</td>
</tr>
<tr>
<td>Rap1</td>
<td>Ras-proximate-1</td>
</tr>
<tr>
<td>RBS</td>
<td>Rap1 binding site</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RSK</td>
<td>ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SRE</td>
<td>serum response element</td>
</tr>
<tr>
<td>STF</td>
<td>short-term facilitation</td>
</tr>
<tr>
<td>STM</td>
<td>short-term memory</td>
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<tr>
<td>US</td>
<td>unconditioned stimulus</td>
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Chapter 1  Introduction

This introduction will review the scientific literature leading to the hypotheses of the experiments that comprise this thesis. Four areas will be of specific focus: 1) early odor preference learning, 2) three distinct phases of memory consisting of short-term, intermediate-term, and long-term memory, 3) the involvement of the PKA pathway and 4) the ERK pathway in these three phases. The last section will outline the undertaken experiments.

1.1 Neonatal odor preference learning

1.1.1 Early odor learning in neonatal rats

In order for a newborn rat to survive, it is imperative that it can recognize and find its mother and nesting environment. The survival of the pup is reliant on its ability to recognize its mother as she is required for protection, food, and thermoregulation. The eyelids of rat pups do not open till approximately postnatal day 13 (Eyars, 1951) and their auditory thresholds are increased until the opening of the auditory meatus also around postnatal day 13 (Crowley and Hepp-Reymond, 1966). Rat pups have highly developed olfactory and tactile senses which allow them to undergo odor learning and memory formation, giving them the ability to find their mother (Johanson and Hall, 1982) and feed
(Pedersen et al., 1982). The pup’s odor learning can be manipulated through using maternal stimuli as pups can generate a preference for a novel odor through its pairing with the scent of the dam’s saliva (Sullivan et al., 1986) or milk (Sullivan and Hall, 1988). Odor learning even occurs in utero as seen with pairings of a novel odor with ethanol (Abate et al., 2002) or opioids (Smotherman and Robinson, 1994). The ability to learn odor preferences early in life is seen within many animal species, including human infants (Sullivan et al., 1991b; Marlier et al., 1998; Schaal et al., 1998), and it can shape the behaviour of the animal in adulthood (Fillion and Blass, 1986). The pup appears to acquire odor preference through the associative learning paradigm of classical conditioning.

Classical conditioning of odor preference can be carried out by pairing a novel odor (conditioned stimulus, CS) with tactile stimulation (unconditioned stimulus, US). Tactile stimulation, such as stroking the pup with a paint brush, mimics the maternal care of the dam when it licks the pup. This causes the pup to form a preference or approach for an odor that is normally aversive (Sullivan et al., 1986). The pairing of the CS with the US for 10 minutes a day from postnatal days (PND) 1-18 generated a long-term odor preference for the novel odor measured on PND 19. After odor preference learning occurred, subsequent presentations of the learned odor were associated with enhanced 2-deoxyglucose (2-DG) uptake in the dorsal lateral and ventral lateral quadrants of the olfactory bulbs, which are odor-specific reception areas of the bulb for peppermint, the odor used in these studies (Sullivan and Leon, 1986). Multiple
trial odor preference learning can result in a permanent memory (Fillion and Blass, 1986; Shah et al., 2002). Reducing the number of training trials can still produce a long-term odor preference on PND 19. However, these training trials needed to occur during a specific time of development. Odor preference training carried out from PND 1-8 led to long-term odor preference being expressed on PND 19, while odor preference training occurring on PND 1-4 or PND 8-12 did not cause memory formation. This suggests that a critical developmental period for odor preference memory formation occurs between PND 4-8 (Woo and Leon, 1987).

1.1.2 Single trial odor preference memory

Since neonatal rats are primed for odor learning and memory formation particularly between PND 4-8 (Woo and Leon, 1987), an examination of single trial odor preference learning was conducted within this developmental period on PND 6 through classical conditioning of a single pairing of a novel odor (CS) with tactile stimulation (US). This caused the formation of a preference for the novel odor that lasted as long as 24 h (Sullivan and Leon, 1987). Examination of the olfactory bulb revealed enhanced 2-DG uptake in the learned odor-specific areas 24 h later, demonstrating the same metabolic characteristic displayed with multiple trial training (Sullivan et al., 1991a; Sullivan and Leon, 1987; Sullivan and Leon, 1986). However, a single trial pairing of an aversive US on PND 6, such as a shock or tail pinch, with a novel odor also generates a long-term preference
for the odor (Camp and Rudy, 1988; Wilson and Sullivan, 1994; Sullivan et al., 2000a). This stereotypical behavioural response to a variety of unconditioned stimuli appears related to the pup’s reliance on the mother at this early stage of development, such that the pup will develop a preference for maternal and novel odors associated with either pleasurable or abusive stimuli (Sullivan et al., 2000a). The mechanism of this unusual generalization is apparently a function of a lack of amygdalar participation at these ages causing reduced aversive learning, as both stroking and shock function as an US where the association of olfactory stimulation with somatosensory stimulation causes odor preference learning and memory formation to occur. Using this behaviourally simple mammalian memory model gives us a tool to explore the cellular and molecular mechanisms of not only odor preference memory, but mammalian memory in general.

1.1.3 Examining the US of neonatal odor preference learning

As described the association of the CS of novel odor and the US of tactile stimulation can cause the formation of odor preference memory. The tactile stimulation can either be stroking the pup with a soft paint brush (McLean et al., 1999; McLean et al., 1993; Sullivan et al., 1991a), or applying foot or tail shock (0.5 mA) (Camp and Rudy, 1988; Okutani et al., 1999; Sullivan et al., 2000a). Electrophysiology studies revealed that stroking or shock causes activation of the locus coeruleus (Nakamura et al., 1987). The response of locus coeruleus...
neurons to stroking in pups is marked and prolonged while little or no response would be seen in an adult. There are ascending noradrenergic projections to the olfactory bulb from the locus coeruleus present at birth (McLean and Shipley, 1991). The pairing of pharmacological stimulation of the locus coeruleus and novel odor exposure also results in an odor preference (Sullivan et al., 2000b), directly implicating the involvement of the locus coeruleus in neonatal odor preference learning. A subcutaneous (s.c.) injection of propranolol (a general β-adrenoceptor antagonist) before the US locus coeruleus activation (Sullivan et al., 2000b) or tactile stimulation (Sullivan et al., 1989; Sullivan et al., 1991a) inhibits the formation of odor preference memory, demonstrating that β-adrenoceptor activation is necessary for neonatal odor preference learning. Further examination found that β-adrenoceptor activation was not only necessary but sufficient in generating neonatal odor preference memory. A s.c. injection of isoproterenol (Iso, a general β-adrenoceptor agonist), as the sole unconditioned stimulus, paired with novel odor exposure resulted in the formation of a long-term odor preference (Sullivan et al., 1991a; Langdon et al., 1997; Price et al., 1998). Since Iso is a general β-adrenoceptor agonist, a further analysis of the subtypes of noradrenergic receptors responsible for neonatal odor preference learning was completed. It was found that the activation of only the β1-adrenoceptors or α1-adrenoceptors paired with novel odor exposure caused the formation of a long-term preference for the odor (Harley et al., 2006). This suggests that in the experiments demonstrating odor preference memory using Iso as an US, the US is mediated by the activation of β1-adrenoceptors.
1.1.4 Neonatal odor preference learning occurs within the olfactory bulb

Once β-adrenoceptor activation was established as an US for neonatal odor preference memory, it was of interest to localize the critical site for receptor activation. The activation of the locus coeruleus by the US of stroking or shock results in an increase of norepinephrine release within the olfactory bulbs (Rangel and Leon, 1995). When this increased bulbar norepinephrine activity was disrupted, by blocking β-adrenoceptors in the olfactory bulbs through a bulbar infusion of propranolol, odor preference memory formation was inhibited (Sullivan et al., 1992). Pairing the CS of novel odor exposure with the US of activating β-adrenoceptors within the olfactory bulbs, through a bulbar infusion of Iso, resulted in the formation of odor preference memory (Sullivan et al., 2000b). These results suggest that the effective US is the increase of bulbar norepinephrine resulting in the activation of β-adrenoceptors within the olfactory bulbs. Further, these studies demonstrate that the olfactory bulbs are a neural area where the pairing of the unconditioned and conditioned stimuli converges to generate odor preference memory.

1.1.5 β-adrenoceptor activation demonstrates an inverted U-curve response in the acquisition and consolidation of neonatal odor preference memory

The generation of neonatal odor preference memory through the association of the CS of novel odor exposure and the US of a s.c. injection of Iso,
is dose-dependent. An inverted U-curve dose response is seen with the s.c. injection of Iso, such that an optimal level of β-adrenoceptor activation is required for learning to occur. The optimal s.c. dose of Iso for generating 24 h neonatal odor preference memory is 2 mg/kg, and higher doses of 4 mg/kg or 6 mg/kg and a lower dose of 1 mg/kg do not produce learning (Langdon et al., 1997; Sullivan et al., 1991a; Sullivan et al., 1989). This dose sensitivity for β-adrenoceptor activation is also seen with the US of stroking. Receiving stimulation from both unconditioned stimuli, at levels that would normally produce learning on their own, is detrimental to learning formation demonstrating that the unconditioned stimuli are additive. Specifically, the combination of a s.c. injection of 2 mg/kg Iso and a normal magnitude of stroking is not associated with learning, while receiving a suboptimal dose of each of these unconditioned stimuli together generates odor preference learning (Sullivan et al., 1991a; Sullivan et al., 1989). This suggests that the activation of β-adrenoceptors from either a s.c. injection of Iso or stroking initiates common molecular substrates that are regulated both positively and negatively by receptor activity in the formation of neonatal odor preference memory.

β-adrenoceptor activation is not only required for the acquisition of neonatal odor preference memory, but also its consolidation. A s.c. injection of propranolol up to 1 h after odor preference learning prevents long-term odor preference memory. The additional activation of β-adrenoceptors, through a s.c. injection of Iso immediately after learning, also causes a long-term odor
preference memory impairment (Wilson et al., 1994). However, β-adrenoceptor activation is not required for the retrieval of neonatal odor preference memory as a propranolol injection 1 h before 24 h odor preference testing had no effect on memory recall (Sullivan and Wilson, 1991).

1.1.6 The advantage of single trial neonatal odor preference learning as a memory model

The study of mammalian memory has been strongly directed by hypotheses based on memory mechanisms that were discovered in invertebrates, such as Aplysia. However, unlike the well-mapped neural connections that are involved in long-term sensitization and facilitation in Aplysia, a drawback of many mammalian models is the complexity of their neural networks resulting in difficulty in representing what has been learned at a cellular level (McLean and Harley, 2004). Also many mammalian memory models require multiple training or learning trials for memory formation and consolidation to occur, such as mastery of the Morris water maze. This makes it difficult to specifically localize the activity of molecules that may be involved in memory formation, as with multiple trials there may be multiple stimuli (both spatial and non-spatial occurring at temporally disparate times) causing learning changes (O'Keefe, 1999). Multiple trial learning also makes it difficult to examine phases of memory such as short-term and intermediate-term memory (Abel and Nguyen, 2008a), which I will discuss later.
The advantage of using the neonatal rat odor preference memory model is that it is a relatively simple neural network. During the first 10 days of life, the connections between the olfactory bulbs and the amygdala or hippocampus are immature (Schwob and Price, 1984; Sullivan et al., 2000a; Thompson et al., 2008), possibly being a reason that odor preference memory can be localized within the olfactory bulbs (Sullivan et al., 2000b). The neuroanatomical connections in the olfactory bulb that are involved in odor preference memory formation are relatively fewer than in other areas in the brain that are involved in other memory paradigms (McLean and Harley, 2004). It has been possible to localize changes caused by learning to specific olfactory bulb cells. As described earlier, neonatal odor preference learning causes localized enhanced 2-DG uptake in conditioned odor specific areas of the dorsal lateral and ventral lateral quadrants of the olfactory bulbs (Sullivan and Leon, 1986). Molecular markers of odor preference learning have also been localized to mitral cells in these odor specific areas (McLean et al., 1999; Yuan et al., 2003b; Cui et al., 2007), while optical imaging has found that neonatal odor preference learning causes enhanced cellular activity in these odor specific areas (Yuan et al., 2002b).

1.1.7 Functional neuroanatomy of neonatal odor preference learning

As mentioned above in section 1.1.6, neonatal odor preference learning occurs within the main olfactory bulb (MOB) (Wilson and Sullivan, 1994). The MOB receives odor information through the reception of odorant molecules out in the olfactory epithelium of the nose. These odorant molecules activate olfactory
receptor neurons which transmit the information to the olfactory bulb through the olfactory nerve. The olfactory nerve synapses within the glomerular layer of the MOB (Pinching and Powell, 1971b;McLean and Shipley, 1992). Neural signaling is projected out from the olfactory bulbs through the excitatory mitral cells via the lateral olfactory tract (Schoenfeld and Macrides, 1984;Scott, 1986). The mitral cell bodies are contained within the mitral cell layer of the MOB, but have apical dendrites that extend into the glomerular layer and form connections with the axons of the olfactory nerve. The olfactory nerve axons terminate in the MOB by forming an ovoid shaped area called a glomerulus (McLean and Shipley, 1992;Pinching and Powell, 1971a;Pinching and Powell, 1971b;Shepherd, 1972). When odorant molecules are received by receptors in the olfactory epithelium, they cause firing of the olfactory nerve and the release of glutamate into these glomeruli. This causes an increase in mitral cell excitation through activating AMPA and NMDA receptors on the apical dendrites (Ennis et al., 1996;Aroniadou Anderjaska et al., 1997;Ennis et al., 1998). However, there are inhibitory interneurons called periglomerular cells that surround the perimeter of the glomerulus and form dendro-dendritic connections with the apical dendrite of the mitral cells and dendro-axonic connections with the axons of the olfactory nerve. A subset of periglomerular cells release GABA and activate GABA receptors on the presynaptic olfactory nerve axon or postsynaptic mitral cells causing inhibited mitral cell excitation (Keller et al., 1998;Okutani et al., 2003;Aroniadou Anderjaska et al., 2000). Mitral cell excitation can also be inhibited through the inhibitory interneurons called granule cells. The granule cell bodies are found in
the granule cell layer of the MOB, but form dendro-dendritic connections with the lateral dendrites of the mitral cells in the external plexiform layer (Mori et al., 1983; Orona et al., 1983; Scott, 1986; McLean and Shipley, 1992). The lateral dendrites of the mitral cells can excite the granule cells (Shepherd, 1972) through the release of glutamate (Jahr and Nicoll, 1982; Isaacson, 1999; Castro and Urban, 2009; Salin et al., 2001). This causes the granule cells to release GABA and activate GABA receptors on the lateral dendrites of the mitral cells to inhibit their excitation (Okutani et al., 1999; Shepherd, 1972; Jahr and Nicoll, 1982) (see Figure 1.1). It is hypothesized that the regulation of inhibition and excitation by this neural network enables preference for a learned odor to be formed within specific areas of the MOB. Specifically, our memory model of learning an odor preference for peppermint causes increased activity in the glomeruli of the dorsal and ventral-lateral areas of the MOB (Sullivan et al., 1991a; Sullivan and Leon, 1987).

1.1.8 Disinhibition model of neonatal odor preference learning

There is some evidence to suggest that the synaptic changes that occur in the olfactory bulbs after odor preference learning are in the dendro-dendritic connections between the granule cells and the lateral dendrites of the mitral cells (Wilson et al., 1985; Wilson and Leon, 1987; Rumsey et al., 2001). Excitatory and inhibitory synaptic mechanisms are present in the neonatal olfactory bulb.
Figure 1.1 Odorant molecules activate olfactory receptor neurons which transmit the information to the MOB through the olfactory nerve (ON) causing glutamate (Glut) release. The ON input forms an ovoid shaped area called a glomerulus where the axons synapse with the apical dendrites (AD) of the mitral cells (MC) in the glomerular layer. Inhibitory interneurons called periglomerular (PG) cells surround the perimeter of the glomerulus and form dendro-dendritic connections with the apical dendrites and dendro-axonic connections with the olfactory nerve axons. A subset of PG cells release GABA and activate type-B GABA receptors to inhibit mitral cell excitation. Mitral cell excitation is also inhibited through their dendro-dendritic connections with granule cells.
The granule cell bodies are found in the granule cell layer of the MOB, but synapse with the lateral dendrites (LD) of the mitral cells in the external plexiform layer. The mitral cells release glutamate from their lateral dendrites to excite granule cells causing them to release GABA and activate GABA receptors on the lateral dendrites. The mitral cell bodies are contained within the mitral cell layer of the MOB and their neural signaling is projected out through their axons via the lateral olfactory tract.
(Wilson and Leon, 1987; Wilson and Leon, 1986). The dendro-dendritic connections between granule cells and mitral cells are modulated by norepinephrine. Norepinephrine inputs from the locus coeruleus to the olfactory bulb terminate in the granule cell layer (McLean et al., 1989) and alter granule cell excitation (Wilson and Leon, 1988). The activation of the locus coeruleus leads to an initial depression of the granule cells followed by a lasting potentiation. This depression and potentiation sequence is inhibited if β-adrenoceptors are blocked within the olfactory bulbs (Okutani et al., 1998), suggesting that β-adrenoceptor activation on granule cells is involved in controlling their excitation.

Learning a preference for a novel odor causes an alteration in the activity of mitral cells within the odor specific areas. The conditioned odor causes excitability to decrease and inhibitory responses to increase within some of these mitral cells (Wilson et al., 1985; Wilson et al., 1987). This increased inhibition of mitral cells is hypothesized to occur from potentiated synapses between the granule cells and the lateral dendrites of mitral cells. This could be a mechanism to reduce the noise of unspecific mitral cell activity and sharpen the odor conditioned mitral cell signal (Brennan and Keverne, 1997). More recent findings have demonstrated that increased, rather than decreased, mitral cell excitability is also involved in odor learning. Theta burst stimulation of the olfactory nerve (which mimics odorant reception) causes a potentiation of the inhibitory periglomerular cells. β-adrenoceptor activation in the olfactory bulb inhibits these
periglomerular cells and decreases the inhibition on the apical dendrite of the mitral cells. The combination of reduced periglomerular inhibition with olfactory nerve stimulation increases calcium entry into the mitral cell (Yuan, 2009). It is possible that the decreased excitatory responses from the conditioned odor that were first described by Wilson et al., could actually be from the sampling of a large population of mitral cells (Wilson et al., 1985;Wilson et al., 1987). The lateral inhibition of other mitral cells in the surrounding population that aren’t involved in encoding the conditioned odor could be occurring, which would contribute to reducing the surrounding noise and increasing the relevant mitral cell signal as already mentioned. The disinhibition model based on granule cell changes does not address the question of why β-adrenoceptor activation produces an inverted U-curve response or how reduced mitral cell output would produce active preference behavior. Mitral cells need to be active for the formation of olfactory memory (Okutani et al., 1999;Okutani et al., 2003) and the level of this activity could be a determining factor in whether the olfactory memory formed is a preference or aversion for the conditioned odor (Okutani et al., 1999). Okutani et al. found that decreasing GABA-A receptor activity, resulting in increased mitral cell excitability, in the olfactory bulbs resulted in a preference for the paired odor. If GABA-A receptor activity was decreased further pharmacologically, resulting in a further increase of bulbar excitability, then the preference turned into an aversion for the paired odor.
1.1.9 Both CS and US converge on the mitral cell during odor preference learning

In vitro evidence also suggests that an enhancement in mitral cell activity might be the cellular mechanism involved in odor learning. Odors cause the release of glutamate from the olfactory nerve onto the apical dendrite of mitral cells (Aroniadou Anderjaska et al., 1997; Ennis et al., 1998; Ennis et al., 1996). An enhancement in the glutamate response between the olfactory nerve and the apical dendrite of the mitral cell is seen when olfactory learning patterns are given (Yuan et al., 2000). This learning specific enhancement involves glutamatergic NMDA and AMPA receptors (Cui et al., 2011; Yuan et al., 2000) and lasts for 24 h (Yuan et al., 2002b). Initially since odor learning is dependent on the US of β-adrenoceptor activity, investigators looked to anatomical studies to identify the site of US modulation and it appeared that NE fibers were densest near the granule cells as discussed in the disinhibition model above. However, β-adrenoceptors are more abundant on mitral cells (Yuan et al., 2003b) and less evident on granule cells, and a β-adrenoceptor dependent modulation of mitral cell activity has been demonstrated (Yuan, 2009). I will discuss the molecular signaling that occurs during odor learning later. However, in additional support of mitral cell mediation of odor learning effects, changes in molecules involved in odor preference learning, such as the second messenger cyclic adenosine monophosphate (cAMP) (Cui et al., 2007) and the transcription factor cAMP responsive element binding protein (CREB) (Yuan et al., 2003a), have been
found to occur within mitral cells in conditioned odor specific areas of the bulb (McLean et al., 1999; Cui et al., 2007; Yuan et al., 2003b). All of this work suggests that the mitral cells are involved in odor preference learning and are the neuronal location where the CS and US converge for memory formation.

1.2 The molecular signaling cascade of neonatal odor preference learning

1.2.1 The molecular mechanisms of the CS and US

As already described the CS of novel odor exposure causes the release of the excitatory neurotransmitter glutamate from the olfactory nerve onto the apical dendrites of the mitral cells, which would activate AMPA, NMDA, and metabotropic glutamate receptors (Ennis et al., 1996; Aroniadou Anderjaska et al., 1997; Ennis et al., 1998; Ennis et al., 2006; Rumsey et al., 2001; Yuan et al., 2000; Cui et al., 2011). Plasticity experiments in the hippocampus have shown that the activation of NMDA receptors results in increased Ca$^{2+}$ signaling (Blitzer et al., 1995). Similarly, at the cellular level, in the olfactory bulb, the activation of the olfactory nerve paired with β-adrenoceptor activation causes increased Ca$^{2+}$ signaling in mitral cells (Yuan, 2009). Ca$^{2+}$ influx leads to the formation of the second messenger Ca$^{2+}$/calmodulin, which can co-activate adenylyl cyclases (AC) (Blitzer et al., 1995; Choi et al., 1992b; Choi et al., 1992a). AC are enzymes that break down adenosine tri-phosphate (ATP) to form cAMP (Choi et al., 1992a; De Blasi, 1989). The US in neonatal odor preference learning involves the release of the neurotransmitter norepinephrine and the activation of the β-
adrenoceptors within the olfactory bulbs. The activation of these receptors results in the activation of G-proteins and the stimulation of AC also resulting in the formation of cAMP (Choi et al., 1992b; Dittman et al., 1994; De Blasi, 1989) (see Figure 1.2). Since glutamate release from the CS and norepinephrine release from the US can synergistically activate AC to form cAMP, these effects suggest that cAMP is an intracellular converging point of the CS and US and that its downstream pathways could be the molecular mechanisms required for neonatal odor preference learning and memory formation.

Cellular experiments on classical conditioning of the siphon and gill withdrawal reflex in *Aplysia* found that the production of cAMP could be a coincidence detector for the CS and US. The activation of AC was greatest under learning conditions where the pairing of the CS and US was in correct sequence. This occurred through the exposure of the CS of Ca$^{2+}$ preceding the exposure of the US of serotonin. The CS caused the binding of Ca$^{2+}$/calmodulin to AC which accelerated the onset of its activation. This activation was further potentiated and maintained through the US of G-protein activation (Yovell and Abrams, 1992). If the sequence was switched to a non-learning backwards pairing of the US preceding the CS, then the activation of AC was decreased (Abrams et al., 1998).

Genetic mutations knocking out cAMP-producing AC cause long-term memory (LTM) deficits in contextual fear conditioning, passive avoidance and late-phase LTP in mice (Wong et al., 1999), and aversive olfactory learning and
Figure 1.2 The CS of novel odor exposure causes the release of glutamate from the olfactory nerve onto the apical dendrites of the mitral cells, which would activate AMPA and NMDA receptors. Activation of NMDA receptors results in Ca$^{2+}$ influx into the cell. Ca$^{2+}$ influx leads to the formation of Ca$^{2+}$/calmodulin (Cal) leading to the activation of CaMK2, which can have nuclear or synaptic effects. Ca$^{2+}$/Cal can also co-activate AC with the US of β-adrenoceptor (β) activation. The activation of β-adrenoceptors results in the activation of G-proteins (G) and the stimulation of AC. The activation of AC causes the degradation of ATP into cAMP.
memory deficits in *Drosophila* (Davis et al., 1995; Livingstone et al., 1984). The examination of the effects of increasing cAMP levels has found different results depending on the memory model. Increasing cAMP levels within the mouse hippocampus facilitates synaptic plasticity and causes an enhancement in the consolidation and retrieval of fear memory (Isiegos et al., 2008). However, olfactory learning and memory is inhibited in *Drosophila* if cAMP levels are too high (Davis et al., 1995). Our examination of cAMP levels within the olfactory bulbs during and immediately after odor preference learning demonstrated that cAMP undergoes a temporally sensitive peak and trough pattern when learning occurs. The association of the CS with the US causes bulbar cAMP levels to peak immediately after learning and leads to memory formation lasting 24 h. However, when the CS is presented without the US, the cAMP peak is delayed or shifted to 5 min after learning and if the US is too high (6 mg/kg Iso), then there is a linear increase in cAMP levels. Both of these latter conditions are detrimental to neonatal odor preference learning and memory formation (Cui et al., 2007), demonstrating the importance of the temporal characteristics of cAMP signaling in neonatal odor preference learning. Histochemical studies demonstrate a strong localization of the cAMP signal to mitral cells in learning animals (Yuan et al., 2003b).

