INHIBITION OF CALCINEURIN EXTENDS THE DURATION OF EARLY ASSOCIATIVE OLFACTORY MEMORY

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by

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Abstract

The search for which molecular players are involved in the transfer from short term memory to long term memory has led down several different avenues. The role of protein phosphatases in learning and memory has been one well-studied route of investigation. Specifically, protein phosphatase 2B (calcineurin, CN) has received a significant amount of attention due to its promotion of the dephosphorylation of CREB. Researchers have ascertained that over-expression of CN is associated with memory retention deficits and impaired memory consolidation in mice (Mansuy, Mayford, Jacob, Kandel & Bach, 1998; Foster, Sharrow, Masses, Norris, & Kumar, 2001). In contrast, CN inhibition enhances conditioned place preference (Gerdjikov & Beninger, 2005) and contextual learning (Ikegami & Inokuchi, 2000).

The present study hypothesized that an infusion of FK506 (a CN inhibitor) bilaterally into the olfactory bulbs would prevent the dephosphorylation of CREB and, prolong the duration of a conditioned odor preference, which normally only lasts 24 h with a single training trial. On post natal day (PND) 6, rat pups received a 2 mg/kg subcutaneous injection of isoproterenol (ISO), a β-adrenoceptor agonist (the unconditioned stimulus, US), and after a 10 minute exposure to peppermint (the conditioned stimulus; CS) were infused with FK506 or vehicle into the olfactory bulbs. Subsequently, preference for peppermint was assessed 24 hrs, 48 hrs, 72 hrs, 96 hrs and 1 week after training. Immunohistochemistry for pCREB revealed that unilateral infusion of FK506 resulted in an amplification of phosphorylated CREB in the olfactory bulb 40 min after training relative to sham side infusions. Additionally, pups infused bilaterally with FK506 maintained a learned preference for peppermint 48, 72 and 96 hrs after training. These results

support the hypothesis that prolonging CREB phosphorylation with CN inhibition can extend the duration of conditioned olfactory memory.

CN inhibition also modified the conventional inverted U curve obtained when ISO is used to replace stroking, as the US. Under normal conditions, 2 mg/kg of systemic ISO is effective, while higher and lower doses are not able to produce learning. When pups were infused with FK506 to inhibit CN, learning occurred with the low dose of 1 mg/kg ISO, and with the higher dose 6 mg/kg. We assumed that high doses of ISO do not elicit olfactory memory due to excessive phosphatase activity, and that low doses do not provide sufficient cAMP activation. CN inhibition blocks phosphatase activity, thus allowing learning to occur at higher doses of ISO. For the low dose effect, we hypothesize that AC9, a positive regulator of cAMP inhibited by CN, is relieved from inhibition and cAMP production is increased to provide sufficient activity to phosphorylate CREB and produce learning following normally insufficient (1 mg/kg ISO) activation. Alternatively, normally subthreshold phosphorylation elicited by small elevations of cAMP via low dose ISO may be raised to suprathreshold levels by phosphatase inhibition.

Calcineurin inhibitors thus have promise as memory enhancers since they facilitate memory initiation and memory duration from sub- to supra-optimal associative learning conditions.

LIST OF FIGURES

ABBREVIATIONS

5, 7 dHT	5, 7, -dihydroxytryptamine
AC	adenylyl cyclase
ACh	acetylcholine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole
AP5	amino-phophono-valeric acid
BTX	alpha-bungarotoxin
AON	anterior olfactory nucleus
ATP	adenosine triphosphate
BCA	bicinchoninic acid
C^{14}-2-DG	C^{14} 2 –deoxyglucose
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CN	calcineurin
CNS	central nervous system
CR	conditioned response
CRE	cAMP response element promoter site
CREB	cyclic AMP response element binding protein
CS	conditioned stimulus
DA	dopamine
Dn-CREB