### 1.2.2 cAMP activates the protein kinase A (PKA) pathway

The enzyme PKA was originally discovered when it was purified from rabbit skeletal muscle (Walsh et al., 1968; Reimann et al., 1971), but was later
found to also be present in the mammalian brain (Cadd and McKnight, 1989). Through enzyme isolation studies it was determined that PKA could be stimulated by cAMP (Beavo et al., 1974; Miyamoto et al., 1969; Walsh et al., 1968), and it was later shown that the primary action of cAMP is to activate PKA (Dell'Acqua and Scott, 1997; Taylor et al., 1990). The enzymatic structure of PKA, in the absence of cAMP, consists of two regulatory subunits bound together with each containing a bound inactive catalytic subunit (McKnight et al., 1988; Taylor et al., 1990). Each of the PKA regulatory subunits contain two cAMP binding sites, a high affinity and a low affinity site (Taylor et al., 1990). When cAMP binds to the two sites on each regulatory subunit, it causes a conformational change in the enzyme resulting in the release of the catalytic subunits (Gibbs et al., 1992). Without being attached to the auto-inhibitory regulatory subunits, the catalytic subunits are active and free to phosphorylate the serine and threonine sites of other proteins (Bacskai et al., 1993; Taylor et al., 1990; Dell'Acqua and Scott, 1997) (See Figure 1.3).

The catalytic subunits can translocate to the nucleus (Bacskai et al., 1993) resulting in the phosphorylation of the transcription factor cAMP responsive element binding protein (CREB) at serine 133 (Bacskai et al., 1993; Delghandi et al., 2005; Arias et al., 1994), which initiates the transcription of CRE associated genes (Gonzalez et al., 1989; Gonzalez and Montminy, 1989; Yamamoto et al., 1988). PKA can also affect the excitation of the synaptic connections of a neuron through its phosphorylation of the glutamate receptors of the AMPA and NMDA type (see Figure 1.4). Phosphorylation of the GluA1 subunit of the AMPA
**Figure 1.3** The PKA protein in its inactive state consists of two regulatory subunits (R) bound together with each R subunit binding an inactive catalytic subunit (C). Each regulatory subunit contains two cAMP binding sites (CBS). When cAMP binds to these sites it causes a conformational change in the enzyme resulting in the release of the C subunits. Without being attached to the inhibitory regulatory subunits, the catalytic subunits are active and free to phosphorylate the serine and threonine sites of other proteins.
receptor at the PKA specific site serine 845 enhances receptor activity and ion movement through increasing the probability, frequency, and duration of channel opening (Banke et al., 2000; Roche et al., 1996; Ahn and Choe, 2009; Greengard et al., 1991). The phosphorylation of the NMDA receptor by PKA increases permeability (Westphal et al., 1999) and influx of Ca\(^{2+}\) ions into the cell (Skeberdis et al., 2006).

1.2.3 cAMP activates the extracellular signal-regulated kinase (ERK) pathway through exchange protein activated by cAMP (Epac)

The activation of \(\beta\)-adrenoceptors also increases ERK phosphorylation (Watabe et al., 2000; Winder et al., 1999), which has been shown to be important for the formation of a variety of memories as will be discussed later. The activation of the ERK pathway by cAMP occurs through cAMP activating Epac. The Epac protein consists of a N-terminal regulatory subunit and a C-terminal catalytic subunit. The regulatory subunit inhibits the binding of Ras-proximate-1 (Rap1) to the catalytic subunit (Gloerich and Bos, 2010). This inhibition is relieved through the binding of cAMP to the regulatory subunit, which causes the regulatory subunit to shift to the backside of the catalytic subunit, allowing the Rap1 binding site to be exposed (de Rooij et al., 2000) (see Figure 1.5). The activation of Epac by cAMP leads to the phosphorylation of ERK through a chain of molecular events starting with Epac activating Rap1.
Figure 1.4  Activation of β-adrenoceptors (β) causes G-proteins (G) to activate adenylyl cyclase (AC), which breaks down ATP into cAMP. cAMP binds to PKA and releases the catalytic subunits, which can translocate to the nucleus resulting in the phosphorylation of the transcription factor CREB at site serine 133. PKA can also affect the excitation of the synapse through its phosphorylation of the glutamate receptors AMPA and NMDA. PKA also regulates dephosphorylating activity by inhibiting protein phosphatase 1 (PP1) through the activation of inhibitor-1 (I-1).
(de Rooij J. et al., 1998; Kawasaki et al., 1998; Ma et al., 2009), leading to the activation of B-rapidly accelerated fibrosarcoma (B-Raf), mitogen-activated protein kinase kinase (MEK), and ERK (Keiper et al., 2004; Lin et al., 2003b; Gelinas et al., 2008; Vossler et al., 1997; York et al., 1998; Morozov et al., 2003). Through this chain of molecular activation, both ERK1 (44 Kda) and ERK2 (42 Kda) are phosphorylated at threonine 202 and tyrosine 204 (York et al., 1998; Marais et al., 1997; Davis and Laroche, 2006) (see Figure 1.6). When ERK is phosphorylated it can control neuronal activity at both the synapse and the nucleus (Sweatt, 2004). The synaptic localization of ERK occurs through its binding with scaffolding proteins (Krapivinsky et al., 2003; Komiyama et al., 2002), which confers the ability to structurally reorganize the synapse through its manipulations of the cytoskeleton proteins MAP-2 and Tau (Goldin and Segal, 2003; Wu et al., 2001; Vaillant et al., 2002). The inactivation of the cAMP/Epac target Rap1 results in a decrease in membrane-associated ERK, which results in the impairment of hippocampal dependent spatial memory, contextual fear conditioning, and long-term potentiation (LTP) (Morozov et al., 2003).

Phosphorylated ERK has nuclear effects through its translocation from the cytoplasm to the nucleus (Treisman, 1996). Studies in the Aplysia memory model of long-term facilitation (LTF) demonstrate that an increase in cAMP levels are required for the translocation of phosphorylated ERK to the nucleus (Martin et al., 1997b). Once in the nucleus, ERK can effect transcriptional activity through its ability to phosphorylate CREB at serine 133 (Impey et al., 1998a; Roberson et al., 1999; Deak et al., 1998). This occurs through ERK
The Mechanism of Epac Activation

Figure 1.5 The Epac protein consists of a N-terminal regulatory subunit (R) and a C-terminal catalytic subunit (C). Epac in its inactive state consists of the regulatory subunit inhibiting the binding of Rap1 to the catalytic subunit. This inhibition is relieved through the binding of cAMP to the regulatory subunit, which causes the regulatory subunit to shift to the backside of the catalytic subunit, allowing the Rap1 binding site (RBS) to be exposed.
Figure 1.6 The activation of β-adrenoceptors (β) activates G-proteins (G) that activate adenylyl cyclase (AC), which turn ATP into cAMP. cAMP activates the ERK pathway through its activation of Epac. Epac activates Rap1, leading to the activation of B-Raf, MEK, and ERK. Both ERK1 (44 Kda) and ERK2 (42 Kda) are phosphorylated at threonine 202 and tyrosine 204. Phosphorylated ERK can have synaptic or nuclear effects. At the synapse ERK can traffick and insert AMPA receptors and close K⁺ channels. In the nucleus, ERK can affect transcriptional activity through its ability to phosphorylate CREB at serine 133 through the activation of RSK or MSK.
activating ribosomal protein S6 kinase (RSK) or mitogen and stress-activated kinase (MSK) (Thomas and Huganir, 2004; Lonze and Ginty, 2002; Davis and Laroche, 2006) (see Figure 1.6).

1.2.4 The phosphorylation of CREB is involved in memory formation

The phosphorylation of the transcription factor CREB at serine 133 initiates the binding of CREB binding protein (CBP) and P300 to the phosphorylated CREB dimer, resulting in the binding and activation of the cAMP responsive element (CRE) (Johannessen et al., 2004). This binding and resultant activation modulates the transcription of genes that contain CRE motifs initiating the production of CRE driven messenger RNA (mRNA) (Montminy, 1997; Lonze and Ginty, 2002) (see Figure 1.7). In mammals this process regulates the expression of more than 100 genes and triggers transcriptional mechanisms that are crucial for the formation of LTM (Mayr and Montminy, 2001; Lonze and Ginty, 2002).

The activation of CREB is required in *Drosophila* (Yin et al., 1994; Tully, 1991), *Aplysia* (Martin et al., 1997a; Dash et al., 1990; Bartsch et al., 1995), and mammals (Josselyn et al., 2004; Kida et al., 2002) in various forms of synaptic plasticity and LTM formation. The training of animals for long-term hippocampal-dependent memories (Impey et al., 1998b; Taubenfeld et al., 1999; Bernabeu et al., 1997; Mizuno et al., 2002) or the induction of a cellular form of hippocampal
Figure 1.7 The phosphorylation of the transcription factor CREB at serine 133 (Ser133) initiates the binding of CREB binding protein (CBP) and P300 to the phosphorylated CREB dimer. This results in the binding and activation of the cAMP responsive element (CRE), which modulates the transcription of genes that contain CRE motifs resulting in the production of CRE driven messenger RNA (mRNA).
LTM, late phase LTP (L-LTP) (Deisseroth et al., 1996; Impey et al., 1996), results in the phosphorylation of CREB and CREB mediated gene expression in CA1 neurons. The inhibition of hippocampal CREB signaling through a hippocampal infusion of antisense oligonucleotides for CREB, inhibits long-term spatial memory measured in the Morris water maze (Guzowski and McGaugh, 1997). Work in Drosophila (Yin et al., 1994) and mutant mice (Bourtchuladze et al., 1994) expressing dominant negative CREB have also demonstrated the involvement of CREB activity in LTM and LTP (Pittenger et al., 2002).

Increasing the activity of CREB within Drosophila (Yin et al., 1995) or the mammalian hippocampus (Barco et al., 2002) or amygdala (Josselyn et al., 2001) resulted in an enhancement of LTM formation and the induction of L-LTP (Barco et al., 2002). We have found that odor preference learning causes an increase in the phosphorylation of CREB within the mitral cells of the olfactory bulbs 10 min after learning (Yuan et al., 2000; McLean et al., 1999). The formation of long-term odor preference memory is inhibited when CREB activity is reduced in the olfactory bulbs, however, the memory can be restored through increasing β-adrenoceptor activation (4 mg/kg Iso). Increasing CREB activity within the olfactory bulbs during odor preference learning resulted in a shift to the left of the inverted U-curve. An enhancement of odor preference learning was demonstrated where long-term odor preference memory was formed with a lower activation of β-adrenoceptors (1 mg/kg Iso). However, normal activation of β-adrenoceptors (2 mg/kg Iso) now produced an impairment in memory formation.
This suggests that levels of pCREB activity follow an inverted U-curve pattern, much like β-adrenoceptor activation, in odor preference learning and memory (Yuan et al., 2003a).

1.2.5 What is transcription and translation?

The processes of transcription and translation within a neuron are important to defining different phases of memory. The process of transcription occurs within the nucleus of the cell and involves transcribing a certain segment of DNA into mRNA. Pharmacological agents, such as the antibiotic actinomycin-D (ACTI), can be used to inhibit the transcription of mRNA. ACTI has been shown to prevent DNA-dependent RNA synthesis in several systems (Reich et al., 1961; Reich et al., 1962). 0.1 mM of ACTI inhibits mRNA transcription up to 98% (Fulton et al., 2005) by binding to DNA at the transcription initiation complex and by preventing RNA polymerase elongation (Sobell, 1985). After the process of transcription, mRNA leaves the nucleus and requires ribosomes for it to be translated into protein. The process of translation can occur either in the cytoplasm of the soma or the dendrites through mRNA attaching to rough endoplasmic reticulum or free floating ribosomes (Vickers et al., 2005; Bramham and Wells, 2007; Steward et al., 1998; Ostroff et al., 2002; Bradshaw et al., 2003). The antibiotic anisomycin (ANI) is a potent and widely used inhibitor of the process of translation. ANI works through interfering with a ribosomal subunit to disrupt transpeptidation during translation (Grollman, 1967). ANI is a reversible translational inhibitor that at a concentration of 0.1 mM blocks translation by 81%
up to 3 h, but its inhibitory effectiveness is significantly reduced and then completely eliminated by 6 h and 12 h after administration, respectively (Fulton et al., 2005). The use of these pharmacological agents in learning and memory models has permitted an investigation of the dependence of memory on de novo or altered protein synthesis. These tools have supported the suggestion that memories have distinct temporal and molecular characteristics.

1.3 Defining memory phases through molecular signaling

1.3.1 Short-term memory

Short-term memory (STM) is a phase of memory that occurs immediately after learning and lasts for a short duration that can be as small as minutes or as long as 3-4 hours (Hernandez and Abel, 2008; McGaugh, 2000; Schafe and LeDoux, 2000). STM can be generated separately from other phases of memory through varying the amount and pattern of training trials (Botzer et al., 1998; Sutton et al., 2002) and US exposure (Montarolo et al., 1986; Ghirardi et al., 1995). Examining the molecular characteristics of STM revealed that this memory phase does not require the activation of mRNA transcription or protein translation (Castellucci et al., 1986; Montarolo et al., 1986; Schafe and LeDoux, 2000; Duvarci et al., 2008; Crow et al., 1997; Ghirardi et al., 1995; Languille et al., 2009) and further examination through work in Drosophila (Yin et al., 1994) and mammals (Guzowski and McGaugh, 1997; Bourchuladze et al., 1994; Pittenger et
al., 2002) found that downstream activation of the transcription factor CREB is also not required. All of this work suggests that STM is formed and sustained through the alteration of the activity of existing proteins such as second messengers, receptors, or kinases. Supporting this hypothesis is work in Aplysia which demonstrates that an increase in the internal concentration of cAMP results in short-term sensitization and facilitation (Castellucci et al., 1986). A mammalian cellular model of STM, early phase LTP (E-LTP), involves an increase in AMPA receptor phosphorylation and insertion into the cellular membrane (Malenka and Bear, 2004), even though it does not require transcription (Nguyen et al., 1994) or translation (Nguyen and Kandel, 1996).

1.3.2 Long-term memory

The most widely studied phase of memory is LTM that can last anywhere from a day to weeks or months (McGaugh, 2000). It has been suggested that STM transitions into LTM through the process of consolidation, where a labile memory becomes strengthened into a stable longer-lasting form (Arshavsky, 2003; Davis and Squire, 1984). Consolidation is thought to involve protein synthesis consisting of the ensemble of gene expression, transcription, and translation (Artinian et al., 2008; Balderas et al., 2008; Hernandez and Abel, 2008; Wanisch et al., 2008; Gruest et al., 2004; Menzel et al., 2001; Schafe and LeDoux, 2000; Schafe et al., 1999; Bourtchouladze et al., 1998; Desgranges et al., 2008). Work in invertebrates examining both associative (Sangha et al., 2003; Martens et al., 2007; Rosenegger et al., 2008; Wustenberg et al.,
and non-associative (Castellucci et al., 1986; Sweatt and Kandel, 1989; Montarolo et al., 1986; Castellucci et al., 1989; Ghirardi et al., 1995; Lee et al., 2008) LTM has found that protein synthesis is required for their formation. Further examination in mammals revealed similar results, with a requirement for protein synthesis in long-term spatial memory (Artinian et al., 2008; Rodriguez-Ortiz et al., 2008; Lattal and Abel, 2001; Naghdi et al., 2003), object recognition memory (Balderas et al., 2008; Akirav and Maroun, 2006; Rossato et al., 2007; Lima et al., 2009), L-LTP (Vickers et al., 2005; Frey and Morris, 1997; Bradshaw et al., 2003; Cracco et al., 2005; Frey et al., 1993; Nguyen and Kandel, 1996; Nguyen and Kandel, 1997), and contextual (Bourtchouladze et al., 1998; Scharf et al., 2002; Barrientos et al., 2002; Igaz et al., 2002; Fischer et al., 2004; Lattal and Abel, 2001) and auditory fear conditioning (Bailey et al., 1999; Schafe and LeDoux, 2000; Maren et al., 2003; Duvarci et al., 2008). Structures where protein synthesis has been shown to be important in mammalian memories include the hippocampus (Vickers et al., 2005; Artinian et al., 2008; Rodriguez-Ortiz et al., 2008; Balderas et al., 2008; Barrientos et al., 2002; Igaz et al., 2002; Fischer et al., 2004; Naghdi et al., 2003; Rossato et al., 2007), amygdala (Bailey et al., 1999; Schafe and LeDoux, 2000; Maren et al., 2003; Duvarci et al., 2008), prefrontal cortex (Akirav and Maroun, 2006), and insular cortex (Balderas et al., 2008) demonstrating that the involvement of protein synthesis in the formation of different long-term mammalian memories is a widespread feature throughout the brain. The temporal examination of protein synthesis-dependent consolidation suggested that the activation of transcriptional
and translational mechanisms occurs during learning and continues for ~1 h (Fulton et al., 2005; Davis and Squire, 1984; Robinson and Franklin, 2007; Bourtchouladze et al., 1998; Gruest et al., 2004).

It is possible that this period of protein synthesis-dependent consolidation is involved in the strengthening of synaptic connections as long-term synaptic modification and strengthening is dependent on gene expression resulting in mRNA production and protein synthesis (Parsons et al., 2006). In support of this, the formation of many different long-term cellular memories has demonstrated a requirement for the connection between the soma and dendrite of the neuron. However, after LTM formation has occurred, the presence of the soma is no longer required suggesting that cellular LTM is supported and maintained through synaptic modification. It is hypothesized that LTM formation requires transcription that occurs in the nucleus of the soma leading to subsequent translation and strengthening in the synapse (Lukowiak et al., 2003; Martens et al., 2007; Vickers et al., 2005; Frey and Morris, 1997; Bradshaw et al., 2003; Cracco et al., 2005). All of this work suggests that the initial consolidation of LTM occurs during or immediately after learning, leading to changes in molecular signaling in processes that can sustain the memory for a long period of time through synaptic strengthening.

1.3.3 Intermediate-term memory
Initially it was assumed that memory formation transitioned from STM directly into LTM. However, while the molecular mechanisms of STM and LTM were being explored, a novel phase of cellular memory was discovered in *Aplysia*. This phase occurred temporally between short-term facilitation (STF) and long-term facilitation (LTF) and it was named intermediate-term facilitation (ITF). This novel phase of facilitation could be formed independently of STF or LTF through altering the amount of serotonin exposure on the sensory neuron. Specifically, LTF lasted for 24 h and required 100 nM of serotonin, while STF lasting 30 min could be formed independently of LTF through the administration of 10 nM of serotonin. ITF could be formed independently of the other two phases of facilitation through the administration of 50 nM of serotonin. This intermediate phase lasted between 3-6 h after training and had distinct molecular characteristics relative to both STF and LTF. The molecular characteristics of the three phases of facilitation consisted of STF being independent of transcription and translation, with ITF requiring translation, and LTF requiring both transcription and translation (Ghirardi et al., 1995).

This unique phase of cellular memory was later demonstrated behaviourally with the examination of sensitization in *Aplysia* (Sutton et al., 2001; Sutton et al., 2002; Lyons et al., 2008), enhanced excitability in *Hermissenda* (Crow et al., 1999), and operant conditioning in *Lymnaea* (Sangha et al., 2003; Lukowiak et al., 2000). These three behavioural memory paradigms found that intermediate-term memory (ITM) was very similar to ITF due to its ability to be independently formed, lasting at least 3 h after learning, and only
requiring translation. Further examination found that the formation of ITM could occur through the translation of existing mRNA at the synapse, which is distinct from LTM which requires transcription to occur in the soma of the neuron (Lukowiak et al., 2003). ITM could be turned into LTM if the exposure to the stimulus is increased through increasing reinforcing stimuli (Parvez et al., 2006a), predator associated stressors (Martens et al., 2007), or molecular phosphorylation (Rosenegger et al., 2008). The transition into LTM is serially linked to ITM formation as the interruption of ITM inhibits the formation of LTM (Parvez et al., 2006a). The formation of ITM may initiate the molecular mechanisms of soma transcription and translation of the newly produced mRNA to allow the establishment of LTM, accounting for the apparent dependence of LTM on ITM formation (Parvez et al., 2005).

The existence of ITM in mammals has been implied but not directly studied. Specifically, work in novel object recognition has suggested that 4 h ITM is different from 24 h LTM based only on its temporal characteristics. However, these two memory phases have not been independently defined through altering training procedures or molecular signaling, and thus cannot be distinguished from each other yet at the molecular level (Taglialatela et al., 2009). Work on a cellular memory model of long-term depression found that the In vivo formation of this cellular memory within CA1 of the hippocampus through the administration of low frequency stimulation depended on the translation of existing mRNA, but not the synthesis of new mRNA (Manahan-Vaughan et al., 2000). The temporal and molecular characteristics of this cellular memory suggests there could be an
intermediate phase as the translation dependence was found to occur 4.5 h and last to at least 8 h after induction. However, the fact that the memory never encounters a transcription-dependent phase suggests that this may not be a representation of ITM, but may be long-term cellular memory that is supported through the translation of existing mRNA. The requirement for translation of already existing mRNA for the formation of memory has previously been supported in L-LTP studies (Bradshaw et al., 2003; Cracco et al., 2005). However, this L-LTP was only seen to last about 4-5 h (Vickers et al., 2005) similar to the ITM times in invertebrates, instead of a normal 8 h previously used to define L-LTP (Nguyen et al., 1994; Nguyen and Kandel, 1996). Even though the authors define this as L-LTP, it could actually be an intermediate phase of LTP that does not require transcription and decays earlier than L-LTP, demonstrating similar characteristics as ITM in invertebrates (Ghirardi et al., 1995). Initial synaptic activation can cause the localization of already existing mRNA at the active dendrite (Havik et al., 2003; Steward et al., 1998; Steward and Worley, 2002), suggesting that the ability to form ITM within mammalian systems exists. However, the majority of memory models use adolescent to adult animals within their training procedures, which may make it difficult to observe ITM. Studies training animals for long-term taste aversion memory found that blocking translation out to 1 h after learning in PND 3 animals caused LTM impairments. However, as the animals grew older, the translational mechanisms appeared to be accelerated and PND 10 animals only showed impairment when translation was blocked immediately after learning while PND 18 animals only showed
partial impairment even under these conditions. It is possible that this accelerated translational activity in older animals is the reason there have been no observations of mammalian translation-dependent ITM, as not blocking the translation soon enough could leave ITM intact with only LTM being partially or fully inhibited (Languille et al., 2008).

1.3.4 The involvement of PKA in short-term memory

An increase in cAMP levels in *Aplysia* has been linked to the formation of short-term facilitation (Braha et al., 1990; Schacher et al., 1988; Chang et al., 2000) and sensitization (Bernier et al., 1982). Since the primary action of cAMP is to activate PKA (Taylor et al., 1990; Dell'Acqua and Scott, 1997), this would suggest that PKA is involved in cellular and behavioural non-associative short-term invertebrate memory formation. The induction of short-term facilitation or sensitization has been shown to cause the activation of PKA (Muller and Carew, 1998), which is necessary for the formation of both short-term facilitation (Ghirardi et al., 1992; Castellucci et al., 1982; Schacher et al., 1988; Chang et al., 2000; Byrne and Kandel, 1996; Ghirardi et al., 1995) and sensitization (Castellucci et al., 1982; Lee et al., 2008; Antonov et al., 2010). Since this phase of memory is independent of translation and transcription, it suggests that PKA modifies existing proteins to form and sustain STM. This short-term invertebrate memory is sustained through the ability of PKA to decrease $K^+$ channel activity at the presynaptic sensory neuron terminal (Hochner and Kandel, 1992), which causes
increased excitation and neurotransmitter release (Hochner et al., 1986; Klein et al., 1982). However, there is invertebrate STM that is not dependent on the activation of PKA. The formation of both short-term habituation in the crab Chasmagnathus (Romano et al., 1996) and short-term associative olfactory memory in the honeybee (Fiala et al., 1999) does not require PKA activity.

The examination of the requirement for PKA activity for short-term mammalian memory formation found similar results to the invertebrate analysis where, as a function of the memory model, there could be a dependence or independence on the kinase. However, unlike the invertebrate findings, there is more evidence demonstrating that PKA activation is not required for short-term mammalian memory formation. The short-term mammalian cellular memory, E-LTP, does not require the activation of PKA for its formation or maintenance (Nguyen and Kandel, 1996; Abel et al., 1997; Nguyen and Kandel, 1997). Further behavioural analysis revealed that PKA activity in the amygdala is not required for short-term auditory fear (Schafe et al., 1999; Schafe and LeDoux, 2000; Goosens et al., 2000) or conditioned taste aversion (Koh et al., 2003; Koh et al., 2002) memories. The examination of hippocampal-dependent STM demonstrates both independence and dependence on the activation of PKA depending on the task. The formation of short-term contextual fear memory (Abel et al., 1997; Schafe et al., 1999) does not require PKA activation, while the formation of short-term inhibitory avoidance does require hippocampal PKA activity (Vianna et al., 1999; Vianna et al., 2000; Izquierdo et al., 2000b).
PKA could support STM by enhancing synaptic excitation for the entire duration of the STM phase. PKA has the ability to inhibit protein phosphatase 1 (PP1), which is an enzyme that dephosphorylates AMPA receptors leading to their internalization and resulting depression of synaptic potentials (Mulkey et al., 1994; Lee et al., 2000; Man et al., 2007; Kameyama et al., 1998; Lee et al., 1998), through its ability to phosphorylate inhibitor-1 (Blitzer et al., 1998; Blitzer et al., 1995; Greengard et al., 1999; Hemmings, Jr. et al., 1984) (see Figure 1.4). The ability of PKA to decrease the activity of PP1 has been implicated as a mechanism in the formation of E-LTP (Blitzer et al., 1998; Blitzer et al., 1995) possibly through the increased phosphorylation and insertion of AMPA receptors and subsequent potentiation of the synapse (Lee et al., 2000; Man et al., 2007). PKA has the ability to traffic AMPA receptors to the synapse (Zheng and Keifer, 2009) and insert them into the membrane (Esteban et al., 2003; Man et al., 2007) resulting in enhanced synaptic excitation (Han and Whelan, 2009; Bocchiaro et al., 2003; Duffy and Nguyen, 2003; Wang et al., 1991; Greengard et al., 1991). PKA can also enhance synaptic excitation through its direct regulation of AMPA receptor activity. PKA phosphorylation of the GluA1 subunit of AMPA receptors at the serine 845 site causes enhanced receptor activity by increasing the probability, frequency, and duration of channel opening (Banke et al., 2000; Roche et al., 1996; Ahn and Choe, 2009; Greengard et al., 1991). However, when this was explored in neonatal odor preference memory, it was discovered that the phosphorylation of AMPA receptors on the PKA site in the olfactory bulbs of learning animals only lasts for 1 h after learning (Cui et al., 2011), which is not
long enough to sustain memory through the entire short-term phase (see Appendix). The insertion of AMPA receptors was found to be increased in the olfactory bulbs between 1 and 3 h after learning and lasted 24 h in animals that had learned an odor preference (Cui et al., 2011). This is long enough to support the later component of short-term memory, and all of intermediate-term and long-term memory.

1.3.5 The involvement of PKA in intermediate-term memory

The involvement of PKA in mammalian ITM has not been explored. However, it has been implicated in invertebrate intermediate-term cellular and behavioural memory. Intermediate-term facilitation and ITM can be isolated in *Aplysia* as mentioned through decreasing the amount of serotonin exposure to the sensory neuron or the amount of tail shocks given to the intact animal (Sutton and Carew, 2000; Sutton et al., 2004; Ghirardi et al., 1995; Sutton et al., 2001; Antonov et al., 2010). The formation of intermediate-term memories causes an increase in PKA activity (Muller and Carew, 1998; Sutton et al., 2001) that is required for the formation of intermediate-term facilitation and sensitization (Sutton and Carew, 2000; Sutton et al., 2001; Antonov et al., 2010). Since this memory phase is translation dependent it suggests that PKA affects translational mechanisms. PKA has been shown to regulate local translation in the hippocampus (Nayak et al., 1998), which could occur through the ability of PKA to regulate the activities of elongation factors (Sutton and Schuman, 2005). Again ITM could also be sustained through enhancing the excitation of the
synapse by increasing synaptic AMPA receptors. PKA has the ability to traffic AMPA receptors to the synapse (Zheng and Keifer, 2009) and insert them into the membrane (Esteban et al., 2003; Man et al., 2007). The formation of LTP in the hippocampus causes a significant increase in the synthesis of AMPA receptors at the synapse 3 h after induction (Nayak et al., 1998), which is the time period of invertebrate ITM (Muller and Carew, 1998; Sutton et al., 2001; Sutton et al., 2004; Lukowiak et al., 2000; Sangha et al., 2003). This synaptic AMPA receptor synthesis is dependent on PKA activity through its regulation of local translation (Nayak et al., 1998), which could be a possible mechanism for sustaining ITM.

There is also invertebrate cellular and behavioural ITM that does not require PKA activation. These memories are formed through administering reduced serotonin exposure or tail shocks to already active sensory neurons (Shobe et al., 2009; Sutton and Carew, 2000; Sutton et al., 2004). This activity-dependent ITM differs from the previously described ITM as it requires the sensory neuron to be in a stimulated state and demonstrates different molecular characteristics as it does not require translation and is formed and maintained through sustained protein kinase C activation (Shobe et al., 2009; Sutton and Carew, 2000; Sutton et al., 2004).

1.3.6 The involvement of PKA in long-term memory
The exploration of LTM in invertebrates has found that the formation of non-associative long-term facilitation and sensitization causes a significant increase in cAMP of the sensory neuron (Brunelli et al., 1976; Schacher et al., 1988; Braha et al., 1990; Bernier et al., 1982; Khabour et al., 2004). As noted earlier the primary action of cAMP is to activate PKA (Dell'Acqua and Scott, 1997; Taylor et al., 1990), suggesting that PKA activity is involved in the formation of long-term invertebrate memories. In support of this inference the formation of long-term facilitation and sensitization in *Aplysia* requires the activation of PKA (Castellucci et al., 1980; Castellucci et al., 1982; Chain et al., 1999; Bao et al., 1998; Chang et al., 2000). Again the process of creating a LTM causes a significant increase in PKA activity in the sensory neuron (Muller and Carew, 1998; Bergold et al., 1990; Chain et al., 1999; Khabour et al., 2004), which causes the reduction of $K^+$ channel currents leading to enhanced neurotransmitter release at the newly potentiated synapse (Hochner and Kandel, 1992). The examination of associative long-term invertebrate memory demonstrated similar results to the non-associative analysis. Operant conditioning in *Aplysia* causes a significant increase in PKA activity, and the inhibition of this activity impairs LTM formation (Michel et al., 2011). Studies of LTM in the honeybee found that acquiring an associative olfactory memory causes a significant increase in PKA activity (Muller, 2000), and the reduction of this activity by 10-15% impaired LTM formation (Fiala et al., 1999). Further analysis found that PKA activity increases had to occur within a short period after learning, as the inhibition of PKA activity 3 h after learning had no effect on LTM formation (Fiala et al., 1999). Similar
results were found in the pond snail, *Lymnaea stagnalis*, where inhibiting the significant increase in PKA activity that occurred immediately after training in an operant conditioning task, resulted in the impairment of LTM formation (Michel et al., 2008).

The mammalian analysis of PKA activation in LTM formation has produced results similar to what has been described in invertebrates. The induction of long-term mammalian cellular memory, L-LTP, requires the activation of PKA, transcription, and translation (Nguyen and Kandel, 1996; Abel et al., 1997; Nguyen and Kandel, 1997; Frey et al., 1993; Woo et al., 2000; Huang and Kandel, 1994). Mammalian long-term behavioural memory studies found that the acquisition of fear memory (Cammarota et al., 2000; Sindreu et al., 2007) or spatial memory (Vazquez et al., 2000; Mizuno et al., 2002) causes a significant increase in PKA activity. If PKA is inhibited in the amygdala (Jentsch et al., 2002; Schafe and LeDoux, 2000; Koh et al., 2002) before or immediately after learning, it causes an impairment of long-term auditory fear (Schafe and LeDoux, 2000) and conditioned taste aversion (Koh et al., 2002; Koh et al., 2003) memories, while the same inhibition in the hippocampus (Abel et al., 1997; Bernabeu et al., 1997; Sharifzadeh et al., 2005; Ma et al., 2009; Bourchouladze et al., 1998; Ahi et al., 2004) impairs contextual fear (Abel et al., 1997; Schafe et al., 1999; Ma et al., 2009; Bourchouladze et al., 1998; Ahi et al., 2004), inhibitory avoidance (Bernabeu et al., 1997; Bevilaqua et al., 1997; Vianna et al., 1999; Vianna et al., 2000) and spatial (Sharifzadeh et al., 2005) memories. The pharmacological activation of PKA combined with the
CS+US pairing in mammalian memory causes a stronger LTM response (Jentsch et al., 2002; Ma et al., 2009; Bernabeu et al., 1997; Vianna et al., 2000), but the extension of the memory has not been examined. The extensive work in invertebrate and mammalian memory systems demonstrates that PKA activation is required for LTM formation in multiple brain areas and memory models.

In terms of mechanism, as reviewed previously PKA could affect LTM formation through multiple molecular mechanisms. LTM is transcription-dependent and PKA mediates transcriptional activity through translocating to the nucleus and phosphorylating transcription factors (Bacskai et al., 1993). As noted, the catalytic subunits of PKA translocate to the nucleus and phosphorylate CREB on serine 133 (Bacskai et al., 1993; Delghandi et al., 2005; Arias et al., 1994) (see Figure 1.4), which initiates the transcription of CRE associated genes (Gonzalez et al., 1989; Gonzalez and Montminy, 1989; Yamamoto et al., 1988) (see Figure 1.7). Focusing on the activity of PKA within the nucleus, it has been reported that inhibiting nuclear PKA activity causes a reduction in the phosphorylation of CREB and deficiencies in L-LTP formation in CA1 of the mammalian hippocampus (Matsushita et al., 2001). This memory phase also requires the translation of the newly transcribed mRNA. Again PKA can regulate local translation (Nayak et al., 1998) through its regulation of elongation factors (Sutton and Schuman, 2005). The regulation of transcription and translation by PKA is a possible mechanism for the synthesis of synaptic AMPA receptors affecting the induction and sustainability of hippocampal L-LTP (Nayak et al., 1998). The sustainability of LTM is thought to occur through the potentiation of
synaptic connections. Post-synaptic potentiation can occur through increased trafficking (Zheng and Keifer, 2009) and insertion (Esteban et al., 2003; Man et al., 2007) of AMPA receptors by PKA, which could be the molecular processes involved in the formation and maintenance of long-term neonatal odor preference memory (Cui et al., 2011).

1.3.7 The involvement of Epac in long-term memory

cAMP can also drive memory formation through an alternate target besides PKA. The formation of long-term facilitation in crayfish requires cAMP but not PKA activation. This facilitation occurs by the enhancement of glutamate release through the ability of cAMP to activate Epac, which may increase the availability of synaptic vesicles for release (Zhong and Zucker, 2005). Within mammalian long-term cellular memory, postsynaptic Epac activation coupled with low frequency stimulation results in the induction of hippocampal long-term depression. This Epac mediated long-term depression was induced through Epac activating Rap1 resulting in the activation of protein interacting with c-kinase 1 (PICK1) that binds to the GluA2 subunit of the AMPA receptor controlling its internalization (Ster et al., 2009). Similar results were found when exploring hippocampal LTP. LTP elicited through high frequency stimulation, when coupled with Epac activation, enhances the maintenance of the LTP leading to an increased duration. This enhanced LTP is not dependent on PKA, but requires ERK activity and is sustained through the translation of existing
synaptic mRNA (Gelinas et al., 2008). This Epac-mediated LTP mimics LTP induced through β-adrenoceptor activation as both require ERK activation and translation (Gelinas et al., 2008; Gelinas and Nguyen, 2005). This suggests that β-adrenoceptor activation can drive cAMP to work through either the PKA (see Figure 1.4) or Epac/ERK (see Figure 1.6) pathways to affect long-term memory (Gelinas and Nguyen, 2005; Nguyen and Kandel, 1997; Nguyen and Kandel, 1996; Gelinas et al., 2008).

Mammalian behavioural studies confirmed the involvement of Epac in memory. Mutating the G-proteins coupled to β-adrenoceptors caused a reduction in cAMP and the impairment of contextual fear memory. This memory impairment could be rescued through the activation of Epac (Kelly et al., 2008). The Kelly study of contextual fear memory found an enhancement that was initiated when Epac was activated immediately after the US. This Epac activation increased Rap1 activity (Ma et al., 2009) suggesting that the activation of the ERK pathway could be involved, as was seen in cellular memory (Gelinas et al., 2008; Morozov et al., 2003). The findings from all this work demonstrate a link between β-adrenoceptors, cAMP, and Epac activation in the generation of memory.

1.3.8 The involvement of ERK in short-term memory

The ability of ERK to translocate to the nucleus (Martin et al., 1997b; Hu et al., 2004; Fioravante et al., 2006; Shobe et al., 2009; Treisman, 1996; Davis et al., 2000) and regulate the activity of transcription factors (Davis et al., 2000; Impey et
al., 1998a; Roberson et al., 1999; Deak et al., 1998), suggests that it is involved in longer phases of memory. This seemed to be the case when ERK activity was analyzed in invertebrate memory, as short-term facilitation (Martin et al., 1997b) and sensitization (Sharma et al., 2003a; Sharma et al., 2003b) did not require its activation. A mutation in *Drosophila* that results in the reduction of ERK activity demonstrated that there was no interruption of olfactory learning and immediate memory (Moressis et al., 2009). The examination of ERK activity in short-term phases of mammalian memory demonstrated similar results to the invertebrate analysis. The induction and maintenance of E-LTP in the mammalian hippocampus (English and Sweatt, 1997; Wu et al., 1999) and amygdala (Huang et al., 2000) does not require ERK activation. Behavioural memory solidified these results as ERK activity is not required in the hippocampus (Blum et al., 1999; Kelly et al., 2003), amygdala (Schafe et al., 2000), thalamus (Apergis-Schoute et al., 2005), or olfactory bulb (Zhang et al., 2003) for short-term object recognition (Bozon et al., 2003; Kelly et al., 2003), spatial (Blum et al., 1999), fear (Apergis-Schoute et al., 2005; Schafe et al., 2000), or aversive olfactory (Zhang et al., 2003) memories respectively. However, there is work in short-term mammalian cellular and behavioural memory that has demonstrated a requirement for the activation of ERK and, as will be discussed, ERK can affect nonnuclear proteins. The establishment of theta-frequency E-LTP and β1-adrenoceptor-dependent E-LTP requires the activation of ERK (Winder et al., 1999). This is of interest as we know that the activation of β1-adrenoceptors is the US for neonatal odor preference memory (Harley et al., 2006). Short-term
hippocampal dependent fear memory also requires ERK activation. Learning an inhibitory avoidance task causes ERK to become phosphorylated in the CA1 region of the hippocampus and the inhibition of this phosphorylation caused the impairment of short-term fear memory (Igaz et al., 2006). ERK may affect short-term memory through its ability to alter synaptic excitation. ERK is localized at the synapse through scaffolding proteins (Krapivinsky et al., 2003; Komiyama et al., 2002) and has the ability to modulate synaptic excitation through closing post-synaptic K^+ channels (Adams and Sweatt, 2002; Sweatt, 2004; Watanabe et al., 2002). The ERK pathway also affects synaptic excitation through its ability to traffic and insert AMPA receptors into the synapse (Zhu et al., 2002) (see Figure 1.6). ERK activation is required for the induction of theta burst LTP (Selcher et al., 2003; Winder et al., 1999). Of possible relevance is the observation that this mode of stimulation coupled with β-adrenoceptor activation produces a cellular model of neonatal odor preference memory (Yuan, 2009).

1.3.9 The involvement of ERK in intermediate-term memory

The involvement of ERK activation in intermediate-term mammalian memory is unknown. However, work in invertebrate associative (Rosenegger and Lukowiak, 2010) and non-associative (Sharma et al., 2003a; Sharma et al., 2003b; Lyons et al., 2008) memory models have revealed a requirement for ERK activation in the formation of this memory phase. The generation of intermediate-term facilitation (Sharma et al., 2003b) and ITM (Sharma et al., 2003b; Sharma et al., 2003a) causes a significant increase in ERK phosphorylation. When this...
phosphorylation was blocked it resulted in the impairment of both the intermediate-term facilitation (Sharma et al., 2003b) and memory (Rosenegger and Lukowiak, 2010; Lyons et al., 2008; Sharma et al., 2003b; Sharma et al., 2003a).

As described above, the activation of the Epac/ERK pathway initiates a translation-dependent LTP (Gelinas et al., 2008). ERK can regulate translation through its ability to affect the translational factors eIF4E, 4EBP1, and ribosomal protein S6 (Kelleher, III et al., 2004). It also regulates mTor activity, which is associated with the ribosomal activity that is necessary for the translation of new proteins (Kelleher, III et al., 2004; Tang et al., 2002). These are possible molecular mechanisms for how ERK can regulate the formation of translation dependent ITM.

However, there is another form of intermediate-term facilitation (Sutton and Carew, 2000) and memory (Sutton et al., 2004) described in the sensitization Aplysia model, as mentioned earlier, that is generated through the exposure of serotonin or tail shock to an already active sensory neuron. This activity-dependent ITM differs from the previously described ITM as it is independent of translation and requires ERK and PKC activation but not PKA activation (Shobe et al., 2009; Sutton and Carew, 2000; Sutton et al., 2004). This suggests that in Aplysia, ERK activity is required for two distinct intermediate-term memories, however, the formation of translation dependent ITM requires the activation of both ERK and PKA.
1.3.10 The involvement of ERK in long-term memory

The involvement of ERK activity in long-term cellular and behavioural memory in both invertebrates and mammals has been widely hypothesized. As discussed above, this phase of memory requires the activation of transcriptional and translational mechanisms and ERK has the ability to regulate the activities of both. When ERK is phosphorylated it can translocate to the nucleus (Martin et al., 1997b; Hu et al., 2004; Fioravante et al., 2006; Shobe et al., 2009; Treisman, 1996; Davis et al., 2000) and regulate transcription (Davis et al., 2000; Impey et al., 1998a; Roberson et al., 1999; Deak et al., 1998). ERK also regulates somatic and synaptic translation through its modulation of the translational factors eIF4E, 4EBP1, ribosomal protein S6, and mTOR as described above (Kelleher, III et al., 2004).

The examination of ERK in long-term facilitation and sensitization in Aplysia revealed that the induction of these memories causes ERK to become phosphorylated (Sharma et al., 2003b; Hu et al., 2004; Lyons et al., 2006) and the inhibition of this phosphorylation resulted in LTM impairments for both models (Hu et al., 2004; Sharma et al., 2003b; Martin et al., 1997b; Shobe et al., 2009). Further examination revealed that the phosphorylated ERK must translocate to the nucleus of the presynaptic sensory neuron for the formation of long-term facilitation (Martin et al., 1997b; Hu et al., 2004). Additional invertebrate studies in Drosophila discovered that a mutation that causes a significant decrease in
ERK activity in the mushroom bodies results in the impairment of long-term olfactory memory (Moressis et al., 2009).

Long-term mammalian cellular memory models examining ERK activity demonstrated very similar results to the invertebrates. The induction of LTP in CA1 (Winder et al., 1999; Rosenblum et al., 2002), dentate gyrus (Davis et al., 2000; Ying et al., 2002), or the insular cortex (Jones et al., 1999) causes a rapid increase in the phosphorylation of ERK. This increased phosphorylation causes the nuclear translocation of ERK resulting in the phosphorylation of CREB (Davis et al., 2000). This nuclear translocation is required for LTP formation (Patterson et al., 2001) and inhibiting ERK phosphorylation reduced nuclear translocation, CREB phosphorylation, and resulted in impaired LTP (Davis et al., 2000). The inhibition of ERK in the amygdala (Huang et al., 2000) and CA1 (English and Sweatt, 1997; Wu et al., 1999) resulted in significant impairments of LTP, including its induction through the coupling of theta frequency stimulation with β1-adrenoceptor activation (Winder et al., 1999). The extensive amount of work in mammalian cellular memory demonstrates that the activation of ERK is required for the induction of synaptic plasticity in multiple conditions and areas of the brain.

Further examination in mammalian behavioural memory replicated the findings from cellular models, as learning amygdala-dependent Pavlovian fear (Schafe et al., 2000) or hippocampal-dependent contextual fear (Atkins et al., 1998; Runyan et al., 2004) results in the phosphorylation of ERK 1 h after
learning. However, ERK phosphorylation can decay quickly as studies on taste aversion memory found that ERK was phosphorylated in the nucleus tractus solitarius (Swank, 2000) and the insular cortex (Berman et al., 2000) 20-30 min after learning, then returned to basal levels within 1 h. Hippocampal memory through massed training (Blum et al., 1999) and spaced training (Kelly et al., 2003) causes ERK phosphorylation to occur as quickly as 5 min after learning. However, it should be noted that over activation of the ERK pathway has been linked to cellular and behavioural hippocampal dependent memory impairments in the mouse model of neurofibromatosis type 1 (Costa et al., 2002). There is the possibility that ERK activity follows an inverted U-curve for memory formation, much like what is seen with the US of β-adrenoceptor activation in neonatal odor preference memory (Langdon et al., 1997). The inhibition of ERK in the amygdala (Duvarci et al., 2005; Schafe et al., 2000), hippocampus (Eckel-Mahan et al., 2008; Ohno et al., 2001; Zhang et al., 2004; Kelly et al., 2003; Walz et al., 1999), thalamus (Apergis-Schoute et al., 2005), and olfactory bulbs (Zhang et al., 2003) has revealed that its activation is required in different areas of the brain for the formation of different long-term memories.

There are multiple possible mechanisms for how ERK mediates the formation of LTM. ERK could drive LTM formation through its ability to phosphorylate the transcription factor CREB (Impey et al., 1998a; Roberson et al., 1999; Deak et al., 1998; Davis et al., 2000) or its ability to stabilize Fos proteins (Murphy et al., 2002) resulting in robust transcriptional activation. The formation of hippocampal-dependent long-term contextual memory requires CRE-driven
gene expression (Impey et al., 1998b) that is mediated through ERK (Athos et
al., 2002; West et al., 2002). The impairment of long-term aversive olfactory
memory, through inhibiting bulbar ERK, resulted in decreasing CREB
phosphorylation in the olfactory bulbs (Zhang et al., 2003). However, ERK could
also mediate LTM formation through its ability to modify synaptic connections.
ERK is localized at the synapse through scaffolding proteins (Krapivinsky et al.,
2003; Komiyama et al., 2002) where it can structurally reorganize the synapse
(Wu et al., 2001; Goldin and Segal, 2003) through its effects on the cytoskeleton
proteins MAP2 and tau (Vaillant et al., 2002). Long-term facilitation studies in
Aplysia have found that ERK phosphorylation is required for the formation of new
synapses (Bailey et al., 1997). However, ERK can also alter the excitation of
existing synapses through its closure of post-synaptic K⁺ channels (Adams and
Sweatt, 2002; Sweatt, 2004; Watanabe et al., 2002) and its ability to traffic and
insert AMPA receptors into the post-synaptic membrane (Zhu et al., 2002) (see
Figure 1.6).

1.4 Rationale and hypotheses for the present thesis

As previously reviewed, mitral cells are the neuronal target for the
convergence of the CS of novel odor exposure and the US of β-adrenoceptor
activation. There is evidence that enhanced olfactory nerve input coupling to
mitral cell activity occurs in neonatal odor preference learning (Yuan, 2009) with
significant increases in cAMP levels (Cui et al., 2007; Yuan et al., 2003b) and the phosphorylation of CREB (McLean et al., 1999) occurring within mitral cells in conditioned odor specific areas. The requirement for the phosphorylation of CREB for neonatal odor preference memory formation (Yuan et al., 2003a) suggests that the transcription of new mRNA and the translation of new protein is also required. The manipulation of the US of β-adrenoceptor activation causes alterations in bulbar cAMP levels leading to the formation or impairment of odor preference memory (Cui et al., 2007) and promotion of cAMP levels by phosphodiesterase inhibition induces learning with subthreshold stimuli (McLean et al., 2005). This suggests that the downstream targets of cAMP are involved in neonatal odor preference memory formation. The work in this thesis will elaborate on previous work that has defined the phases of memory formation, STM, ITM, and LTM that occur following a single trial of neonatal odor preference training (see Appendix). It will examine the molecular mechanisms involved in the formation of these memory phases with a specific focus on the US signaling by cAMP. The two known pathways of cAMP signaling will be explored, first, direct activation of the PKA pathway, and, second, the indirect activation of the ERK pathway through Epac activation.

Different phases of invertebrate memory are defined based on their independence or dependence on the activation of transcriptional and translational mechanisms (Ghirardi et al., 1995). Recent experiments from this laboratory (See Appendix) assessed the involvement of the activation of transcriptional and translational mechanisms in different phases of neonatal odor
preference memory. We hypothesized that neonatal odor preference memory would demonstrate similar memory phases as those first seen in *Aplysia*, with neonatal odor preference memory having a translation-independent STM, translation-dependent ITM, and transcription- and translation-dependent LTM. Further, the ability to selectively generate ITM without LTM through reducing the US, as seen in invertebrates (Ghirardi et al., 1995), was also assessed. The existence of the three hypothesized phases and their independence and/or dependence on protein synthetic machinery was confirmed.

The first series of experiments in the thesis (Chapter 2) focuses on the involvement of the activation of PKA in the three distinct phases of short-term, intermediate-term, and long-term neonatal odor preference memory. As discussed above, there are studies in both invertebrates (Castellucci et al., 1980; Castellucci et al., 1982; Chain et al., 1999; Bao et al., 1998; Chang et al., 2000) and mammals (Abel et al., 1997; Bernabeu et al., 1997; Sharifzadeh et al., 2005; Ma et al., 2009; Bourtchouladze et al., 1998; Ahi et al., 2004; Schafe et al., 1999; Schafe and LeDoux, 2000) demonstrating the requirement for PKA activation in LTM formation. Its requirement in ITM has only been examined in *Aplysia* (Sutton and Carew, 2000; Sutton et al., 2001; Antonov et al., 2010) and there are studies in invertebrates and mammals demonstrating both the dependence (Vianna et al., 1999; Vianna et al., 2000; Izquierdo et al., 2000b; Castellucci et al., 1982; Lee et al., 2008; Antonov et al., 2010) and independence (Abel et al., 1997; Schafe et al., 1999; Romano et al., 1996; Fiala et al., 1999; Koh et al., 2002; Schafe and LeDoux, 2000) of STM on PKA activation.
Since cAMP is involved in the formation of neonatal odor preference memory (Cui et al., 2007) and its primary action is to activate PKA (Dell'Acqua and Scott, 1997; Taylor et al., 1990), we hypothesize that PKA activation is required for all phases of neonatal odor preference memory formation. The regulation of the phosphorylation of CREB, which is required for neonatal odor preference memory formation (Yuan et al., 2003a), through PKA activity is also examined as PKA has the ability to translocate and phosphorylate this nuclear target (Bacskai et al., 1993; Delghandi et al., 2005; Arias et al., 1994). Activating PKA in the hippocampus and amygdala enhances the formation of mammalian associative memory (Jentsch et al., 2002; Ma et al., 2009; Bernabeu et al., 1997; Vianna et al., 2000). We hypothesize that activating bulbar PKA will not just enhance neonatal odor preference memory formation leading to its extension, but will produce the memory, acting as the US itself.

The second series of experiments (Chapter 3) examines an alternate US pathway that can be activated by cAMP. cAMP can activate Epac (Kawasaki et al., 1998; de Rooij J. et al., 1998), which leads to the activation of the ERK pathway (Keiper et al., 2004; Lin et al., 2003b; Gelines et al., 2008). The activation of Epac (Ma et al., 2009) and ERK (Eckel-Mahan et al., 2008; Ohno et al., 2001; Zhang et al., 2004; Kelly et al., 2003; Walz et al., 1999; Schafe et al., 1999; Apergis-Schoute et al., 2005; Schafe et al., 2000) is required in long-term associative mammalian memory formation. Long-term mammalian aversive olfactory memory causes the phosphorylation of bulbar ERK, which is required for its formation (Zhang et al., 2003). Epac has not been examined in STM and
ITM paradigms. ERK activity is required for short-term mammalian memory (Igaz et al., 2006), but has only been explored in invertebrates for ITM formation (Sharma et al., 2003a; Sharma et al., 2003b; Lyons et al., 2008; Rosenegger and Lukowiak, 2010). We hypothesize that cAMP can also work through Epac to activate the ERK pathway for the formation of all phases of neonatal odor preference memory. We will examine bulbar ERK activity under learning and non-learning conditions and examine if inhibiting this activity impairs memory formation. The ability of Epac activation to be a sufficient US for the formation of neonatal odor preference memory will also be assessed.
Chapter 2

Increased bulbar PKA activity is necessary and sufficient for neonate intermediate- and long-term odor preference memory, but not required for short-term memory

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2.1 Introduction

Rat pup odor preference learning is an adaptive form of learning critical for organisms that require their mother for protection, warmth, and food during early life. Within the adult mammalian brain, odor pathways are extensive and multiple areas are recruited to facilitate odor learning (Rolls et al., 2003; Staubli et al., 1995; Tronel and Sara, 2002). However, the advantage of rat pup odor preference learning is that many components of the odor pathway are immature, resulting in the elements of odor learning being localized to the olfactory bulbs (Sullivan et al., 2000b).

Odor preference learning can be induced in a single trial of 10 min exposure to novel odor (conditioned stimulus, CS) paired with β-adrenoceptor activation in the olfactory bulb (unconditioned stimulus, US) (Sullivan et al., 1991a; Langdon et al., 1997; Price et al., 1998). The combination of β-adrenoceptor activation, which activates coupled G-proteins, with odor exposure, which stimulates calcium entry through NMDA receptors, activates adenylate cyclase leading to the conversion of adenosine tri-phosphate (ATP) to cyclic adenosine mono-phosphate (cAMP) (Rosenberg and Li, 1995; Yovell and Abrams, 1992; Yuan et al., 2003b). Odor preference learning induces a significant phasic increase in cAMP immediately after training (Cui et al., 2007) and the primary action of cAMP is to activate protein kinase A (PKA) (Taylor et al., 1990; Dell'Acqua and Scott, 1997).
PKA is involved in long-term memory formation in both invertebrates (Barco et al., 2006) and vertebrates (Abel and Nguyen, 2008b). The catalytic subunits of PKA phosphorylate multiple learning-relevant substrates such as serine 845 of the AMPA receptor GluA1 subunit (Banke et al., 2000; Ahn and Choe, 2009) and serine 133 of the transcription factor cAMP response element binding protein (CREB) (Arias et al., 1994; Delghandi et al., 2005; Tao et al., 1998).

The phosphorylation of CREB (pCREB) is implicated as a mediator of learning and memory in fruit flies (Tully et al., 2003; Tully and Quinn, 1985; Yin et al., 1995), Aplysia (Abel and Kandel, 1998), and rodents (Brightwell et al., 2007; Colombo et al., 2003; Kudo et al., 2005; Silva et al., 1998). Previous research from our laboratory demonstrated the requirement for an increase in pCREB within the mitral cells of the olfactory bulb 10 min after learning for the formation of long-term odor preference memory (Yuan et al., 2003a; Yuan et al., 2000; McLean et al., 1999). Knowing that odor preference learning causes an increase in cAMP and CREB phosphorylation, we sought, in the present study, to determine the involvement of PKA in odor preference memory.

We generated both intermediate-term and long-term memory with direct PKA activation as the US. These memories were formed independently of short-term odor preference memory suggesting a PKA-independent mechanism for short-term memory and supporting the hypothesis of parallel memory pathways. We found long-term memory blocked by PKA inhibition could be rescued and
formed apparently independent of PKA, suggesting a compensating molecular pathway. This compensating pathway was also associated with CREB phosphorylation solidifying previous results demonstrating the requirement of pCREB for long-term odor preference memory (Yuan et al., 2003a).

2.2 Materials and Methods

2.2.1 Animals

Sprague-Dawley rat pups of both sexes were used. The dams were maintained under a 12 h light/dark cycle, with ad libitum access to food and water. The litters were culled to 12 pups on postnatal day 1 (PND1). No more than one male and one female pup were used for each condition per litter. All experimental procedures were approved by the Memorial University Institutional Animal Care Committee.

2.2.2 Experimental Design

2.2.2.1 Cannula surgery
On PND5, pups underwent guide cannula placement surgery. The guide cannula was constructed of two 23 gauge/6 mm long stainless steel tubes (Small Parts Inc., WA, USA) set in dental acrylic (Lang Dental, IL, USA) 2 mm apart. Each pup was anaesthetized by hypothermia and then placed in an ice bath in a stereotaxic apparatus (Rumsey et al., 2001). A sagittal incision of the scalp was made from the snout to approximately the level of the lamboid suture. Two holes were drilled through the skull over the olfactory bulbs and the dura was removed gently with fine forceps. The guide cannula was lowered into place such that each olfactory bulb had a stainless steel cannula resting on the top of it. The guide cannula was held in place while it was secured to an upside down plastic screw glued to the skull using dental acrylic. The animal was removed from the ice bath and placed on a heating pad where its scalp was sutured around the cannula. When the pup was resuscitated, bitter apple biting deterrent (Grannick's Bitter Apple Company) was applied to the sutures and cannula to prevent the dam from biting at the area. The pup was then placed back with the dam.

2.2.2.2 Odor-preference learning

Experiments were completed in a sound proof dimly lit room with a temperature of 27°C. On PND6, the pups underwent odor-preference training. The training started with the pup receiving a subcutaneous (s.c.) injection of either saline or isoproterenol (2 mg/kg or 6 mg/kg, Sigma). Thirty minutes after
the s.c. injection of saline or isoproterenol, the pup was removed from the dam and placed in a clean weigh boat for 10 min. The pup was then placed on peppermint scented bedding (500 ml of bedding containing 300 μl of peppermint extract and allowed to air out for 5-10 min for the evaporation of alcohols) for 10 min, where it was allowed to freely move. After the odor exposure, the pup was placed back with the dam.

2.2.2.3 Inhibiting PKA during odor-preference learning

Pups underwent odor-preference learning as described above except 10 min after the isoproterenol or saline injection, the animal received a 1 μl infusion of either saline or Rp-cAMPS (22 mM, dissolved in saline, Sigma) into both olfactory bulbs. The infusion was administered by lowering a 30 gauge/7 mm long stainless steel infusion cannula into each of the guide cannula. The infusion cannula was attached to polyethylene tubing which was, in turn, connected to a 10 μl Hamilton syringe (Hamilton Company). One microliter of solution was infused into each olfactory bulb over a 4 min period. The infusion cannulae were left in the guide cannulae for an additional two min to allow the solution to diffuse throughout the olfactory bulb. The animal was placed back with the dam after the olfactory bulb infusion.

2.2.2.4 Activating PKA to generate odor preference learning
On PND6, odor preference training started with the pups receiving an olfactory bulb infusion (described above in section 2.2.2.3) of either saline or Sp-cAMPs (4.5, 9, 18, or 36 μg/0.5 μl, dissolved in saline, Alexis Biochemicals). Twenty minutes later, the pup was placed on peppermint scented bedding (described above in section 2.2.2.2) for 10 min, where it was allowed to freely move. After odor exposure, the pup was returned to the dam.

### 2.2.2.5 Odor-Preference Testing

For PKA inhibition studies, pups were tested 3, 5, or 24 h after odor exposure for short, intermediate, and long-term odor preference memory respectively. With PKA activation studies, pups were tested 3, 5, 24, 48, 72, or 96 h after odor exposure for short, intermediate, long-term, and extension of odor preference memory, respectively. Each pup was tested at one time point only. The pup was removed from the dam and placed in a clean weigh boat and transferred to a separate testing room that was dimly lit, 27°C, and sound proof. The animal was lowered into the testing chamber (36 cm x 20 cm x 18 cm), which consisted of a stainless steel box with a small grid floor that was covered in a 1000 μm polypropylene mesh (Small Parts Inc., WA, USA), that was separated into two halves by a small neutral zone (no bedding underneath, 2.5 cm x 20 cm). One half of the floor lay over natural bedding, while the other half lay over peppermint-scented bedding. The animal was placed into the neutral zone and the examiner, who was blind to the treatment, recorded the time the animal spent...
over the peppermint scented bedding compared to the natural bedding for a total of 1 min. The animal was given five 1 min trials, switching the direction (away or towards the examiner) the animal faced each time.

2.2.3 Sample Collection /PKA Activity Assay

The pups were given the odor-preference learning procedure. The animals were then killed by decapitation at 5 different time points (immediately before odor exposure, 5 min into odor exposure, immediately after odor exposure, 5 min after odor exposure, and 10 min after odor exposure). The olfactory bulbs were removed from the skull within 2 min of decapitation, frozen on dry ice, and stored at -80°C in microcentrifuge tubes until they were assayed for PKA activity. Olfactory bulbs were homogenized in a microcentrifuge tube with 100 μl of ice cold extraction buffer (PBS containing 1 mM EGTA, 1 mM EDTA, 0.01% Triton X-100, 5 mM DTT, 0.5 mM PMSF, 10 mg/ml Chymostatin, 10 mg/ml Leupeptin, 10 mg/ml Antipain, 10 mg/ml Pepstatin, & 10 mg/ml Aprotinin) using a tissue grinder. The tubes were quickly vortexed and stored on ice, then transferred to a rotator at 4°C for 30 min. The tubes were then centrifuged at 14,000 RPM for 15 min. The supernatant solution was carefully transferred to a clean centrifuge tube and stored on ice, while the pellet was discarded. The protein content of the supernatant solutions was assayed using a Bicinchoninic Acid protein assay kit (Pierce). PKA content was normalized by protein content in each sample. PKA activity in the supernatant was measured
by using a PKA activity assay kit (Millipore/Upstate). Briefly, 20 μg of protein from the supernatant was mixed in a microcentrifuge tube with Kemptide, PKC/CaMK inhibitor cocktail, and Magnesium/ATP cocktail containing [γ-32P]ATP. Background controls contained everything except the supernatant, while negative samples contained everything plus PKA inhibitor cocktail (PKI). Each sample was incubated at 30°C for 5 min and assayed in duplicate by blotting the mixture on P81 paper squares (Clegg et al., 1987). The paper squares were washed 5 X 2 min in 0.75% phosphoric acid and allowed to air dry for 90 min. The squares were then submerged in scintillation fluid in scintillation tubes and read in a scintillation counter for the counts per minute (CPM). PKA activity (expressed as pmol [γ-32P]ATP/min/μg of protein) was calculated relative to the background activity without added supernatant (Isiegas et al., 2006).

2.2.4 Immunohistochemistry

Pups underwent cannula surgery and the odor-preference learning (2 mg/kg, 6 mg/kg Iso or saline) procedures as described above. However, during the olfactory bulb infusion, the pup received Rp-cAMPS (22 mM) in one olfactory bulb while being given saline in the other olfactory bulb. Each pup was perfused 10 min after the peppermint odor exposure, as this was determined to be a critical time point for the phosphorylation of CREB (McLean et al., 1999; Yuan et al., 2000) and the increase in PKA activity. The perfusion procedure was completed as previously reported by this lab (McLean et al., 1999).
Immunohistochemical procedures followed a standard protocol for this laboratory as published previously (McLean et al., 1999; Yuan et al., 2003b). Briefly, 30 μm sections were melted on slides with two alternating slide sets collected. Rabbit phosphorylated CREB (Ser133, Cell Signaling Technology) and phosphorylated PKA substrate (Cell Signaling Technology) antibodies were diluted to 1:500 or 1:1000 in 0.1M phosphate buffered saline with 0.2% Triton X-100, 0.02% sodium azide, and 2% normal goat serum and placed on the tissue for 48 h in a humidified chamber at 4°C. The tissue was removed from the cold room and processed further using the avidin-biotin complex technique (Vectastain Elite, Vector Labs). All sections from the same experiment had identical incubation times in the antibodies and identical exposure to chromogen solutions.

2.2.5 Image Analysis

The density of pCREB staining was analyzed using a Bioquant image analysis system (R&M Biometrics). Slides were coded so the observer was blind to the experiment. The glomerular, mitral cell, and granule cell layers were encircled by visualizing sections of the olfactory bulb on a computer screen using a 4X objective on a Leitz microscope connected to a CCD camera. Light intensity from the microscope was always set at an optical density (OD) of 220. Background correction was performed to provide an even field of illumination. Relative optical density was determined by the optical density of the background region (reading taken from the olfactory nerve layer) subtracted from the optical
density of the area of interest on the tissue (i.e. glomerular, mitral cell, or granule cell layer) divided by the optical density of the background tissue. The relative optical density was obtained from all regions of the main olfactory bulb.

### 2.2.6 Statistical Analysis

A one-way analysis of variance (ANOVA) was used in the animal behaviour analysis to compare drug effects. A Dunnett's post hoc analysis was used to compare the various behavioural training groups to the non-learning saline control group. For the PKA activity assay, the isoproterenol experimental groups were normalized to the saline control within the same time point and analyzed using a one sample T-test. For the relative optical density analysis of immunohistochemistry a repeated measures ANOVA was used followed by Bonferroni post hoc analysis. Statistical significance was taken as \( p < 0.05 \) for all tests.

### 2.3 Results

#### 2.3.1 PKA activity increases 10 min following odor preference training

Single trial odor preference training induces an oscillatory peak in olfactory bulb cAMP levels at the end of the 10 min odor presentation (Cui et al., 2007).
Phosphorylation of PKA substrates CREB (McLean et al., 1999; Yuan et al., 2000; Yuan et al., 2003a), NMDA receptor subunit N1, and AMPA receptor subunit GluA1 (Cui et al., 2011) are highest 10 min following the end of odor presentation and the cAMP peak (Cui et al., 2007). In the present study PKA activity was assessed using a multi-substrate phosphorylation assay in groups sacrificed immediately prior to odor presentation and at 5 min intervals during and up to 10 min following odor presentation. Animals receiving odor+saline served as the baseline standard in each assay run. PKA activity was significantly elevated 10 min following odor presentation in the odor+2 mg/kg isoproterenol group (the learning condition, 36% increase) relative to odor+6 mg/kg isoproterenol (non-learning condition, 6% increase) with PKA activity normalized to the non-learning saline control. No other time points differed (Fig. 2.1). The result replicates the temporal pattern of PKA activity indexed by the PKA substrate phosphorylation measures already described.

2.3.2 Inhibition of PKA disrupts intermediate-term and long-term odor preference memory with no effect on short-term memory

Work from this laboratory has found three different phases of neonatal odor preference memory (Grimes et al., 2011). Short-term memory up to 3 h is translation-independent, while intermediate-term memory occurs around 5 h and is translation-dependent and transcription-independent. Long-term memory at 24 h is both translation- and transcription-dependent. To examine the causal role of
PKA in these distinct phases of odor preference memory, Rp-cAMPs, a competitive inhibitor of PKA, was infused into the olfactory bulbs 10 min after the s.c. injection of isoproterenol and 30 min before odor presentation. PKA inhibition disrupted normal odor preference (2 mg/kg isoproterenol as the US) 24 h after training (Fig. 2.2A) and 5 h after training (Fig. 2.2B), while pups tested 3 h after training displayed odor preference memory with a normal inverted U curve relationship between isoproterenol dosages (Fig. 2.2C).

In rat pups given 6 mg/kg isoproterenol as the US, which normally do not learn (Langdon et al., 1997;Yuan et al., 2000), Rp-cAMPs produced a 24 h odor preference (Fig. 2.2A). In the 3 h memory group (Fig. 2.2C) and 5 h memory group (Fig. 2.2B), no odor preference occurred in the 6 mg/kg isoproterenol group even with prior intrabulbar Rp-cAMPs (Fig. 2.2C). The inhibition of intermediate-term and long-term odor preference memory by Rp-CAMPs suggests intermediate-term and long-term memories require PKA activation, while short-term memory uses alternate molecular pathways. This is the first test of the role of the cAMP/PKA/CREB cascade in short and intermediate-term mammalian odor preference memory.

### 2.3.3 PKA activity in the olfactory bulb under training conditions is strongest in the glomerular and mitral cell layers and is significantly inhibited by infusion of Rp-cAMPs
PKA activity patterns have not been previously described in the olfactory bulb. As seen in Figure 2.3C, PKA activity assessed by a multiple phosphorylated substrates antibody (Sindreu et al., 2007) and immunohistochemistry at 10 min after training, was highest in the glomeruli (gl) and in the mitral cells (mcl). Mitral cell dendrites also exhibited reactivity in the region of lateral dendritic spread (inner external plexiform layer, epl). Reactivity was lower in the granule cell region (gcl). High levels of AKAP-150, a PKA anchoring protein, have been described in the glomeruli and in the lateral mitral cell dendritic area in rats (Glantz et al., 1992) and in the mitral cell bodies in mice (Ostroveanu et al., 2007), which is consistent with the picture here for PKA. A high level of PKA in the granule cell layer reported for AKAP-150 in rats (Glantz et al., 1992), but not mice (Ostroveanu et al., 2007) was not observed. MAP2, which may be the main neuronal PKA anchoring protein (Zhong et al., 2009) has a similar distribution to that seen here for PKA (Philpot et al., 1997) and is located in glomerular mitral cell dendrites at the EM level (Kasowski et al., 1999).

Unilateral infusion of Rp-cAMPs into one bulb versus saline in a control bulb in pups trained with a 2 mg/kg learning dose of isoproterenol produced a significant decrease in PKA activity in all regions of the Rp-cAMPs infused bulb (Fig. 2.3A). In a second set of comparisons, pups received 6 mg/kg isoproterenol. Despite the higher dose of isoproterenol, Rp-cAMPS induced a significant decrease in all regions of the infused bulb relative to the saline infused bulb 10 min after training (Fig. 2.3B). This result suggests Rp-cAMPs inhibited PKA throughout different levels of β-adrenoceptor activity.
2.3.4 Olfactory bulb infusion of Rp-cAMPs decreases CREB phosphorylation, but increased β-adrenoceptor activation rescues CREB phosphorylation

Since phosphorylation of CREB on serine 133 is required for odor preference learning (McLean et al., 1999; Yuan et al., 2003a), it was hypothesized that PKA inhibition would be associated with lower levels of CREB phosphorylation, particularly in non-learning groups. Immunohistochemistry on olfactory bulbs taken 10 min after training from pups given odor + 2 mg/kg isoproterenol revealed a decrease in pCREB in the Rp-cAMPs infused bulb relative to the saline infused bulb as predicted (Fig. 2.4A). Experiments conducted with odor + 6 mg/kg isoproterenol revealed elevated pCREB staining density in the Rp-cAMPs bulb relative to the saline infused bulb (Fig. 2.4B) demonstrating the association of the restoration of learning in the 6 mg/kg condition and elevated phosphorylation of CREB.

2.3.5 Activating PKA generates intermediate-term and long-term memory without short-term memory

The blocking effect of PKA inhibition on odor preference memory at 24 h supports a causal role for PKA in odor preference learning. A causal role was further probed by attempting to induce learning with PKA activation. The PKA activator, Sp-cAMPs, was infused at varying concentrations into the olfactory
bulbs 20 min before odor exposure. Three hours after training there was no evidence of odor preference memory at any concentration of Sp-cAMPS paired with odor (Fig. 2.5A). However, 5 h after training and 24 h after training all groups of pups given a concentration of 9 μg/0.5 μl or higher, showed odor preference memory (Fig. 2.5B and Fig. 2.5C). The lowest concentration of Sp-cAMPs used (4.5 μg/0.5 μl) was ineffective. This pattern of results is consistent with the inactivation data. Both inhibition and activation manipulations suggest PKA has a causal role in intermediate-term and long-term odor preference memory. PKA is neither necessary, nor sufficient, for short-term odor preference memory. These results support a model of independent and parallel memory mechanisms for short-term and longer-term odor preference learning.

2.3.6 Increasing PKA activity extends the duration of odor preference memory

Increases in cAMP levels using phosphodiesterase inhibition have previously been shown to extend the duration of single trial odor preference memory in the rat pup, which is normally limited to 24 h (McLean et al., 2005). In the present study, extended memory durations were assessed when directly activating PKA with varying concentrations of Sp-cAMPS as the US. The two highest concentrations (18 μg/0.5 μl and 36 μg/0.5 μl) were associated with significant odor preference for peppermint 48 h (Fig. 2.6A) and 72 h (Fig. 2.6B) after training. There was no evidence of odor preference at 96 h (Fig. 2.6C) with
the concentrations used. Enhanced activation of PKA alone is sufficient to extend memory duration for odor preference.

2.4 Discussion

2.4.1 PKA activation can act as an US

PKA activity is required for long-term memory formation across many mammalian models (Abel and Nguyen, 2008b). In typical associative memory, the activation of PKA has been shown to promote long-term memory when CS+US pairings are given, but not to create it (Ma et al., 2009; Viola et al., 2000; Muller, 2000). Here we were able to generate associative mammalian memory with direct PKA activation as the sole US. This underscores the requirement of cAMP signaling through PKA within the olfactory bulbs for the formation of odor preference memory. Rat pups are primed for odor memory formation and retrieval for their survival. Special features of early locus coeruleus (Moriceau and Sullivan, 2004; Sullivan et al., 2000b; Rangel and Leon, 1995) and amygdala (Thompson et al., 2008; Moriceau et al., 2006; Sullivan et al., 2000a) functioning result in locus coeruleus activation and/or norepinephrine acting as the US for early odor preference learning. Isoproterenol in the olfactory bulbs will produce preference learning in older pups beyond the critical period (Moriceau and Sullivan, 2004). It will be interesting to examine direct PKA
activation within the olfactory bulbs of older rats in this context. This could provide a memory model and tool to define downstream mechanisms of memory over the lifespan.

2.4.2 PKA activation does not exhibit an inverted U-curve response and can lead to memory extension

When PKA activity was increased with higher activator doses, an inverted U-curve response did not occur. An inverted U-curve is found with β-adrenoceptor activation (Langdon et al., 1997). Consistent with the inverted U-curve for learning was the finding here that PKA activation 10 min following training did not occur with 6 mg/kg isoproterenol as the US. However higher doses of our PKA activator, instead of being less effective, both initiated and extended memory. Memory enhancement through increasing PKA activity has been found in other mammalian memory models (Izquierdo et al., 2000a; Bernabeu et al., 1997; Vianna et al., 2000; Jentsch et al., 2002; Ma et al., 2009), but these also required an additional US and memory extension was not probed. With punishment, memory can be so robust that extension is hard to measure (Izquierdo et al., 2000a). This is an advantage of our model where single trial training just produces 24 h memory, while multiple trials lead to longer memory (Woo and Leon, 1987), permitting us to relate memory extension to altered molecular events within the olfactory bulb.
We hypothesize that these changes in molecular activity would alter synaptic strength in order to cause memory formation and extension. Activating PKA can deliver AMPA receptors to the synapse (Zheng and Keifer, 2009), increase excitation of existing synapses (Banke et al., 2000; Han and Whelan, 2009; Bocchiaro et al., 2003) and activate silent synapses (Ma et al., 1999). Investigation of synaptic changes between the olfactory nerve and mitral cells within odor learning areas (Sullivan and Leon, 1987; Sullivan et al., 1989; Sullivan et al., 1991a; McLean et al., 1999) could illuminate the cellular modifications required for memory formation and extension.

2.4.3 Intermediate-term and long-term memories, but not short-term memory, depend on PKA activation

PKA activation within the olfactory bulbs was not sufficient or necessary for short-term odor preference memory. Inhibiting PKA activity inhibited intermediate-term and long-term memory, but not short-term memory. Activating PKA reinforced these results as it generated intermediate-term and long-term memory without short-term memory. Work in both invertebrate (Fiala et al., 1999; Michel et al., 2008; Muller and Carew, 1998; Romano et al., 1996) and vertebrate (Ma et al., 2009; Abel et al., 1997; Bernabeu et al., 1997; Locatelli et al., 2002; Sharifzadeh et al., 2005; Jentsch et al., 2002; Izquierdo et al., 2000a) memory models have shown that PKA activity is implicated selectively in long-term memory. Some forms of intermediate-term memory are PKA-dependent
within invertebrates (Sutton et al., 2001; Sutton and Carew, 2000), but this is the first demonstration in an associative mammalian memory model. Knowing our intermediate-term memory is translation-dependent (Grimes et al., 2011), suggests PKA could be an important kinase for regulating translation within the olfactory bulb. PKA has been found to regulate local translation in the hippocampus (Nayak et al., 1998) possibly through its regulation of elongation factors (Sutton and Schuman, 2005).

Other short-term mammalian memory models reinforce the present pattern of results in demonstrating PKA activity is not required for short-term memory (Abel et al., 1997; Schafe and LeDoux, 2000; Koh et al., 2003; Goosens et al., 2000) but there is some support for PKA in short-term memory (Runyan and Dash, 2005; Vianna et al., 2000). An alternative mechanism to covalent modification of existing proteins by PKA is the activation of calcium pathways. Ca$^{2+}$/calmodulin dependent kinase 2 (CaMK2) has autophosphorylating capabilities (Lisman et al., 2002; Miller and Kennedy, 1986; Rich and Schulman, 1998; Yang and Schulman, 1999) and can cause receptor insertion (Lisman and Zhabotinsky, 2001; Hayashi et al., 2000), trafficking (Lisman et al., 2002; Hayashi et al., 2000; Malinow and Malenka, 2002), and phosphorylation at Ser831 of the AMPA receptor (Barria et al., 1997a; Derkach et al., 1999; Barria et al., 1997b; Mammen et al., 1997). Unpublished data from our laboratory demonstrate a causal role for this kinase in odor preference memory and it is activated earlier than PKA. The involvement of autophosphorylated CaMK2 in short-term memory
has been shown in other memory models (Zhao et al., 1999; Takahashi et al., 2009; Antonov et al., 2010).

Another possibility for short-term memory support and/or for the long-term memory seen with higher doses of isoproterenol, both occurring in the presence of PKA inhibition, is the activation of Epac. Epac is activated by cAMP increases and activates Rap1 (de Rooij J. et al., 1998; Kawasaki et al., 1998; Ma et al., 2009) which increases the phosphorylation of ERK (Gelinas et al., 2008; Morozov et al., 2003; Lin et al., 2003b; Keiper et al., 2004). Epac can initiate a cellular model of short-term memory, early phase LTP (Gelinas et al., 2008) and ERK is also implicated in some forms of short-term mammalian memory (Igaz et al., 2006). Thus, an increase in the activities of the ERK pathway through β-adrenergic receptor activation and the calcium pathway through novel odor exposure could be responsible for memories occurring in the presence of PKA inhibition.

### 2.4.4 The role of CREB

Mammalian memory models have demonstrated the requirement for the phosphorylation of CREB, for long-term but not short-term memory (Bourtchuladze et al., 1994; Guzowski and McGaugh, 1997; Lamprecht et al., 1997; Kogan et al., 1997; Pittenger et al., 2002; Josselyn et al., 2001). The examination of CREB phosphorylation in the olfactory bulbs here also suggests this transcription factor is required for long-term, but not short-term memory.
formation. The rescuing of long-term memory in the presence of bulbar Rp-cAMPS through increased β-adrenoceptor activation is accompanied by increased CREB phosphorylation 10 min after learning, when PKA activity is inhibited. This suggests an alternate molecular pathway is responsible for CREB phosphorylation. We know cAMP levels are linearly increased with 6 mg/kg isoproterenol (Cui et al., 2007). It is possible that this could increase Epac activity and stimulate ERK. Such a parallel pathway could restore the phosphorylation of CREB and long-term memory while PKA is inhibited. PKA activation appears to require a pulsatile cAMP signal (Cui et al., 2007). CREB phosphorylation is likely not required for intermediate-term memory, consistent with its dependence on translation, not transcription.

2.4.5 Summary

Our work suggests that different phases of a mammalian odor preference memory are formed via different molecular pathways that work in parallel. We determined that an increase in PKA activity is required 10 min after learning for the formation of 5 h intermediate-term and 24 h long-term memory. However, 3 h short-term memory does not require a PKA activity increase. Inhibition of PKA also decreased CREB phosphorylation. However, when long-term memory was rescued through increased β-adrenoceptor activation, CREB phosphorylation was restored apparently without an increase in PKA activity. This underscores the requirement of CREB phosphorylation for our long-term memory (Yuan et al., 81)
2003a; Yuan et al., 2000; McLean et al., 1999), which can apparently also occur through a pathway independent of PKA. Of particular interest was the ability to generate an associative memory by pairing odor and PKA activation directly. The lack of an inverted U curve with PKA activation suggests memory strength and duration may be directly related to PKA activation strength and duration.
Figure 2.1 Learning causes a significant increase in PKA activity 10 min after learning. Pups were subcutaneously injected with varying doses of isoproterenol and sacrificed for removal of olfactory bulbs at different time points during odor preference training. Olfactory bulb tissue was assayed for PKA activity and data was normalized to respective saline control. A one sample two-tail T-test demonstrated that only learning animals (2 mg/kg Isoproterenol + odor) demonstrated a significant increase in PKA activity compared to saline controls. This increase occurred ten minutes after odor presentation, the same time point that an increase in the phosphorylation of CREB occurs. N=5 pups per group. *p<0.05. Error bars indicate SEM.
Figure 2.2 PKA activity is required for 5 h intermediate-term and 24 h long-term memory, but not 3 h short-term memory, while 24 h long-term memory is formed through increased β-adrenoceptor activity. Pups received an olfactory bulb infusion of Rp-cAMPs 10 min after the s.c. injection of isoproterenol. A, Pups were tested 24 h after learning for long-term odor preference memory. A one way ANOVA F(4, 36)=13.339, p<0.0001; N=8-9 per group and Dunnett’s post hoc test comparing experimental groups to the saline/saline non-learning control revealed that inhibiting PKA activity in the olfactory bulbs disrupts long-term memory formation. However, long-term memory was formed with increasing β-adrenoceptor activation. B, 5 h intermediate-term memory is also reliant on PKA activity. A one way ANOVA F(4,15)=24.255, p<0.0001; N=4 and Dunnett’s post hoc test comparing experimental groups to the saline/saline non-learning control revealed that PKA inhibition in the olfactory bulb disrupts intermediate-term memory. However, the memory was not rescued through increased β-adrenoceptor activity. C, 3 h short-term memory does not rely on PKA activity. A one way ANOVA F (4,21)=34.398, p<0.0001; N=5-6 and
Dunnett’s post hoc test comparing experimental groups to the saline/saline non-learning control revealed that inhibiting PKA had no effect on short-term memory formation and the experimental groups demonstrated an inverted U-curve response to β-adrenoceptor activation, which has been demonstrated in previous work without olfactory bulb manipulations (Grimes et al., 2011). *p<0.05, **p<0.01. Error bars are SEM.
Figure 2.3 Olfactory bulb infusion of Rp-cAMPs decreases PKA immunohistochemical activity across multiple layers of the olfactory bulb. A/C, Pups received an olfactory bulb infusion of saline in one bulb and Rp-cAMPs in the other bulb 10 min after a s.c. injection of 2 mg/kg isoproterenol. They were sacrificed 10 min after odor training, which is the time point of significant increase in PKA activity (Fig. 2.1). A repeated measures ANOVA F(5,3)=93.032, p<0.0001 and Bonferroni post hoc test comparing the density of immunoreactivity in the bulbs between conditions revealed a significant decrease in PKA activity in the glomerular layer (gl, N=4,***p<0.001), mitral cell layer (mcl, N=4,***p<0.001), and granule cell layer (gcl, N=4,***p<0.001). B/D, Supra-optimal
\( \beta \)-adrenoceptor activation (6 mg/kg Iso) also produced visible reduction in PKA activity ipsilateral to the Rp-cAMPs infusion. The reduction was found to be significant using a repeated measures ANOVA \( F(5,3) = 35.154, p < 0.0001 \) and Bonferroni post hoc test revealing a decrease in PKA activity in the glomerular layer (gl, \( N = 4, **p < 0.01 \)), mitral cell layer (mcl, \( N = 4, **p < 0.01 \)), and granule cell layer (gcl, \( N = 4, *p < 0.05 \)). Error bars are SEM. Scale bar is 200 \( \mu \)M.
Figure 2.4 Olfactory bulb infusion of Rp-cAMPs decreases the phosphorylation of CREB (pCREB) immunohistochemical expression across multiple layers of the olfactory bulb (A,C). However, pCREB is elevated when long-term memory is rescued by increased β-adrenoceptor activation (B,D). A/C, Pups received an olfactory bulb infusion of saline in one bulb and Rp-cAMPs in the other bulb 10 min after a s.c. injection of 2 mg/kg isoproterenol. Pups were sacrificed 10 min after training, which is the time point of significant bulbar pCREB increase (McLean et al., 1999; Yuan et al., 2000). Olfactory bulb sections were subsequently processed for pCREB immunohistochemistry. A repeated measures ANOVA F(5,4)=15.036, p<0.0001 and
Bonferroni post hoc test comparing the Rp-cAMPs infused olfactory bulb to the saline infused olfactory bulb revealed a significant decrease in pCREB expression in the glomerular layer (gl, N=5, *p<0.05), mitral cell layer (mcl, N=5, **p<0.01), and granule cell layer (gcl, N=5, **p<0.01). B/D, When we examined increased β-adrenoceptor activation that rescued long-term memory, there was an elevation in the phosphorylation of CREB. Pups underwent the same procedure as described above (fig. 2.4A) except they received a s.c. injection of 6 mg/kg isoproterenol instead of 2 mg/kg. A repeated measures ANOVA F(5,4)=18.498, p<0.0001 and Bonferroni post hoc test comparing the Rp-cAMPs infused olfactory bulb to the saline infused olfactory bulb revealed a significant increase in CREB phosphorylation in the glomerular layer (gl, N=5, **p<0.01), mitral cell layer (mcl, N=5, **p<0.01), and granule cell layer (gcl, N=5, **p<0.01). Error bars are SEM. Scale bar is 200 μM.
**Figure 2.5** Activating PKA as an US generated 5 h intermediate-term and 24 h long-term memory, without 3 h short-term memory. Pups received an olfactory bulb infusion of Sp-cAMPs 20 min before odor exposure. **A,** Pups were tested 3 h after training for short-term odor preference memory. A one way ANOVA $F(4,28)=1.657, p=0.188; N=6-7$ and Dunnett’s post hoc test comparing experimental groups to the saline non-learning control revealed that activating PKA in the olfactory bulbs is not sufficient to generate short-term memory. The examination of intermediate-term memory at 5 h (B) and long-term memory at 24 h (C) demonstrated opposite results. In **B** a one way ANOVA $F(4,28)=37.686, p<0.0001; N=6-7$ and Dunnett’s post hoc test comparing experimental groups to the saline non-learning control for 5 h intermediate-term memory revealed that the three higher doses of Sp-cAMPs produced odor preference memory and in **C** a one way ANOVA $F(4,26)=53.759, p<0.0001; N=5-7$ and Dunnett’s post hoc test comparing experimental groups to the saline non-learning control for 24 h long-term memory similarly revealed that activating PKA within the olfactory bulbs as an US at all three higher doses was adequate to generate 24 h long-term memory. No inverted U-
curve response occurred with increasing Sp-cAMPS concentration. **p<0.01. Error bars are SEM.
**Figure 2.6** Increased PKA activity as an US generated memory extension that lasted 72 hours after learning. Pups received an olfactory bulb infusion of Sp-cAMPs 20 min before odor exposure. **A,** Pups tested 48 h after training exhibited an odor preference if given the two highest doses of Sp-cAMPs. A one way ANOVA F(4,21)=22.143, p<0.0001; N=4-6 and Dunnett’s post hoc test comparing experimental groups to the saline non-learning control revealed that increased PKA activity in the olfactory bulbs extended normal 24 h long-term memory. **B,** Pups tested 72 h after training also demonstrated memory extension at the two highest doses of Sp-cAMPs. Again a one way ANOVA F(4,24)=21.947, p<0.0001; N=5-7 and Dunnett’s post hoc test comparing experimental groups to the saline non-learning control revealed a significant odor preference. **C,** However, memory extension was not seen 96 h after training with these doses. A one way ANOVA [F(4,25)=0.3751, p=0.8241; N=5-8 per group] was not significant. **p<0.01.** Error bars are SEM.
Chapter 3

The activation of the Epac/ERK pathway is required for specific phases of associative mammalian olfactory memory
3.1 Introduction

We use the well defined neonatal rat odor preference memory model to examine the molecular mechanisms involved in the formation of three distinct phases of associative mammalian memory consisting of a translation-independent 3 h STM, a translation-dependent 5 h ITM, and translation- and transcription-dependent 24 h LTM (see Appendix). This neonatal memory model is an adaptive form of learning critical for organisms that depend on the dam for thermoregulation, protection, and food during early life. Although adult odor learning may require extensive odor pathways and the recruitment of multiple neural areas (Rolls et al., 2003; Staubli et al., 1995; Tronel and Sara, 2002), the advantage of neonatal odor preference learning is that the elements of learning are localized to the olfactory bulbs due to the immature development of the odor pathways at this stage of life (Sullivan et al., 2000b).

A single 10 min trial consisting of novel odor exposure (conditioned stimulus, CS) paired with β-adrenoceptor activation in the olfactory bulb (unconditioned stimulus, US) induces neonatal odor preference learning (Sullivan et al., 1991a; Langdon et al., 1997; Price et al., 1998). The US demonstrates an inverted U-curve in which β-adrenoceptor activation needs to be optimal (s.c. injection of 2 mg/kg Iso) for learning to occur, as lower and higher activity causes learning impairments (Yuan et al., 2000; Langdon et al., 1997; McLean et al., 2005; Price et al., 1998; Sullivan et al., 1991a). The activation of β-adrenoceptors,
which activates coupled G-proteins, paired with odor exposure, which stimulates calcium entry through NMDA receptors, results in the activation of adenylyl cyclase leading to the conversion of adenosine tri-phosphate (ATP) to cyclic adenosine mono-phosphate (cAMP) (Choi et al., 1992b; Dittman et al., 1994; De Blasi, 1989; Rosenberg and Li, 1995; Yovell and Abrams, 1992; Yuan et al., 2003b) (see figure 1.2). Optimal β-adrenoceptor activation paired with novel odor generates neonatal odor preference learning and induces a significant phasic increase in cAMP immediately after training (Cui et al., 2007). This β-adrenoceptor activation is involved in the formation of all phases of odor preference memory (see Appendix). Since PKA is not involved in the formation of short-term neonatal odor preference memory (Chapter 2), there is a possibility that cAMP works through an alternate target and pathway to generate STM.

A possible alternate target for cAMP is the exchange protein activated by cAMP (Epac). When cAMP binds to the regulatory subunit of Epac, it causes the regulatory subunit to shift and expose the Rap1 binding site on the catalytic subunit (Gloerich and Bos, 2010; de Rooij et al., 2000) (see figure 1.5). Epac is involved in hippocampal-dependent cellular (Gelinas et al., 2008) and behavioural memory (Kelly et al., 2008; Ma et al., 2009). Its activation can promote long-term associative memory when it is coupled with an additional US (Ma et al., 2009). Epac works through Rap1 (de Rooij J. et al., 1998; Kawasaki et al., 1998) to activate the extracellular signal-regulated kinase (ERK) pathway (Lin et al., 2003b; Keiper et al., 2004; Gelinas et al., 2008) (see figure 1.6). The
phosphorylation of ERK is increased immediately after training for hippocampal-dependent memories (Blum et al., 1999; Kelly et al., 2003). ERK phosphorylation has been shown to be required for short-term (Igaz et al., 2006) and long-term (Duvarci et al., 2005; Schafe et al., 2000; Eckel-Mahan et al., 2008; Ohno et al., 2001; Zhang et al., 2004; Kelly et al., 2003; Walz et al., 1999; Zhang et al., 2003) associative mammalian memory. However, its requirement in ITM has only been examined in invertebrates (Sharma et al., 2003b; Sharma et al., 2003a; Lyons et al., 2008; Rosenegger and Lukowiak, 2010). We hypothesize that cAMP can work through Epac to activate the ERK pathway to generate all phases of neonatal odor preference memory.

We found that generating neonatal odor preference memory caused an increase in bulbar ERK phosphorylation immediately after learning that lasted for 10 min. The increased activation of β-adrenoceptors with a higher dose of isoproterenol, which is detrimental for memory formation, causes a second period of increased ERK phosphorylation occurring 1 h after learning. We were able to generate 24 h neonatal odor preference memory through direct activation of bulbar Epac paired with odor exposure, demonstrating that Epac can function as an US. The inhibition of bulbar ERK activity caused ITM and LTM impairments, with no effect on STM suggesting the requirement for an ERK-independent mechanism for STM and supporting the hypothesis of parallel memory pathways. Finally, LTM inhibited by blocking bulbar ERK activity can be rescued through increasing β-adrenoceptor activation, as was previously seen in PKA studies.
This rescued memory does not occur if bulbar ERK and PKA are simultaneously inhibited, demonstrating that these two pathways can cooperate and compensate for each other.

3.2 Materials and Methods

3.2.1 Animals

This study used Sprague-Dawley rat pups of both sexes. The dams had ad libitum access to food and water and were maintained under a 12 h light/dark cycle. On postnatal day 1 (PND1), the litters were culled to 12 pups. No more than one male and one female pup were used for each condition per litter. All experimental procedures were approved by the Memorial University Institutional Animal Care Committee.

3.2.2 Cannula surgery

Pups underwent guide cannula placement surgery on PND5. The guide cannula consisted of two 23 gauge/6 mm long stainless steel tubes (Small Parts Inc., WA, USA) set in dental acrylic (Lang Dental, IL, USA) 2 mm apart. Each pup was anaesthetized by hypothermia and then placed in an ice bath in a stereotaxic apparatus (Rumsey et al., 2001). A sagittal incision of the scalp was
made from the snout to approximately the level of the lamboid suture. Two holes were drilled through the skull over the olfactory bulbs and the dura was removed gently with fine forceps. The guide cannula assembly was lowered into place until each olfactory bulb had a stainless steel cannula resting on the top of it. An upside down plastic screw was glued to the skull, which was secured to the guide cannula assembly using dental acrylic. The animal was removed from the ice bath and placed on a heating pad where its scalp was sutured around the assembly. After the pup was resuscitated, bitter apple biting deterrent (Grannick’s Bitter Apple Company) was applied to the sutures and cannula to prevent the dam from biting. The pup was then placed back with the dam.

3.2.3 Odor-preference learning

Experiments were completed in a sound proof dimly lit room with a temperature of 27°C. On PND6, the pups underwent odor-preference training, which started with the pup receiving a subcutaneous (s.c.) injection of either saline or isoproterenol (2 mg/kg or 6 mg/kg, Sigma). Thirty minutes after the s.c. injection of saline or isoproterenol, the pup was removed from the dam and placed in a clean weigh boat for 10 min. The pup was then placed on peppermint scented bedding (500 ml of bedding containing 300 µl of peppermint extract and allowed to air out for 5-10 min for the evaporation of alcohols) for 10 min, where it was allowed to freely move. After the odor exposure, the pup was placed back with the dam.
3.2.4 Inhibiting ERK during odor-preference learning

Pups underwent odor-preference learning as described above in section 3.2.3 except 10 min before the isoproterenol or saline injection, the animal received a 0.5 μl infusion of either 50% DMSO and ACSF or 5.3 mM U0126 (this compound inhibits ERK through specifically inhibiting its upstream activator MEK, Cayman Chemical, MI, USA), which is dissolved in 50% DMSO and ACSF (10 mM glucose, 2 mM CaCl₂, 126 mM NaCl, 1.2 mM MgCl₂, 25 mM NaHCO₃, 2.5 mM KCl, 1.2 mM NaH₂PO₄, Ph=7.35) (Favata et al., 1998; Duvarci et al., 2005; Apergis-Schoute et al., 2005; Schafe et al., 2000), into both olfactory bulbs. The infusion was administered by lowering a 30 gauge/7 mm long stainless steel infusion cannula into each of the guide cannula. The infusion cannulae were attached to polyethylene tubing which was, in turn, connected to two 10 μl Hamilton syringes (Hamilton Company, NV, USA). 0.5 μl of solution was infused into each olfactory bulb over a 4 min period. The infusion cannulae were left in the guide cannulae for an additional two min to allow the solution to diffuse throughout the olfactory bulb. The animal was placed back with the dam after the s.c. injection.

For the inhibition of bulbar ERK and PKA simultaneously, the same procedure was carried out as described above except 10 min before the s.c. injection of either saline or Iso, the animal received a 0.5 μl infusion of either 50%
DMSO and ACSF or a cocktail solution of 5.3 mM U0126 and 22 mM Rp-cAMPs dissolved in 50% DMSO and ACSF.

3.2.5 Activating Epac to generate odor preference learning

On PND6, odor preference training started with the pups receiving an olfactory bulb infusion (described above in section 3.2.4) of either saline or the highly selective Epac agonist 8-pCPT-2'-O-Me-cAMP (8-pCPT, 0.5, 2.5, 5, or 10 μg/0.5 μl, dissolved in saline, Axxora Biochemicals, CA, USA) (Christensen et al., 2003; Ma et al., 2009; Gelinas et al., 2008). Twenty minutes later, the pup was placed on peppermint scented bedding (described above in section 3.2.3) for 10 min, where it was allowed to freely move. After odor exposure, the pup was returned to the dam.

3.2.6 Odor-Preference Testing

For ERK inhibition studies, pups were tested 3, 5, or 24 h after odor exposure for short-, intermediate-, and long-term odor preference memory respectively. Each pup was tested at one time point only. For simultaneous inhibition of ERK and PKA or Epac activation studies, pups were tested 24h after odor exposure for long-term odor preference memory. The pup was removed from the dam and placed in a clean weigh boat and transferred to a separate testing room that was dimly lit, 27°C, and sound proof. The animal was lowered
into the testing chamber, which consisted of a stainless steel box with a small grid floor that was covered in a 1000 μm polypropylene mesh (Small Parts Inc., WA, USA), that was separated into two halves by a small neutral zone (no bedding underneath). One half of the floor lay over natural bedding, while the other half lay over peppermint-scented bedding. The animal was placed into the neutral zone and the examiner recorded the time the animal spent over the peppermint-scented bedding compared to the natural bedding for a total of 1 min. The animal was given five 1 min trials, switching the direction (away or towards the examiner) the animal faced each time.

3.2.7 Sample collection and protein determination

Olfactory bulb protein for each group that was analyzed was collected from the same litter and determined in the same plate and analyzed in the same gel and same membrane for Western blot analysis. The procedure started with the pups receiving the odor-preference learning procedure described in section 3.2.3. The animals were sacrificed by decapitation at 5 different time points (immediately before odor exposure, immediately after odor exposure, 10 min after odor exposure, 30 min after odor exposure, and 60 min after odor exposure). The olfactory bulbs were removed from the skull within 2 min of decapitation, frozen on dry ice, and stored at -80°C in microcentrifuge tubes until the day of the Western blot procedure. Olfactory bulbs were homogenized in a microcentrifuge tube with 100 μl of ice cold extraction buffer (PBS containing
0.1% SDS, 10% NP-40, 20 mM Tris, 10% glycine, and 1.37 mM sodium chloride with 2 μg/ml leupeptin, 2 mM PMSF, 19 μg/ml aprotinin, 1 mM sodium vanadate, and 2.1 mg/ml sodium fluoride) using a tissue grinder. The tubes were quickly vortexed and stored on ice, then transferred to a rotator at 4°C for 30 min. The tubes were then centrifuged at 14,000 RPM for 15 min. The supernatant solution was carefully transferred to a clean centrifuge tube and stored on ice, while the pellet was discarded. The protein content of the supernatant solutions was assayed using a Bicinchoninic Acid protein assay kit (Pierce, IL, USA). ERK content was normalized by protein content in each sample. The phosphorylation of ERK was assessed in the supernatant through Western blotting.

3.2.8 Western blotting

50 μg of protein was used from the lysates and was prepared using 4 μl of 5X sample buffer (10% SDS, 0.05% bromophenol blue, 0.3 M Tris-HCl, 50% glycerol, and 0.5 M dithiothreitol added just before use) and deionized water was added to bring the total volume up to 20 μl. The samples were then boiled for 5 min. The gels were 7.5% SDS-PAGE, and one color-coded molecular weight ladder (10 μl, Invitrogen, CA, USA) and all the samples were loaded into separate lanes. After loading the samples and molecular weight ladder, the gel apparatus was attached to a Bio-Rad power supply set at 60 mA for 10 min. The current was then reset to 30 mA for ~1h or until the blue line of the sample buffer ran off the gel. The running buffer that the gel was submerged in during this time
consisted of 25 mM Tris, 250 mM glycine, and 3.5 mM SDS at pH 8.8. After the blue line of the sample buffer ran off the gel, the proteins in the gel were transferred to a nitrocellulose membrane (Amersham, PA, USA) for 1 h by being transported to a 4°C cold room and submerged in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) in a transfer apparatus that was set at 350 mA. After the transfer, the nitrocellulose membranes were transported back to room temperature and rinsed 3 x 5 min in TBST (Tris-Buffered Saline Tween-20, 20 mM Tris, 137 mM sodium chloride, 0.1% Tween 20). After rinsing, the membranes were immersed and agitated in 5% milk made in TBST for 1 hr, followed by 3 x 5 min rinses in TBST. The blots were incubated with agitation in a 4°C cold room in 10 ml of 5% milk in TBST containing phosphorylated ERK1/2 (Thr202/Tyr204, 1/1000; Cell Signaling Technology, MA, USA) overnight. The next day, the membranes were removed from the 5% milk and antibody solution and rinsed 5 x 5 min in TBST, followed by a 1 h incubation in goat-anti-rabbit IgG-horseradish peroxidase (1:10000, Pierce, IL, USA) made up in 5% milk in TBST. The membranes were then rinsed 3 x 5 min in TBST, followed by a 5 min immersion in SuperSignal West Pico Chemiluminescent Substrate (Pierce, IL, USA). The membranes were then analyzed in the ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences, QE, Canada). Briefly, the membrane was placed on the Epi tray and set to position 1 (105 mm x 70 mm) and the door of the imager was closed. Next, the detection method of chemiluminescence was selected and the focus adjusted. The exposure type "precision" was selected and exposure time was set to auto with exposure
sensitivity set at high. The exposure images were digitized and saved to the hard drive. The membranes were then exposed to film (AGFA, Mortsel, Belgium) for representative pictures. The images from the biomolecular imager were analyzed using ImageQuant analyzing software which gives optical density readings for all bands on the membrane. These numbers were entered into an Excel program which normalized the experimental groups to the non-learning saline control. The percentages for each condition were analyzed using InStat. After the analysis, the membranes were rinsed in TBST for 3 x 5 min, followed by an immersion in stripping buffer (Restore, Thermo Scientific, UT, USA) with agitation for 15 min at room temperature. The membranes were then rinsed 3 x 10 min in TBST and incubated with agitation in a 4°C cold room in 5% milk in TBST containing total ERK1/2 (1:1000; Cell Signaling Technology, MA, USA) overnight. The next day, the procedures for incubating in secondary antibody and analysis were completed as described above.

3.2.9 Statistical Analysis

A one-way analysis of variance (ANOVA) was used in the animal behaviour analysis to compare drug effects. A Dunnett’s post hoc analysis was used to compare the various behavioural training groups to the non-learning saline control group. For the Western blot experiments, the isoproterenol experimental groups were normalized to the non-learning saline control and analyzed using a repeated measures ANOVA and Dunnett’s post hoc analysis.
comparing the experimental groups to the non-learning saline control. Statistical significance was taken as \( p<0.05 \) for all tests.

3.3 Results

3.3.1 A single early increase in bulbar ERK phosphorylation is associated with odor preference learning

As cAMP undergoes a single phasic increase at the end of training in the olfactory bulbs of pups that learn an odor preference (Cui et al., 2007), we examined the activation of the Epac/ERK pathway in odor preference memory through measuring the phosphorylation of ERK within the olfactory bulbs of learning (2 mg/kg Iso + odor) and non-learning (6 mg/kg Iso + odor) animals. Pups underwent the sample collection and protein determination and Western blotting procedures as described above in sections 3.2.7 and 3.2.8. Repeated measures ANOVAs (Fig. 3.1A: \( F(6,24)=10.546, P<0.0001 \); Fig. 3.1B: \( F(4,16)=3.264, P=0.0388 \)) revealed there was a significant difference in ERK1 phosphorylation between learning and non-learning animals compared to the non-learning saline control (s.c. injection of saline without odor). A Dunnett’s post-hoc test (*\( p<0.05 \), **\( p<0.01 \)) revealed that learning animals (2 mg/kg Iso + odor) had a significant increase in the phosphorylation of ERK1 immediately after training relative to the non-learning saline control (Fig. 3.1A). This increase
remained elevated for 10 min after training, but then returned to control levels 30 min after training where it remained (Fig. 3.1B). However, this differed from non-learning animals (6 mg/kg Iso + odor) where the significant increase in the phosphorylation of ERK1 occurred immediately after training, remained elevated for 10 min (Fig. 3.1A), and then became elevated again 60 min after learning (Fig. 3.1B). There was also a difference in the phosphorylation of ERK2 as repeated measures ANOVAs (Fig. 3.1C: $F(6,24)=7.182, P=0.0002$; Fig. 3.1D: $F(4,16)=4.134, P=0.0173$) revealed a significant difference between learning and non-learning animals compared to the non-learning saline control. A Dunnett’s post-hoc test (*$P<0.05$, **$P<0.01$) revealed that learning animals had a significant increase in the phosphorylation of ERK2 immediately after training relative to the non-learning saline control (Fig. 3.1C). This increase in phosphorylation was not present 10 min later and remained at control levels (Fig. 3.1D). However, this differed from non-learning animals where the significant increase in the phosphorylation of ERK2 occurred immediately after training, stayed elevated for 10 min (Fig. 3.1C) and then became elevated again 60 min after training (Fig. 3.1D).

3.3.2 Inhibition of ERK disrupts intermediate-term and long-term odor preference memory with no effect on short-term memory, while ERK and PKA can compensate for each other to rescue long-term memory
We examined the requirement for the phosphorylation of bulbar ERK through inhibiting ERK in the olfactory bulbs before learning took place as described above in section 3.2.4. We found that inhibiting bulbar ERK had no effect on 3 h STM (one-way ANOVA, $F(4,25)=19.424, P<0.0001$), with the results demonstrating a normal inverted U-curve response (see Appendix). A Dunnett’s post-hoc test (**P<0.01) revealed that the learning control (2 mg/kg Iso + vehicle infusion + odor) and ERK-inhibited learning animals (2 mg/kg Iso + U0126 infusion + odor) significantly differed from the non-learning control (saline injection + vehicle infusion + odor), while ERK inhibited non-learning animals (0 mg/kg Iso + U0126 infusion + odor & 6 mg/kg Iso + U0126 infusion + odor) did not (Fig. 3.2A). The examination of 5 h ITM demonstrated the requirement for bulbar ERK activation (one-way ANOVA, $F(4,25)=12.872, P<0.0001$). Both ERK inhibited learning and non-learning animals did not significantly differ from the non-learning control (Dunnett’s post-hoc, **P<0.01) (Fig. 3.2B). LTM demonstrated a similar result to ITM, where learning was inhibited with the inhibition of bulbar ERK activity (one-way ANOVA, $F(4,15)=59.917, P<0.0001$). Dunnett’s post-hoc analysis (**P<0.01) revealed that ERK-inhibited learning animals did not differ from the non-learning control. However, increased β-adrenoceptor activation, which normally does not produce learning (Yuan et al., 2000; Langdon et al., 1997), caused learning and LTM formation to occur as animals receiving a U0126 infusion with 6 mg/kg Iso and odor significantly differed from the non-learning control (Fig. 3.2C). Previous work found that inhibiting PKA disrupts LTM unless increased β-adrenoceptor activation occurs,
which causes the phosphorylation of CREB to increase while PKA is inhibited (Chapter 2). We hypothesize that cAMP could work through the Epac/ERK pathway to drive transcription and LTM formation when PKA is inhibited. The cooperation and compensation between these two pathways could be occurring in this instance as well, with elevated cAMP from increased β-adrenoceptor activation (Cui et al., 2007) working through the PKA pathway while ERK is inhibited. We examined this cooperation and compensation between the PKA and ERK pathways by conducting a LTM experiment as described above in section 3.2.4, but the animals received an infusion containing both U0126 and 22 mM Rp-cAMPS (a PKA antagonist, Sigma, MO, USA) to block ERK and PKA simultaneously. The one-way ANOVA ($F(4,27)=12.950, P<0.0001$) and Dunnett’s post-hoc analysis (**$P<0.01$) revealed no difference between any of the experimental groups and the non-learning control, even when β-adrenoceptor activation was increased (Fig. 3.2D), suggesting that cAMP works through both the PKA and ERK pathways to generate memory, even under non-physiological conditions, demonstrating both pathways’ ability to compensate while the other is inhibited.

### 3.3.3 Epac is a sufficient US to generate odor preference memory

cAMP stimulates the ERK pathway through its ability to activate Epac (Keiper et al., 2004;Lin et al., 2003b;Gelinas et al., 2008) and we examined this through bulbar infusions of the selective agonist for Epac, 8-pCPT (Christensen
et al., 2003; Ma et al., 2009; Gelinas et al., 2008). A one-way ANOVA
\( F(4,26)=7.412, P=0.0004 \) and Dunnett’s post-hoc analysis (**P<0.01) revealed
that all experimental groups significantly differed from the non-learning control.
This shows that the activation of bulbar Epac as the sole US results in the
generation of odor preference memory at four increasing different concentrations
(Fig. 3.3). This demonstrates that cAMP can activate Epac to generate odor
preference memory and that activating bulbar Epac results in a similar pattern as
seen with direct activation of bulbar PKA (Chapter 2).

3.4 Discussion

The purpose of the present study was to determine if cAMP can work
through the Epac/ERK pathway to generate odor preference memory. We found
that the activation of Epac can generate odor preference memory and ERK
activity is required for the formation of ITM and LTM but not STM. Also, the
activity of ERK occurs immediately after learning and is only associated with
learning if it decreases subsequently and remains at control levels in later
samples, demonstrating the requirement for a temporal pattern of activity similar
to bulbar cAMP (Cui et al., 2007). It is also interesting to discover that the
inhibition of ERK and PKA simultaneously eliminates LTM formed through
increased \( \beta \)-adrenoceptor activation suggesting both molecular pathways are
normally co-operating in generating odor preference learning and either can compensate for the lack of the other, but at least one is necessary.

3.4.1 A short period of bulbar ERK phosphorylation occurs immediately after odor preference learning

The phosphorylation of ERK is increased by many different types of mammalian learning. Sampling ERK phosphorylation at multiple times after learning found that it occurs 1 h after learning in robust fear memory models (Atkins et al., 1998; Schafe et al., 2000; Runyan et al., 2004). However, immediate ERK phosphorylation occurs in hippocampal-dependent memory (Kelly et al., 2003; Blum et al., 1999) and aversive odor learning causes bulbar ERK to be phosphorylated for about 60 min after learning (Zhang et al., 2003). We found that several kinds of molecular signaling occur within a 10 min period after one trial odor preference learning (Yuan et al., 2000; Cui et al., 2007; Grimes et al., 2011; Grimes et al., 2010), which is what is seen with ERK phosphorylation in learning animals in the present study. ERK can activate the transcription factor CREB (Impey et al., 1998a; Roberson et al., 1999; Deak et al., 1998; Davis et al., 2000), which is required for neonatal odor preference learning (Yuan et al., 2003a) and could be a target of the immediate increase of bulbar ERK phosphorylation seen here. However, a non-learning dose of supra-optimal Iso causes a second period of ERK phosphorylation which could increase CREB phosphorylation further. We have found that too much CREB activity is
detrimental to odor preference learning (Yuan et al., 2003a). It is possible that
the second period of increased ERK activity in non-learning animals receiving
supra-optimal US, increases CREB activity at a later time period which could be
detrimental to learning. However, another possible mechanism is that ERK
hyperactivity causes increased GABA release which results in learning
impairments that can be rescued by inhibiting ERK or GABA-dependent activity
(Costa et al., 2002; Cui et al., 2008; Guilding et al., 2007). It is possible that the
increased β-adrenoceptor activity which increases bulbar ERK activity could
potentiate GABA release from granule or periglomerular cells leading to
decreased mitral cell excitation and learning impairments. Increasing GABA
effects from granule or periglomerular cells is detrimental to olfactory memory
formation (Okutani et al., 1999; Okutani et al., 2003). The hyperactivity of ERK
and increased GABAergic activity has been linked to cognitive impairments in
rodents and humans (Guilding et al., 2007; Cui et al., 2008; Costa et al., 2002; Li et
al., 2005; Krab et al., 2008; Ehninger et al., 2008). The pattern of bulbar ERK
activity here is similar to bulbar cAMP signaling in learning animals (Cui et al.,
2007), where ERK phosphorylation is increased immediately after and 10 min
after learning. This would suggest that cAMP signaling rapidly enhances ERK
activity through Epac, however, we cannot say if ERK phosphorylation follows an
identical phasic pattern as cAMP as we did not measure ERK phosphorylation 5
min after learning.
3.4.2 The activation of Epac as a sufficient US implicates an alternate molecular pathway for odor preference memory formation

We examined whether cAMP could activate the ERK pathway for odor preference memory formation solely through the activation of the cAMP target Epac (Kawasaki et al., 1998; de Rooij J. et al., 1998), which leads to the activation of the ERK pathway (Keiper et al., 2004; Lin et al., 2003b; Gelinas et al., 2008) (see figure 1.6). We tested this possibility through bulbar infusions of the selective Epac agonist, 8-pCPT (Christensen et al., 2003; Ma et al., 2009; Gelinas et al., 2008). We found that the activation of bulbar Epac was a sufficient US to generate long-term odor preference memory, demonstrating that cAMP can activate Epac to induce odor preference memory formation. Epac has been demonstrated as a molecular mechanism involved in US signaling in hippocampal-dependent memory (Ma et al., 2009), however, it has never been shown that Epac activation alone is a sufficient US. The activation of bulbar Epac results in a similar behavioural pattern as activating bulbar PKA (Chapter 2). The lack of a dose-dependent inverted U-curve, which is normally seen with β-adrenoceptor activation (Yuan et al., 2000; Langdon et al., 1997), with Epac activation could be due to the possibility that activating downstream molecules such as Epac could bypass the activation of molecules that decrease cAMP signaling such as phosphodiesterases (McLean et al., 2009; McLean et al., 2005) and cause protein dephosphorylation such as phosphatases (Christie-Fougere et al., 2009; Genoux et al., 2002; Malleret et al., 2001; Woo et al., 2002).
Phosphodiesterases may be upregulated with increased β-adrenoceptor activation (Vacas et al., 1985; Ye and O'Donnell, 1996) and we have found that inhibiting the activity of the phosphatase calcineurin causes learning to occur even in the presence of supra-optimal β-adrenoceptor activity (Christie-Fougere et al., 2009) suggesting that increased β-adrenoceptor activity could cause a shift from essential cAMP signaling and increased phosphorylation to detrimental cAMP signaling and dephosphorylation.

3.4.3 Intermediate-term and long-term memories depend on ERK activation

As Epac is involved in neonatal odor preference memory formation, we examined the involvement of the downstream ERK pathway in all phases of neonatal odor preference memory through inhibiting bulbar ERK activity. The inhibition of bulbar ERK resulted in impaired intermediate-term and long-term memory. Work in invertebrate associative (Rosenegger and Lukowiak, 2010) and non-associative (Sharma et al., 2003a; Sharma et al., 2003b; Lyons et al., 2008) memory models has shown a requirement for ERK activation for the formation of ITM. As this memory phase requires translation (see Appendix), ERK could be involved in ITM formation through its ability to mediate the activities of the translational factors eIF4E, 4EBP1, ribosomal protein S6, and mTor (Kelleher, III et al., 2004), giving ERK the ability to directly regulate the translation of existing mRNA which is required for ITM formation (see Appendix).
ERK activity is also required in the amygdala (Duvarci et al., 2005; Schafe et al., 2000), hippocampus (Eckel-Mahan et al., 2008; Ohno et al., 2001; Zhang et al., 2004; Kelly et al., 2003; Walz et al., 1999), thalamus (Apergis-Schoute et al., 2005), and olfactory bulbs (Zhang et al., 2003) for the formation of different long-term mammalian memories. LTM requires the activation of transcriptional and translational mechanisms (see Appendix) and ERK has the ability to regulate the activities of both. When ERK is phosphorylated it can translocate to the nucleus (Martin et al., 1997b; Hu et al., 2004; Fioravante et al., 2006; Shobe et al., 2009; Treisman, 1996; Davis et al., 2000) and regulate transcription (Davis et al., 2000; Impey et al., 1998a; Roberson et al., 1999; Deak et al., 1998) through its ability to stabilize Fos proteins (Murphy et al., 2002), causing robust transcriptional activation. The nuclear translocation of ERK can also result in the phosphorylation of CREB (Davis et al., 2000), causing transcription through CRE-driven gene expression (Athos et al., 2002; West et al., 2002) (see figure 1.7). The inhibition of ERK phosphorylation reduces its nuclear translocation, CREB phosphorylation, and results in impaired long-term mammalian cellular memory (Davis et al., 2000). ERK can also affect LTM memory formation through its ability to regulate synaptic mechanisms. ERK is localized at the synapse through scaffolding proteins (Krapivinsky et al., 2003; Komiyama et al., 2002) where it can regulate local synaptic translation through its modulation of the translational factors eIF4E, 4EBP1, ribosomal protein S6, and mTOR as described above (Kelleher, III et al., 2004). ERK can also alter synaptic excitation through its closure of post-synaptic K⁺ channels (Adams and Sweatt, 2002).
2002; Sweatt, 2004; Watanabe et al., 2002) and its ability to traffic and insert AMPA receptors into the post-synaptic membrane (Zhu et al., 2002) (see figure 1.6).

Previous work found that inhibiting PKA disrupts LTM unless increased β-adrenoceptor activation occurs, which causes the phosphorylation of CREB to increase while PKA is inhibited (Chapter 2). We hypothesize that cAMP could also work through the Epac/ERK pathway to drive transcription and LTM formation when PKA is inhibited. The present study found that increased β-adrenoceptor activation, which normally does not lead to learning (Yuan et al., 2000; Langdon et al., 1997), caused learning and LTM formation to occur when bulbar ERK was inhibited. The uniqueness of LTM is its requirement for transcription (see Appendix) and the fact that the phosphorylation of CREB was restored when this learning occurred in the PKA inhibited condition (Chapter 2) suggests that ERK or PKA may drive this LTM through their common ability to phosphorylate the transcription factor CREB when the other kinase is inhibited. Supporting this idea is the inhibition of memory seen when both kinases are inhibited simultaneously in the olfactory bulbs, demonstrating a cooperation and compensation between the PKA and ERK pathways in which activity in at least one is required for learning. Moreover, normal levels of activity in only one pathway are not sufficient to compensate. This suggests that the extended period of elevated cAMP with increased β-adrenoceptor activation (Cui et al.,
2007) is needed to sufficiently stimulate the uninhibited pathway alone to drive adequate transcription for LTM formation.

The formation of ITM cannot occur through this increased stimulation of just one cAMP pathway. The inhibition studies on PKA (Chapter 2) and ERK have demonstrated that when either one of the pathways is inhibited, then ITM is inhibited at all concentrations of Iso tested. We were able to generate ITM using a PKA agonist as the US (Chapter 2) demonstrating that cAMP signaling is involved in ITM formation. I hypothesize that Epac activation as the US will also generate ITM and should be explored in the future. The work thus far suggests that both the PKA and ERK pathways need to be activated to generate ITM and that activating PKA and Epac together at different concentrations may give us the ability to selectively generate ITM without STM or LTM. Since ITM requires translation of existing mRNA (see Appendix), it is possible that the regulation of local translation factors to form ITM requires the activation of both PKA and ERK.

3.4.4 The ERK pathway is not involved in STM formation

As PKA is not involved in short-term odor preference memory formation (Chapter 2), we hypothesized that STM could be supported through the activation of the Epac/ERK pathway. Epac can initiate a short-term cellular memory, early phase LTP (Gelinas et al., 2008), and ERK is implicated in some forms of short-term mammalian memory (Igaz et al., 2006). However, we found that bulbar
ERK activation is not required for STM which also demonstrated a normal inverted U-curve response (see Appendix & Chapter 2). This is supported with findings from other mammalian memory models (Apergis-Schoute et al., 2005; Schafe et al., 2000; Blum et al., 1999; Kelly et al., 2003; Schafe et al., 1999), including aversive short-term odor memory (Zhang et al., 2003), which demonstrate that STM can form independently of ERK. Since STM does not require transcription or translation (see Appendix), an alternative mechanism for STM formation is the covalent modification of existing proteins by the Ca$^{2+}$ pathway. Ca$^{2+}$/calmodulin dependent kinase 2 (CaMK2), which is also implicated in long-term odor preference memory (de Jong et al., 2008), has autophosphorylating capabilities (Lisman et al., 2002; Miller and Kennedy, 1986; Rich and Schulman, 1998; Yang and Schulman, 1999) and can cause AMPA receptor insertion (Lisman and Zhabotinsky, 2001; Hayashi et al., 2000), trafficking (Lisman et al., 2002; Hayashi et al., 2000; Malinow and Malenka, 2002), and phosphorylation of its Ser831 site (Barria et al., 1997a; Derkach et al., 1999; Barria et al., 1997b; Mammen et al., 1997). The involvement of autophosphorylated CaMK2 in short-term memory has been shown in other memory models (Zhao et al., 1999; Takahashi et al., 2009; Antonov et al., 2010).

3.4.5 Summary

Our work suggests that different phases of a mammalian odor preference memory are formed via different molecular pathways that work in parallel. We
determined that a 10 min increase in ERK activity is required immediately after learning for the formation of 5 h intermediate-term and 24 h long-term memory, but not 3 h short-term memory. When long-term memory was rescued through increased β-adrenoceptor activation, it could be eliminated through the simultaneous inhibition of bulbar ERK and PKA. This demonstrates the cooperation and compensation that these pathways can exhibit under non-physiological conditions to restore memory formation. Of particular interest was the ability to generate an associative memory by pairing odor and Epac activation directly. The lack of an inverted U curve with Epac activation suggests memory strength and duration may be directly related to the strength of Epac activation and its duration.
Figure 3.1 Odor preference learning causes immediate activation of ERK that decays quickly, while non-learning animals demonstrate two periods of ERK activation. A/B. The phosphorylation of ERK1 increases immediately after learning and remains elevated for 10 min in learning animals (2 mg/kg Iso + odor), after which it returns to normal control levels. However, in non-learning animals (6 mg/kg Iso + odor) the phosphorylation of ERK1 increases at two separate time periods. It increases immediately after learning and remains elevated for 10 min after which it returns to normal control levels, but then becomes elevated again 1 h after learning. C/D. The phosphorylation of ERK2 increases immediately after learning and returns to normal control levels 10 min later in learning animals. However, in non-learning animals the phosphorylation of ERK2 increases at two separate time periods. It increases immediately after learning and remains elevated for 10 min after which it returns to normal control levels, but then becomes elevated again 1 h after learning. E/F. A representative immunoblot of phosphorylated ERK1/2 (1:1000, Cell Signaling) under learning (2 mg/kg Iso + odor) and non-learning (6 mg/kg Iso + odor) conditions. G/H. A representative immunoblot of total ERK1/2 (1:1000, Cell Signaling) under learning and non-learning conditions demonstrates no change in total ERK between conditions and across all time points. n=5 for all groups. Data are expressed as phosphorylated ERK normalized to total ERK and expressed as percentage relative to saline non-learning control ran in the same experiment. Error bars are SEM. *P<0.05, **P<0.01.
Figure 3.2 The activation of bulbar ERK is required for 5 h ITM and 24 h LTM, but not 3 h STM, while ERK and PKA can compensate for each other to rescue LTM. 

A, An olfactory bulb infusion of U0126 50 min before learning does not affect 3 h STM formation (n=6 for all groups). This ERK inhibition had no effect on learning (2 mg/kg Iso + odor), resulting in a normal inverted U-curve response. 

B, An olfactory bulb infusion of U0126 50 min before learning causes the inhibition of 5 h ITM formation (n=6 for all groups). This ERK inhibition disrupted normal learning (2 mg/kg Iso + odor)
resulting in ITM being reduced to non-learning control levels. C, The olfactory bulb infusion of U0126 50 min before learning also caused the inhibition of 24 h LTM formation (n=4 for all groups). The inhibition of bulbar ERK before learning disrupts normal learning (2 mg/kg Iso + odor) and inhibits LTM formation. However, learning for 24 h LTM is restored with increased β-adrenoceptor activation (6 mg/kg Iso). D, An olfactory bulb infusion of U0126 and Rp-cAMPs 50 min before learning causes the inhibition of 24 h LTM formation (sal/sal, n=6; 2 mg/kg Iso/sal, n=6; sal/U0126+Rp-cAMPs, n=6; 2 mg/kg Iso/U0126+Rp-cAMPs, n=7; 6 mg/kg Iso/U0126+Rp-cAMPs, n=7). The inhibition of both bulbar ERK and PKA before learning inhibits normal learning (2 mg/kg Iso + odor) and learning that is achieved with increased β-adrenoceptor activation (6 mg/kg Iso) when either ERK or PKA is inhibited. Data are expressed as mean +/- SEM. **P<0.01.
Figure 3.3 Epac activation is a sufficient US to generate LTM. An olfactory bulb infusion of 8-pCPT 20 min before odor exposure generates 24 h LTM (sal, n=7; 0.5 µg 8-pCPT, n=6; 2.5 µg 8-pCPT, n=5; 5 µg 8-pCPT, n=6; 10 µg 8-pCPT, n=7), demonstrating the bulbar Epac activation is a sufficient US to generate neonatal odor preference memory. No inverted U-curve response occurred which is what was seen with the activation of another cAMP target, PKA, as the US (Chapter 2). Data are expressed as mean +/- SEM. **P<0.01.
Chapter 4

Summary and General Discussion

4.1 Neonatal odor preference learning forms three distinct phases of memory

There is evidence in invertebrates demonstrating that not all phases of memory are the same in terms of molecular initiation. STM and LTM are different due to independence or dependence on protein synthesis, respectively. Another interesting aspect of the temporal analysis of memory is the report of a novel intermediate-term phase of memory that is dependent on translation, but independent of transcription. Further analysis found that these three memory phases could be generated selectively by altering the concentration of serotonin exposure (Ghirardi et al., 1995). ITM within mammalian models has been implied but not directly tested (Taglialatela et al., 2009). We found that neonatal odor preference memory consists of at least three distinct memory phases as was seen in the invertebrate analysis. Specifically, the bulbar infusions of ACTI and ANI dissected neonatal odor preference memory as predicted, demonstrating a translation-independent STM that lasts for about 3 h, a translation-dependent ITM that occurs around 5 h after training, and a transcription- and translation-dependent LTM lasting for 24 h. Infusing ANI at different times after training showed that the activation of learning-critical protein synthesis mechanisms occurs during training and lasts for about 1 h after, whereas an infusion of ANI 3
h after training has no effect in inhibiting LTM. To bring this mammalian model of memory even closer to invertebrate memory models, we tested our ability to selectively generate ITM without LTM. Decreasing the US of β-adrenoceptor activation through a s.c. injection of 1.5 mg/kg Iso causes the formation of a 5 h ITM without the presence of a 24 h LTM (see Appendix).

Neonatal odor preference memory requires the translation of new proteins to induce an ITM, while the transcription of new mRNA is required for LTM. It is important to now ask what proteins are critical for the formation of both translation- and transcription-dependent memory. They need not be the same. A proteomics approach could be helpful here since changes are confined to the bulb and control conditions can be quite specific.

4.2 The PKA and ERK pathways are required for longer protein synthesis dependent phases of memory

The formation of long-term neonatal odor preference memory requires a specific temporal increase in cAMP levels occurring immediately after learning (Cui et al., 2007) and the phosphorylation of CREB occurring 10 min after learning (Yuan et al., 2003a; Yuan et al., 2000). cAMP directly activates the PKA pathway (Dell'Acqua and Scott, 1997; Taylor et al., 1990), while it indirectly activates the ERK pathway through its activation of Epac (Keiper et al., 2004; Lin et al., 2003b; Gelinas et al., 2008). Learning within invertebrate (Muller and
Carew, 1998; Bergold et al., 1990; Chain et al., 1999; Khabour et al., 2004) and mammalian (Vazquez et al., 2000; Mizuno et al., 2002; Cammarota et al., 2000; Sindreu et al., 2007) memory models causes the activation of PKA, which is required for LTM formation (Abel et al., 1997; Bernabeu et al., 1997; Sharifzadeh et al., 2005; Ma et al., 2009; Bourchouladze et al., 1998; Ahi et al., 2004). Epac activation is also involved in long-term mammalian cellular (Gelinas et al., 2008) and behavioural (Ma et al., 2009) memories, while mammalian hippocampal-dependent memory causes an increase in the phosphorylation of ERK immediately after learning (Blum et al., 1999; Kelly et al., 2003), which is also required for LTM formation (Eckel-Mahan et al., 2008; Ohno et al., 2001; Zhang et al., 2004; Kelly et al., 2003; Walz et al., 1999; Zhang et al., 2003).

The involvement of PKA in STM is demonstrated in invertebrates (Ghirardi et al., 1992; Castellucci et al., 1982; Schacher et al., 1988; Chang et al., 2000; Byrne and Kandel, 1996; Ghirardi et al., 1995) but is less clearly implicated in mammals where short-term memory testing has demonstrated both their independence (Schafe et al., 1999; Schafe and LeDoux, 2000; Goosens et al., 2000) and dependence (Vianna et al., 1999; Vianna et al., 2000; Izquierdo et al., 2000b) on PKA activity. The requirement for ERK activity in short-term mammalian memory is also indecisive as different memory models are dependent (Igaz et al., 2006) or independent (Kelly et al., 2003; Blum et al., 1999; Izquierdo et al., 2000a; Schafe et al., 1999; Schafe et al., 2000; Zhang et al., 2003) of this activity. PKA (Sutton and Carew, 2000; Sutton et al., 2001; Antonov et al., 2010) and ERK (Sharma et al., 2003a; Sharma et al., 2003b; Lyons et al., 2012).
2008; Rosenegger and Lukowiak, 2010) have only been explored in invertebrates for the formation of ITM, which has demonstrated their requirement.

Odor preference training caused a significant increase in bulbar PKA activity 10 min after training, which is the same time point as bulbar CREB phosphorylation (Yuan et al., 2000). Inhibiting PKA activity selectively impaired ITM, LTM, and bulbar CREB phosphorylation. However, the LTM could be restored through increased β-adrenoceptor activation, which caused the restoration of CREB activity without the activation of PKA. This suggests that an alternate compensating pathway is involved in this memory formation. The exploration of the alternate Epac/ERK pathway found that neonatal odor preference learning caused a significant increase in bulbar ERK phosphorylation immediately after training. Further, if ERK activity was inhibited then ITM and LTM were impaired but there was no effect on STM. However as with the inhibition of PKA, we see the restoration of LTM through increasing β-adrenoceptor activation while ERK is inhibited. When we inhibited ERK and PKA simultaneously, memory restoration was eliminated demonstrating that each pathway can compensate for the other inhibited pathway to restore LTM.

These antagonist studies suggest that the three phases of short-term, intermediate-term, and long-term odor preference memory are truly separate entities. We were able to generate STM without ITM and LTM and LTM without STM and ITM. The fact that ITM could not be generated under any Iso concentration tested when either the PKA or ERK pathway was inhibited.
suggests that both pathways need to be activated to some degree for ITM formation. Future focus should be on the generation of ITM without STM or LTM, which I hypothesize, requires a small amount of activation of the PKA pathway and the ERK pathway together.

Activating bulbar PKA is a sufficient US resulting in the generation of ITM and LTM, but not STM. Further, this PKA activation also extended odor preference memory to 72 h after learning. This work further demonstrated that PKA is not involved in the alteration of existing proteins for the formation and support of STM. It is selectively involved in the formation of longer phases of memory that require protein synthesis, suggesting that PKA may regulate this synthesis. The examination of the ability of cAMP to activate Epac to generate neonatal odor preference memory found that the activation of bulbar Epac is a sufficient US to generate neonatal odor preference memory. This suggests that both PKA and ERK are involved in the regulation of protein synthesis suggesting that the US of cAMP signaling is very precise in its involvement in the formation of longer protein synthesis dependent phases of memory. This leads me to propose that the formation of STM may be reliant on the activity of the calcium pathway, which is influenced by the CS of novel odor exposure.
Increased Activity | 3 h STM | 5 h ITM | 24 LTM
--- | --- | --- | ---
PKA = 10 min After Odor | PKA Independent | PKA Dependent | PKA Dependent
ERK = Immediately After Odor | ERK Independent | ERK Dependent | Epac & ERK Dependent

**Figure 4.1** The summary table shows the current evidence of the involvement of two molecular pathways in the olfactory bulb for three distinct phases of neonatal odor preference memory. Bulbar ERK and PKA activity are increased immediately after and 10 min after odor preference training in learning animals, respectively. Short-term memory formation does not depend on the activation of the PKA or ERK pathways. Intermediate-term memory and long-term memory both depend on the activation of the PKA and ERK pathways. The two molecular pathways of PKA and Epac/ERK are involved in longer protein synthesis dependent memory phases but not shorter protein synthesis independent memory.
4.3 General Limitations

Specific limitations for the experiments undertaken in this thesis were discussed in each chapter, however, there are some general overall limitations that merit discussion.

4.3.1 The assumption that the molecular signaling involved in neonatal odor preference learning occurs through β1-adrenoceptor activation

Several experiments were completed using a s.c. injection of a general β-adrenoceptor agonist (Iso) as the US. We know from past studies that the activation of β1-adrenoceptors or α1-adrenoceptors, but not β2-adrenoceptors or α2-adrenoceptors, is sufficient to generate neonatal odor preference memory (Harley et al., 2006). From this past research we assume that Iso is working through the β1-adrenoceptors to generate neonatal odor preference memory. However, we have not directly tested this hypothesis by administering a specific β1-adrenoceptor agonist, such as dobutamine, as the US for the undertaken molecular studies. Thus, there could be different molecular signaling occurring from the activation of both β1 and β2 adrenergic receptors due to the s.c. injection of Iso compared to the activation of β1-adrenoceptors alone.

4.3.2 Does the molecular signaling that is involved in neonatal odor preference learning also apply to adolescent and adult memories?
The work we have completed is in neonatal rats. We hypothesize that the molecular signaling we have discovered in neonatal odor preference learning and memory formation would be very similar for olfactory learning in adult animals that are not dependent on the dam. There is work that demonstrates that the development of auto-inhibitory α2-adrenoceptors in the locus coeruleus prevents the formation of odor preference learning in these older animals. However, manipulating β-adrenoceptor signaling directly in the olfactory bulb is necessary and sufficient for generating odor preference learning in these older animals (Moriceau and Sullivan, 2004), suggesting that the molecular signaling we have described downstream of this receptor activation is still involved in older animals. However, this has not been directly tested. There could be changes in receptor and molecular dynamics in adult animals which we are unaware of. However, our ability to generate odor preference memory through the direct bulbar activation of PKA or Epac now gives us a valuable molecular tool to test the downstream signaling for memory formation in mature animals.

4.3.3 Does neonatal odor preference memory form serially or in parallel?

The serial processing of ‘consolidating’ short-term memories into long-term memories is a popular ideology in the study of memory. This would suggest that STM must occur to form ITM and ITM must occur to form LTM. However, there is work that suggests that memory phases do not form in a serial fashion, but that they are separate entities that form in parallel to each other (Izquierdo et
al., 1998; Emptage and Carew, 1993; Lukowiak et al., 2000; McGaugh, 2000). Our work seems to agree with the later theory as we were able to generate STM without LTM and vice versa. Specifically, inhibiting either PKA or ERK caused STM to be present without ITM or LTM. This still could suggest a serial processing mechanism as it might not be enough molecular activation for longer phases of memory, but enough for STM. However, if we increased β-adrenoceptor activation, then we were able to generate LTM without STM or ITM (Chapter 2 & 3). PKA agonist studies demonstrated that we were able to generate ITM and LTM without STM (Chapter 2) suggesting that these three phases of neonatal odor preference memory may not be linked and are separate entities that form in parallel to each other. I will mention that the final piece that would solidify this idea is if we were able to generate ITM without STM or LTM. Altering the level of β-adrenoceptor activation causes the formation of ITM without LTM (see Appendix), suggesting that ITM is its own separate phase, however, STM was not tested. We hypothesize that these three memory phases are sensitive to cAMP signaling and focusing on its downstream targets may be the key to isolating them. Since PKA or ERK inhibition eliminates ITM at all Iso concentrations tested, it suggests that both pathways need to be activated for ITM formation. Activating PKA and Epac together at different concentrations may give us the ability to selectively generate ITM without STM and LTM and give us the definitive result demonstrating that these three memory phases are truly independent of each other.
4.3.4  Does neonatal odor preference learning only require one period of molecular activity?

The process of ‘consolidating’ short-term memories into long-term memories occurs over hours after learning (McGaugh, 2000). There is variation in identifying the number of consolidation periods that occur after learning to solidify LTM. Invertebrate (Epstein et al., 2003) and mammalian (Richter et al., 2005) memory models have demonstrated two phases of protein synthesis dependent-consolidation occurring immediately and 3-6 h after learning, while the same was shown with PKA activity in mammalian memory (Vianna et al., 2000; Bernabeu et al., 1997). We have examined PKA, ERK, and translational activity at varying time points after neonatal odor preference learning. We found that PKA activity is increased 10 min after learning, while ERK and translational activity is increased immediately after learning and decays 10 min or 1 h later, respectively. We suggest that only this single period of molecular activity occurs to initiate 24 h neonatal odor preference memory. However, we did not explore PKA activity after its increase so we do not know if its activity is maintained, returns to baseline, or appears again at a second time point. With the examination of ERK and translational activity, we do not know if there is a second period of activation that occurs around 3-6 h after learning. The two periods of molecular activity that have been seen in previous models appear robust and required for the formation and duration of their memories. Work in invertebrates
(Fulton et al., 2005) and mammals (Robinson and Franklin, 2007) revealed that LTM can be formed from a single consolidation period occurring immediately after training. It is possible that the pairing of the CS and US during neonatal odor preference learning is enough stimulation to generate one phase of molecular activity that is sufficient to generate all phases of odor preference memory. However, we cannot say this definitively without examining molecular signaling at later time points (e.g. 3h and 5h) after learning.

**Future directions**

4.4 The formation and maintenance of short-term neonatal odor preference memory could occur through the CS pathway

The examination of short-term neonatal odor preference memory has revealed that the US pathway of cAMP signaling seems not to be involved. It is possible that the covalent modifications needed for synaptic strengthening and STM formation and maintenance occur through the CS pathway of Ca\(^{2+}\) signaling. The generation of neonatal odor preference memory causes the activation of NMDA receptors (Yuan et al., 2000) and increased Ca\(^{2+}\) influx into the mitral cells (Yuan, 2009). Examining the molecular activity required for STM formation has revealed that the downstream target of Ca\(^{2+}\)/calmodulin, CaMK2, is required for some forms of STM (Zhao et al., 1999; Takahashi et al.,
CaMK2 could sustain covalent modifications for STM formation through its ability to autophosphorylate (Lisman et al., 2002; Miller and Kennedy, 1986; Rich and Schulman, 1998; Yang and Schulman, 1999), which causes an extended period of activation without elevated levels of Ca$^{2+}$/calmodulin. When CaMK2 is activated, it binds to NMDA receptors which results in anchoring AMPA receptors to the synapse (Lisman and Zhabotinsky, 2001; Hayashi et al., 2000). Activated CaMK2 also phosphorylates AMPA receptors at serine 831 (Barria et al., 1997a; Derkach et al., 1999; Barria et al., 1997b; Mammen et al., 1997), which causes their insertion (Lisman and Zhabotinsky, 2001; Hayashi et al., 2000) resulting in the synapse becoming potentiated (Malinow and Malenka, 2002). AMPA receptor activity in neonatal odor preference memory has revealed that the PKA-specific phosphorylation of AMPA receptors does not sustain activity long enough for 3 h STM. However, it was found that the insertion of AMPA receptors into the synapse could be the mechanism for both later STM and LTM formation in neonatal odor preference learning (Cui et al., 2011). CaMK2 could be the kinase responsible for this increased membrane insertion due to the molecular characteristics described above and the fact that it has been shown to be required for long-term neonatal odor preference memory formation (de Jong et al., 2008), which is dependent on the insertion of AMPA receptors into the membrane (Cui et al., 2011). The examination of CaMK2 activity for STM formation should be examined in neonatal odor preference learning. CaMK2 could phosphorylate AMPA receptors for a longer period of time than PKA, or it could increase the post-synaptic
localization and insertion of AMPA receptors, and either or both could extend synaptic potentiation for the formation of short-term neonatal odor preference memory.

4.5 Modifying synaptic connections could maintain long-term neonatal odor preference memory

4.5.1 Neonatal odor preference memory could be sustained through synaptic PKA activity counteracting phosphatase activity

Exploration of the apical dendrites of mitral cells could lead to the discovery of the molecular mechanisms of synaptic strengthening and neonatal odor preference memory formation. The formation of LTF in Aplysia causes immediate and significantly greater increases in cAMP levels and PKA activity in the processes of the cell compared to the soma (Bacskai et al., 1993). PKA is localized at the synapse through its binding to anchoring proteins, which also bind and localize protein phosphatases (Dell'Acqua et al., 2006). Protein phosphatase 1 (PP1) can counteract the activity of PKA causing the dephosphorylation of synaptic proteins leading to the inhibition of memory formation. However, PKA can counteract PP1 by phosphorylating and activating the endogenous inhibitor of PP1, inhibitor-1 (Blitzer et al., 1998; Blitzer et al., 1995; Greengard et al., 1999; Hemmings, Jr. et al., 1984). At the synapse PKA
can counteract PP1 activity leading to the induction of LTP (Blitzer et al., 1998; Blitzer et al., 1995), while the knockout of hippocampal PKA resulted in impaired L-LTP in the CA1, which was rescued through the inhibition of PP1 (Woo et al., 2002). Dephosphorylation of inhibitor-1 by calcineurin caused increased activation of PP1 and the induction of long-term depression (Mulkey et al., 1994), while the genetic inhibition of calcineurin enhanced the expression of LTP (Malleret et al., 2001). Our examination of calcineurin revealed that decreasing its activity enhanced odor preference memory formation leading to its extension (Christie-Fougere et al., 2009). This could occur through increased inhibitor-1 activity and decreased PP1 activity, as learning and memory enhancement was also seen with the direct reduction of PP1 activity (Genoux et al., 2002). PKA can regulate both positive and negative molecular signaling at the synapse, and this synaptic regulation likely needs to be optimally tuned to initiate neonatal odor preference memory formation. Also, the memory enhancement seen with decreasing PP1 activity could be a mechanism underlying memory extension with increased bulbar PKA activity.

Another means by which PKA can modify the synapse is through the formation of a synaptic tag. After transcription and translation occurs within the neuron, the newly synthesized mRNAs and proteins are delivered throughout the cell but are only functionally incorporated in synapses that have been tagged by previous synaptic activity to potentiate and strengthen the synapse for LTM formation. Weak synaptic activation that causes the induction of E-LTP, forms a synaptic tag for about 3 hours which causes the synapse to capture the
synthesized mRNAs and proteins, leading to its potentiation and formation of L-LTP (Frey and Morris, 1997; Frey and Morris, 1998; Barco et al., 2002). Work in Aplysia (Casadio et al., 1999) and mice (Barco et al., 2002) has demonstrated that PKA is involved in this molecular tagging signal to mark stimulated synapses for the capture of newly synthesized products of gene expression. The inhibition of PKA blocked the synaptic capture of L-LTP, while the activation of PKA produced this synaptic capture and maintained a persistent facilitation at the synapse (Young et al., 2006). PKA could form this synaptic tag through its reduction of PP1 as this caused an increase in CREB phosphorylation (Genoux et al., 2002), which lead to CRE-driven gene expression and the production of proteins that can tag the synapse (Barco et al., 2002). Synaptic tagging should be examined within the apical dendrites of mitral cells that are activated by the learned odor, as this could be both a molecular and cellular mechanism for the formation of long-term neonatal odor preference memory.

4.5.2 Post-synaptic excitation can be regulated through K⁺ channel and NMDA receptor activity

The strengthening of a synapse could also involve the effects that intracellular kinase signaling has on K⁺ channels. Voltage-dependent K⁺ channels of the Kv4.x family regulate dendritic membrane depolarization through regulating the effects of dendritic back propagating action potentials (Birnbaum et al., 2004). K⁺ channels are subject to modulation through both PKA and β-
adrenoceptor activation, however, these modulations are dependent on ERK activation such that the inhibition of ERK causes increased K⁺ channel activity reducing the excitation of the synapse. ERK decreases the activity of K⁺ channels through phosphorylating the alpha subunit, which alters the channel properties causing the requirement for a greater depolarization to open (Adams et al., 2000; Yuan et al., 2002a; Morozov et al., 2003; Watanabe et al., 2002; Birnbaum et al., 2004). K⁺ channels can modulate NMDA receptor activity through controlling the membrane potential that the NMDA receptor detects. The reduction of K⁺ channel activity enhances membrane excitation resulting in increased NMDA receptor activation (Watanabe et al., 2002; Birnbaum et al., 2004). The NMDA receptor is activated during neonatal odor preference learning (Yuan et al., 2000). Increased CREB activity, which is required for neonatal odor preference memory (Yuan et al., 2003a), causes an increase in NMDA receptor expression (Marie et al., 2005). PKA is coupled to NMDA receptors, through an A-kinase anchoring protein (AKAP), allowing it to readily modulate receptor channel activity (Westphal et al., 1999). Inhibiting PKA within the hippocampus reduces both the calcium permeability of NMDA receptors and calcium signaling in active dendritic spines. This PKA regulation of NMDA receptor currents was more prominent in neurons cultured from immature animals (Skeberdis et al., 2006), suggesting that NMDA receptor regulation by PKA may be an important molecular mechanism for neonatal odor preference learning.

Neonatal odor preference learning is likely to cause synaptic enhancement between the olfactory nerve and the apical dendrite of the mitral
cell. We know that β-adrenoceptor, PKA, ERK, and CREB activation are all required for long-term odor preference memory. It is possible that these mechanisms enhance the olfactory nerve and mitral cell synapse through decreasing K⁺ channel activity and increasing NMDA receptor activity. Cellular modeling of neonatal odor preference memory has shown that activation of the olfactory nerve in combination with β-adrenoceptor activation causes an increased influx of Ca²⁺ into the mitral cells (Yuan, 2009) and increased mitral cell firing (Lethbridge et al., 2011). This suggests that neonatal odor preference learning causes enhanced NMDA receptor activation on the post-synaptic mitral cell dendrites, which could occur through the molecular signaling of PKA, ERK, CREB, and K⁺ channel activity.

4.5.3 New synaptic connections can be established through the activation of silent synapses

The maintenance of long-term neonatal odor preference memory could occur through the formation of new synaptic connections. Work in *Aplysia* demonstrated that pre-existing presynaptic varicosities that are empty of synaptic vesicles become active during LTF. These empty varicosities are filled with synaptophysin and synaptic vesicles 3-6 h after the induction of LTF. These newly activated presynaptic varicosities account for approximately 32% of newly activated synapses seen 24 hours after LTF induction (Kim et al., 2003). However, within the mammal, silent synapses refer to excitatory glutamatergic
synapses whose postsynaptic membrane contains no AMPA receptors. Even though the membrane contains NMDA receptors, the lack of AMPA signaling at the synapse renders it silent under normal conditions (Malinow et al., 2000). However, this silence can be eliminated through synaptic stimulation, which activates silent synapses through the insertion of AMPA receptors into the membrane. The activation of CaMK2 causes it to bind to NMDA receptors resulting in additional sites to anchor AMPA receptors (Lisman and Zhabotinsky, 2001; Hayashi et al., 2000). When CaMK2 is active, it phosphorylates AMPA receptors (Barria et al., 1997a; Derkach et al., 1999; Barria et al., 1997b; Mammen et al., 1997), which causes their insertion (Lisman and Zhabotinsky, 2001; Hayashi et al., 2000) and renders the synapse not only active but potentiated (Malinow and Malenka, 2002). The activation of these new silent synapses may involve the synthesis of new CaMK2 (Ouyang et al., 1999; Huang et al., 2002) and AMPA subunits (Nayak et al., 1998), possibly through the activation of CREB (Marie et al., 2005). CaMK2 (de Jong et al., 2008), CREB (Yuan et al., 2003a), and AMPA receptor insertion (Cui et al., 2011) are required for long-term neonatal odor preference memory, suggesting that the activation of silent synaptic connections through these mechanisms could be a cellular change that occurs for the formation, maintenance, and extension of LTM.

4.6 Transcription could be regulated through histone acetylation
Histone acetylation is the process of transferring acetyl groups to histones, which causes the release of DNA from the histone complex resulting in the activation of transcription (Levenson and Sweatt, 2006). Work in *Aplysia* has shown that facilitatory stimuli leads to acetylation, while inhibitory stimuli causes deacetylation and that histone acetylation is involved in the maintenance of LTF (Guan et al., 2002). Mammalian contextual fear conditioning also leads to an ERK-dependent histone acetylation in CA1 of the hippocampus as the activation of ERK in CA1 caused histone acetylation, while its inhibition blocked it (Levenson et al., 2004). The activation of CREB recruits CREB binding protein (CBP) which acts as an acetyl-transferase and leads to histone acetylation in both *Aplysia* (Guan et al., 2002) and mammals (Alarcon et al., 2004). CBP activity is required for hippocampal dependent L-LTP and long-term spatial and contextual fear memory (Wood et al., 2005), while eliminating the ability of CBP to act as an acetyl-transferase impairs long-term memory formation (Korzus et al., 2004). The enhancement of histone acetylation through the inhibition of the deacetylating enzyme, histone deacetylase (HDAC), facilitated the induction of LTF (Guan et al., 2002). Mammalian studies have shown similar results with HDAC inhibitors enhancing LTP induction in the Schaffer collateral pathway of the hippocampus (Alarcon et al., 2004; Levenson et al., 2004), and the formation of hippocampal (Levenson et al., 2004) and amygdala-dependent (Yeh et al., 2004) fear conditioning. As histone acetylation is a mechanism of LTM formation and maintenance in a broad range of memory models, it is a possible novel mechanism that should be studied within the MOB for the formation of neonatal
odor preference memory. Since neonatal odor preference memory is reliant on both ERK (Chapter 3) and CREB (Yuan et al., 2003a) activity, this highlights possible bulbar molecular mechanisms that can regulate histone acetylation, the enhancement of transcription, and LTM formation.

4.7 Other transcription factors besides CREB could be involved in neonatal odor preference memory formation

Neonatal odor preference learning requires the phosphorylation of CREB (Yuan et al., 2003a), which has the ability to activate other transcription factors (Conkright et al., 2003; Zhang et al., 2005) leading to multiple transcriptional mechanisms working together. Other transcription factors such as Ets-like protein (Elk-1), c-fos, Zif268, or NF-kb can also be involved in the transcriptional regulation of long-term synaptic changes and memory (Sgambato et al., 1998; Albensi and Mattson, 2000; Izquierdo and Cammarota, 2004; Tischmeyer and Grimm, 1999; Davis et al., 2000; Davis and Laroche, 2006). We have shown that the activation of ERK is required for ITM and LTM formation (Chapter 3). The phosphorylation of ERK directly activates the transcription factor Elk-1, which leads to serum response element (SRE) activation (Marais et al., 1993; Hipskind et al., 1994; Wasylyk et al., 1998). The promoter region of the Zif268 gene contains 2 CRE sites and 6 SRE sites (Beckmann and Wilce, 1997), demonstrating that Zif268 can be controlled by PKA or ERK though the activation of CREB or by ERK through the activation of Elk-1 (Bozon et al., 2003; Davis and
Laroche, 2006; Thomas and Huganir, 2004). The induction of hippocampal LTP causes the rapid phosphorylation of ERK and its translocation to the nucleus resulting in Elk-1 phosphorylation. Inhibiting ERK phosphorylation resulted in decreased Elk-1 and CREB phosphorylation and inhibited LTP-dependent transcriptional activation of Zif268 in dentate gyrus granule cells, which resulted in impaired LTP induction (Davis et al., 2000). The parallel activation of CREB and Elk-1 by ERK has been seen in the hippocampus after one-trial avoidance learning (Cammarota et al., 2000), while the retrieval of fear memory causes the activation of Zif268 in the CA1 or amygdala (Hall et al., 2001). As neonatal odor preference memory causes and requires the activation of bulbar ERK (Chapter 3) and CREB (Yuan et al., 2003a; Yuan et al., 2000), special focus should be given to the molecular regulation of the transcription factors Elk-1 and Zif268 as a requirement for these factors has also been shown in a wide range of mammalian memories.

4.8 Concluding remarks

The data presented in this thesis extends the existing literature regarding mammalian memory formation. We have found three distinct phases of mammalian memory that follow the molecular characteristics described previously in invertebrate models. We have extended this analysis by examining the enzymatic activity that is involved in the formation of these distinct memory
phases. From this work we have shown that memory does not necessarily transition in a serially dependent fashion from STM into LTM. The fact that we are able to generate ITM and LTM without STM, STM without ITM or LTM, and LTM without STM and ITM suggests that these memory phases undergo formation separately and parallel to each other. As noted, evidence suggesting separate and parallel mechanisms for the formation of both STM and LTM is the ability to generate LTM without the presence of STM. We were able to do this on two separate occasions through the selective generation of LTM by inhibiting bulbar PKA or ERK and increasing β-adrenoceptor activation. We also found that this LTM formation occurred through the activation of the other uninhibited compensating pathway. These results in combination with the activation of bulbar PKA not generating STM suggests that the PKA and ERK molecular pathways are involved in the formation of longer-term memories that require protein synthesis. To better understand the role of each pathway it would be useful to look at STM and ITM when both pathways are blocked. That remains for a future study. Hopefully, this work will inspire people to examine the less studied memory phases of STM and ITM in mammals, as it is essential to clarify how mammalian memory forms over time. Further analysis of the involvement of the PKA and Epac/ERK signaling pathways in protein synthesis regulation may reveal the mechanisms for LTM formation and maintenance. Defining this molecular signaling may lead to specific targets that can be manipulated for the rescue and restoration of memory that is in an inhibited or impaired state.
A.1 Introduction

In 1995 Kandel's group, investigating cAMP/PKA-dependent synaptic sensitization in *Aplysia* cell cultures (Ghirardi et al., 1995), reported a novel intermediate phase of sensitization that was blocked by disruption of protein translation using anisomycin, but that was not blocked by disruption of protein transcription using actinomycin D. This intermediate form could be selectively demonstrated using serotonin concentrations higher than those that elicited short term sensitization, which was insensitive to any disruption of protein synthesis, and lower than those that induced long-term sensitization (24 h), which could be disrupted by both the transcription inhibitor actinomycin D and the translation inhibitor, anisomycin. Subsequently, behavioral sensitization with three distinct time phases (short-term memory, STM; intermediate-term memory, ITM; long-term memory, LTM) was demonstrated in intact *Aplysia*, with ITM outlasting STM by more than an hour (Sutton et al., 2001). The same unconditioned stimulus protocols delivered in a reduced preparation showed ITM was sensitive to inhibition of protein translation, but not protein transcription (Parvez et al., 2006b). These were the first mechanistic studies of multiphasic memory. A protein synthesis-independent ITM has also been described in *Aplysia* and is distinguished, in part, from translation-dependent ITM by dependence on protein kinase C rather than protein kinase A (Sutton et al., 2004).
Associative memory also exhibits a protein translation-dependent ITM. This has been characterized in some detail using operant conditioning in the pond snail (Lymnea) (Parvez et al., 2006a). Following 30 min of training an operant ITM is seen at 3 h, which is blocked by the translation blocker anisomycin, but not the transcription blocker actinomycin D. With one hour of training, memory is seen at longer intervals, up to and including 24 h, and this LTM is blocked by both translation and transcription inhibitors of protein synthesis (Sangha et al., 2003). When the soma critical for inducing pond snail LTM is removed, operant ITM is still observed, providing additional evidence that translation in neurites provides the support for ITM (Scheibenstock et al., 2002). ITM has also been reported in a classical conditioning paradigm using light as the conditioned stimulus (CS) in Hermissenda. Again this ITM is translation, but not transcription, dependent as revealed by selective protein synthesis inhibitors (Crow et al., 1999).

The discovery of protein synthesis machinery and mRNAs near synaptic sites (Steward and Levy, 1982), which are regulated, as first proposed, by synaptic activation (for a recent review see (Bramham and Wells, 2007) and behavioral work with learning and memory in mutant mice with impaired local mRNA targeting (Miller et al., 2002) point to a role for local protein translation in mammalian memory. However, translation dependent ITM has not yet been described in vertebrates. Translation-dependent ITM in rodents has been assumed in a novel object recognition paradigm, but has not been tested with a translation inhibitor (Taglialatela et al., 2009).
The neonate rat shows both short-term and long-term odor preference memory initiated by activation of the cAMP/PKA cascade. Neonate rat odor preference learning depends on the activation of β-adrenoceptors in the olfactory bulb (Sullivan et al., 2000b; Harley et al., 2006). Activation of β-adrenoceptors (as the unconditioned stimulus, US), paired with a novel odor (as the conditioned stimulus, CS), is both necessary and sufficient for the expression of a preference for the odor 24 h later (Sullivan et al., 1991a). We (Langdon et al., 1997; Rumsey et al., 2001; Yuan et al., 2003a) and others (Moriceau and Sullivan, 2004) have shown that manipulations limited to the olfactory bulb are sufficient to induce or prevent early odor preference learning.

In the present experiments, intrabulbar infusions of protein synthesis inhibitors for translation (anisomycin) and transcription (actinomycin) given immediately after training were used to explore the protein synthesis requirements of 3 h, 5 h and 24 h odor preference memory in rat pups. We also varied the dose of the US (isoproterenol, Iso) to probe the induction of ITM without LTM in this model.

A.2 Materials and Methods

A.2.1 Subjects
Subjects were Sprague-Dawley pups. Litter effects were controlled by using one male and one female for a training condition within a litter. Animals were maintained on a 12 h light/dark cycle with food and water available *ad libitum*. All experiments were approved by the Institutional Animal Care Committee of Memorial University and followed the standards of the Canadian Council on Animal Care.

A.2.2 Surgery

On postnatal day (PND) 5, pups were anesthetized by hypothermia and the skull over the olfactory bulbs exposed. A small plastic screw (Small Parts Inc., USA) was glued upside down caudally and two holes 2 mm apart were drilled over the central portion of the bulbs. A pair of 23 gauge stainless steel guide cannulae, 6 mm long, was lowered until it rested on the surface of the olfactory bulbs. Dental acrylic secured the cannula assembly to the plastic screw. A biting deterrent gel (Four Paws Ltd., NY) was applied to the sutured skin to minimize maternal disturbance. Pups were returned to the dam within 30 min from the start of surgery.

A.2.3 Training

On PND 6 pups were removed from the dam to receive a 50 μl s.c. injection of either sterile saline or 1, 1.5 or 2 mg/kg Iso (Sigma Chem) and then
returned to the home cage for 30 min. The pup was then placed into a clean weigh boat for 10 min away from the dam. Next, it was placed in a polycarbonate cage (30 cm x 19 cm x 13 cm) containing peppermint-scented bedding (0.3 ml peppermint extract in 500 ml fresh bedding) for 10 min in a separate room maintained at 27°C. In many experiments, the 10 min odor exposure was followed by olfactory bulb infusion of saline, anisomycin, or actinomycin D as described below. After infusion, the pup was returned to the dam.

A.2.4 Macromolecular synthesis inhibitor infusions

Insect pins blocking the 23 gauge guide cannula were replaced with 7 mm long 30 gauge stainless steel tubes attached to PE 20 polyethylene tubing and a 10 μl Hamilton syringe secured in a syringe pump (Razel Instruments). Bilateral infusion of 1.25 μl of a blocker into each bulb was made over a two min period either immediately after training (anisomycin, actinomycin D) or 1 or 3 h after training (anisomycin). The infusion cannulae were left in place for an additional 2 min to allow the solutions to diffuse throughout the olfactory bulbs.

Anisomycin (100 μg/μl, Alexis Biochemicals, San Diego, USA) was prepared by mixing 3.2 mg with 14.8 μl of phosphate buffered saline (PBS), followed by 1.15 μl of equimolar HCl and an additional 14.8 μl of PBS. The pH was readjusted to 7.4. Prior to infusion, frozen aliquots of anisomycin were thawed on ice and sonicated for 20 min to ensure the compound was dissolved.
and mixed. The choice of infusion concentration was based on the work of Schafe and LeDoux (Schafe and LeDoux, 2000). Their work had shown a similar concentration injected in a smaller volume and targeting the lateral amygdala blocked fear memory when given immediately after a single auditory fear conditioning trial. However, with their concentration, Schafe and LeDoux also reported failure of learned freezing at 1 h, but not at 4 h. They attributed this early failure to protein synthesis-dependent impairment of sensory processing at 1 h. Thus, there was some concern here that impairment of olfactory function might compromise the separation of STM and LTM effect in the rat pup. However, rat pups infused with anisomycin in the olfactory bulbs immediately after learning exhibited intact STM, allaying these concerns.

A careful investigation of protein synthesis inhibition with an intrahippocampal dose of 62.5 μg/0.5/μl anisomycin (Wanisch and Wotjak, 2008) provides evidence of more than 90% inhibition of protein synthesis 30 min after infusion with significant depression of protein synthesis continuing for 6 h and recovery to control levels by 9 h.

Actinomycin D (2.5 μg/μl, Alexis Biochemicals, San Diego, USA) was prepared fresh by dissolving 1 mg of actinomycin in 200 μl of sterile saline and 200 μl of 50% dimethyl sulfoxide followed by 30 min sonication to dissolve and mix the compound prior to infusion. This is similar to the dose (5 μg/0.8μl) used by Yang and Lu (Yang and Lu, 2005) in the amygdala which blocked d-cycloserine facilitation of extinction and had also been shown to block fear
conditioning memory at 24 h (Lin et al., 2003a), but not extinction itself (Lin et al., 2003a; Yang and Lu, 2005). Otani et al. (Otani et al., 1989) had shown that 5 µg/µl actinomycin D infused at 2 µl/min in the lateral ventricle produces 95% inhibition of RNA synthesis in the dorsal dentate gyrus measured at the end of 30 min, the earliest time point assessed. A drawback of microgram per microliter concentrations of actinomycin D is the possibility of toxic effects at 24 h (e.g. see (Rizzuto and Gambetti, 1976b); using 10 µg). However, these effects are not typically seen at early time points and for the current study it was important to ensure substantial suppression of RNA synthesis in the first hours after training. Proven nontoxic nanogram per microliter doses of actinomycin D produce only ~40% suppression of RNA synthesis 2 h after injection, the earliest time point tested (Bailey et al., 1999). Finally, in the Yang and Lu study with 5 µg/.8µl infusions, rats exhibit normal extinction levels 24 and 48 h after actinomycin D and normal potentiation of extinction by cycloserine after an actinomycin D infusion that blocked earlier cycloserine potentiation suggesting no permanent loss of function due to their concentration, which is somewhat higher than that used in the present study.

A.2.5 Testing

Pups were tested 1, 3, 5 or 24 h after odor preference training. Pups from each litter were only tested at one time point and training groups were run one at a time. Pups were given an odor preference test in a quiet room at 27°C.
A stainless steel testing box (36 x 20 x 18 cm) with a mesh bottom was centered over two trays. The trays were 2 cm apart, creating a neutral zone in the center. One tray contained 500 ml of fresh bedding while the other contained 500 ml of peppermint scented bedding prepared at the same concentration used during training. The tester was blind to the previous training procedure given to the pup.

Each pup underwent five one minute trials, starting in the neutral zone and alternately facing towards or away from the tester with each subsequent trial. When the pup's snout and one paw moved from the neutral zone to either the peppermint or control zone, a timer for that side was started. Pups were given thirty second rest periods between trials. Summation of the time spent over the peppermint side divided by the total time active (time spent over peppermint plus time spent over control) gave the percent time over peppermint. Thus, preference for the conditioned odor was measured by the percent of time spent by the pup over the peppermint odor.

A.2.6 Statistics

A one-way analysis of variance (ANOVA) determined the difference among experimental groups and the Dunnett’s post hoc test was used for comparisons with the Iso + saline (plus odor) group as the control.
A.3.1 Immediate post-training anisomycin

Interference with protein-translation by intrabulbar anisomycin infusion immediately after the 10 min training trial blocked expression of ITM and LTM, but not STM. Memory was tested at four time points with separate groups of pups: 1 h, 3 h, 5 h and 24 h (see Figure A.1 for percent time spent by pups over peppermint odor in memory tests). Odor preference memory in the anisomycin-infused groups was normal when tested 1 h or 3 h after training. ANOVAs for short term memory tests were: 1 h groups, \( F_{[3, 14]} = 18.91, p < 0.0001 \), and 3 h groups, \( F_{[3, 24]} = 10.95, p < 0.0001 \). The non-learning control group (saline + saline infusion) in the 1 h and 3 h tests was significantly different (\( p < 0.05 \)) from the learning control group (2 mg/kg Iso + saline infusion). Finally, pups given odor and a 2 mg/kg Iso injection + anisomycin were not significantly different from the learning group (odor and 2 mg/kg Iso+saline, \( p > 0.05 \)) in the 1 h and 3 h tests. This result also suggests odor sensing itself was not impaired by anisomycin.

Odor preference memory was blocked in the immediate anisomycin-infused groups tested at 5 h or 24 h after training (Fig. A.1). ANOVAs for longer term memory tests were: 5 h group, \( F_{[3, 26]} = 23.55, p < 0.0001 \), and 24 h group, \( F_{[3, 14]} = 17.59, p < 0.0001 \). Again, the non-learning control pups spent significantly less time over the peppermint than the learning control pups (\( p < 0.01 \)). However, now the 2 mg/kg Iso injection + anisomycin infusion condition
differed significantly from the learning control (p < 0.01) and did not show evidence of odor preference. Odor preference memories appear to switch from not requiring post-training olfactory bulb protein translation for memories of 3 h or less to requiring post training translation for memories of 5 h or longer.

**A.3.2 Anisomycin 1 h or 3 h post-training**

Interference with protein-translation by intrabulbar anisomycin infusion beginning 1 h or 3 h after training indicates the requirement of 24 h term memory for bulbar protein synthesis is diminished 1 h after training and no longer present at 3 h (Fig. A.2). The inhibition of protein synthesis in the olfactory bulbs of pups at 1 h post-training (Fig. A.2A) produced odor preferences at 24 h (~57% time over peppermint) that fell between those of learning (Iso+saline, 75% time over peppermint) and non-learning controls (saline+saline ~24%, saline+anisomycin ~32% time over peppermint) \((F_{[3, 20]} = 6.16, p < 0.005)\). Four of the 2 mg/Iso+anisomyin pups spent 68-89% of the test time over the conditioned odor, a normal memory result, while 3 pups spent 4-44% of the time over the conditioned odor, a range characteristic of non-learning. This suggests one hour after training is near the limit of the interval in which new protein synthesis (via translation) must occur for 24 h memory.

When anisomycin was infused 3 h after training (Fig. A.2B), odor preference memory was normal in the group receiving the protein synthesis
inhibitor ($F_{[3, 12]} = 25.33, p < 0.0001$). The 2 mg/kg Iso+saline learning group (spending ~75% time over peppermint) differed significantly from the saline+saline non-learning control group (spending ~20% time over peppermint, while the 2 mg/kg Iso injection + anisomycin experimental group (spending ~66% time over peppermint) did not differ from the learning group. The non-learning control group for anisomycin (saline+ansiomycin spending ~27% time over peppermint) did differ from the learning group as expected (non-trained pups do not like the smell of peppermint). This suggests the proteins required for LTM are synthesized prior to 3 h after training. It also argues that the earlier lack of 24 h memory with immediate post training anisomycin was not due to an odor detection impairment caused by a delayed effect of anisomycin.

A.3.3 Immediate post-training actinomycin D

Interference with protein transcription by intrabulbar actinomycin D infusion immediately after the 10 min training trial prevented expression of LTM, but not ITM. Transcription (Fig. A.3A) is not required for 5 h ITM, ANOVA ($F_{[3, 20]} = 13.13, p < 0.0001$). The Iso + actinomycin group was not significantly different from the learning (Iso + saline) group when pups were tested for odor preference 5 h after training. These results imply that memory seen 5 h after training can be supported through the translation of existing mRNA within the olfactory bulb. It also demonstrates that this dose of actinomycin D did not prevent normal olfactory discrimination at 5 h after delivery.
The inhibition of transcription through olfactory bulb infusions of actinomycin D immediately after training did prevent 24 h memory (Fig. A.3B) with the ANOVA showing a significant group effect ($F_{[3, 42]} = 3.75, p < 0.05$). All conditions examined (saline + saline, Iso + actinomycin, or saline + actinomycin) differed significantly from the learning control ($p < 0.05$). Twenty-four hour odor preference memory requires transcription as well as translation (anisomycin experiments).

A.3.4 An intermediate unconditioned stimulus elicits intermediate-term memory

Varying the strength of the unconditioned stimulus resulted in a dose-dependent length of memory for the conditioned odor. One-way ANOVA revealed a group effect for odor preference memory for both 5 h ($F_{[3,40]} = 3.82, p < 0.05$) and 24 h ($F_{[3,15]} = 53.32, p < 0.0001$) memory. Post hoc Dunnett’s tests revealed that a dose of 1.5 mg/kg Iso and 2.0 mg/kg Iso produced odor preferences significantly different from the non-learning control (Fig. A.4A) at 5 h, while only the 2 mg/kg Iso dose induced an odor preference memory at 24 h (Fig. A.4B).

A.4 Discussion

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These results are consistent with the hypothesis that a protein translation-dependent intermediate term memory exists in a cAMP/PKA-dependent mammalian learning model, paralleling what was described first in invertebrates. Translation-dependent, but not transcription-dependent, cAMP-initiated long-term synaptic plasticity has also been identified in mammalian hippocampus. The activation of β-adrenoceptors in CA1 induces a translation-dependent long-term potentiation mediated by Epac rather than by the cAMP/PKA cascade (Gelinas et al., 2008). A translation-dependent long-term depression has also been reported in CA1 (Manahan-Vaughan et al., 2000). Whether Epac (as in the Gelinas experiments) or PKA (as in the earlier Aplysia experiments) plays the pivotal role in the present ITM remains to be investigated.

The ability to elicit an ITM, without a LTM, shown here with an intermediate dose of isoproterenol, also replicates earlier findings in the invertebrate models (Lukowiak et al., 2000; Sutton et al., 2002). Since we can now elicit an ITM in isolation, it will be possible to probe whether or not ITM can be boosted to LTM (for example by 24 h spaced presentations) suggesting a serial component to this multi-phasic memory (as in the pond snail (Parvez et al., 2005) or whether the two processes operate in parallel (see Izquierdo et al., 2002).

Recently, systemic anisomycin given to 6 day rat pups immediately after an hour of odor+mild shock pairings (14 total), which also produces an odor preference, was shown to prevent memory at 24 h as seen here (Languille et al.,
2009), but anisomycin did not affect preference memory at 4 h (see also (Schafe and LeDoux, 2000) for a similar effect with fear conditioning). The choice of a 5 h testing point in the present study may have been fortuitous for detecting translation-dependent odor preference memory since translation dependence was not seen at 3 h, while 4 h was not tested. Nonetheless, the odor preference training paradigm used here and that used by Languille et al. differ in other outcomes. The memory they induce with repeated shock lasts at least 6 days since pups tested at 12 d show memory, while our single 10 min odor pairing no longer influences odor preference 48 h after training unless steps are taken to boost memory (Christie-Fougere et al., 2009; McLean et al., 2005). Additionally, the odor preference Languille et al. induce reverses to an odor aversion by day 12. The avoidance memory seen with shock pairings at day 12 was also blocked by anisomycin immediately after training on day 6 suggesting both the appetitive and aversive memory are part of the same process (Languille et al., 2009).

Extending the present odor + Iso (or tactile stimulation) training using spaced trials to induce a multi-week memory does not lead to reversal of the original odor preference (Sullivan et al., 1989; Woo and Leon, 1987).

Temporally, the important translation events for LTM here are confined to ~ 1 h after training since anisomycin given at 1 h post training had mixed effects on LTM; with a subgroup of pups failing to learn, while others were successful. There was no significant effect of anisomycin given at 3 h on LTM (see also Languille et al. (2009) as discussed earlier). A similar short time window for ‘consolidation’ using anisomycin has been reported in pond snail (Fulton et al., 160
In conditioned taste aversion learning in 3 day old rat pups, anisomycin prevents 24 h memory when given 1 h, but not 6 h, after training (Gruest et al., 2004). Interestingly, in rat pups, the 'consolidation' window for conditioned taste aversions shows a developmental progressive decrease in the temporal window for anisomycin interference. While effective 1 h after training on day 3, it is only disruptive immediately after training on day 10, and by day 18, even with immediate injection, anisomycin disruption of LTM is only partial (Languille et al., 2008). Thus, translation-dependent protein synthesis may occur quite rapidly with learning in adult rodents and the very brief time window for disruption could account for the failure to observe mammalian translation-dependent ITM previously.

We set out, initially, to characterize the role of protein synthesis in our memory model. Our results appear consistent with a canonical view of that role, i.e. a role in LTM but not STM, and with the update that there is a translation-, but not transcription-dependent, ITM (see Fig. A.6 for a summary diagram). However, there is currently a controversy about whether or not protein synthesis has any unique role in learning and memory (for a recent review see (Gold, 2008). The antibiotic tools used each have other effects that could provide alternate explanations of their actions.

Of particular importance for our rat pup model is evidence that local infusion of anisomycin in the amygdala causes a dramatic increase in monoamines including norepinephrine, followed by a marked reduction, and that
infusing norepinephrine prior to an inhibitory learning task produces the same amnesia (at 48 h) seen with pretraining anisomycin (Canal et al., 2007). The memory block by anisomycin can be reduced by propranolol implicating β-adrenoceptor over-activation in the amnesia (Canal et al., 2007). A β-adrenoceptor agonist, isoproterenol, is the US in our rat pup odor preference learning and varying dosages produce an inverted U curve for effectiveness in inducing learning. When isoproterenol is given at doses higher than the optimal 2 mg/kg (e.g., 4 or 6 mg/kg), LTM is not seen (Langdon et al., 1997).

Overactivation of β-adrenoceptors by anisomycin could account for a loss of LTM. However, as seen here, and in Languille et al. 2009 (and in other fear conditioning models, e.g., Schafe and LeDoux, 2000), anisomycin does not disrupt STM. We tested STM with a 6 mg/kg Iso US that would not produce LTM. Unlike the pattern of results with anisomycin, this high dose of a β-adrenoceptor agonist prevented STM (Fig. A.5). It appears unlikely that anisomycin is acting solely via over-activation of β-adrenoceptors in our model.

This is only one of a number of alternative explanations (e.g., electrophysiological changes (Sharma, 2010), activation of apoptotic cascades (see for review (Alberini, 2008) and/or necrotic tissue effects, (Rizzuto and Gambetti, 1976a)), which might explain differences in the temporal effects of the two antibiotics used here, rather than their selective roles in blocking translation and transcription. A conclusive demonstration of selective translational and transcriptional dependence of learning events would require ruling out alternative
explanations. We suggest, however, as proposed by others ((Rudy, 2008), see also (Rosenegger et al., 2010)), that a proteonomic approach in our model will be more fruitful in illuminating the biology of memory than further explorations of antibiotic effects. Translation-dependent memory events should be associated with changes in the production and/or levels of proteins produced by pre-existing mRNAs. The antibiotic data here suggest such proteins occur in the rat pup olfactory bulb and the narrow time window (~1 h) for the putative critical translation events provides a critical temporal target for analysis.
Figure A.1 Behavioral results showing the effect of translation block immediately after odor preference training on subsequent odor memory of rat pups at subsequent testing times. A timeline for the injection, odor training, infusion and testing is given above the graphs. Pups were given an s.c. injection of isoproterenol (Iso, 2 mg/kg) or saline 40 min before training and intrabulbar infusion of anisomycin (ANI) or saline (Sal) immediately after training. Memory was examined (A) 1 h, (B) 3 h, (C) 5 h or (D) 24 h after training. Each pup was tested only once in order to avoid possible extinction effects. Each graph was analyzed by one way ANOVA. Post hoc analysis was by
Dunnett's comparison to the learning controls (2 mg/kg Iso + odor). *, p<0.05; **, p<0.01.
Figure A.2 Effect of delaying translation block on memory. A timeline for the injection of US injection, odor training, infusion of anisomycin (ANI) and test time is given above the graph. Each pup was tested only once. A. Delaying anisomycin infusion to 1 h post-training resulted in a non-significant effect on memory. B. Delay of anisomycin infusion until 3 h post-training had no effect on 24 h memory of the peppermint (CS) odor. Each graph was analyzed by one way ANOVA. Post hoc analysis was by Dunnett’s comparison to the learning controls (2 mg/kg Iso + odor). These data suggest that most critical translation is completed by 1 h after the commencement of memory consolidation. The data also suggest that anisomycin infusion had no long lasting or non-specific detrimental effect on memory. Post-hoc comparisons are against the learning control (2 mg/kg Iso + odor). *, p<0.05; **, p<0.01.
Figure A.3 The effect on memory by infusing a transcription blocker immediately after odor preference training was examined at 5 h (A) or 24 h (B) post-training. The timeline of training, transcription blocker infusion and odor preference testing is given above the graph. Each pup was tested only once. Infusion of actinomycin (ACTI) had no effect on 5 h memory (A) but significantly blocked 24 h odor preference memory (B). Each graph was analyzed by one way ANOVA. Post hoc analysis was by Dunnett’s comparison to the learning controls (2 mg/kg Iso + odor). *, p<0.05; **, p<0.01.
Figure A.4 The effect on ITM (5 h) and LTM (24 h) of pups given variable doses of the unconditioned stimulus, isoproterenol (Iso). The time-line of training, odor preference testing and testing is given above the graph. Each pup was tested only once. A. Testing pups 5 h following paired CS (odor) and US (1.5, or 2 mg/kg Iso) resulted in significant odor preference compared to pups given odor only. B. In contrast, the 1.5 mg/kg US dose was ineffective in enabling 24 h LTM while the 2 mg/kg dose was effective. Each graph was analyzed by one way ANOVA. Post hoc analysis was by Dunnett’s comparison to the non-learning control group (odor only) *, p<0.05; ***, p<0.001.
Figure A.5 Supra-optimal level of β-adrenoceptor activation (6 mg/kg Iso) at the time of pairing with the conditioned stimulus odor interferes with odor memory 3 h later while 2mg/kg Iso induces significant odor preference compared to the non-learning (0 mg/kg Iso paired with odor); ANOVA p<0.5. This, in addition to the fact that anisomycin did not inhibit STM at 3 h (Figure A.1B), suggests that the effect of anisomycin is on LTM but not STM. Further, it suggests that anisomycin use in this model does not cause a surge in norepinephrine release that interferes with memory. The finding that infusion of anisomycin 3 h after training does not interfere with 24 h memory (see Figure A.2B) provides further evidence that the drug infusion is acting on protein translation during a critical period of consolidation immediately after training. *, p<0.05
Figure A.6 The summary table shows the current evidence for mammalian translation-dependent intermediate-term odor preference memory (ITM), which is absent following immediate post-training bulbar infusion of the translation inhibitor, anisomycin. Long-term memory (LTM) depends on protein synthesis within the first hour since partial LTM is seen with anisomycin infusion at that interval and LTM is intact with anisomycin infusion 3 h post-training. An immediate infusion of the transcription inhibitor actinomycin, does not alter ITM, but LTM is absent, demonstrating the distinction between translation-dependent ITM and transcription-dependent LTM in this model. Finally a weaker unconditioned stimulus, 1.5 mg/kg Iso can induce an ITM at 5 h but is insufficient to initiate an LTM at 24 h.
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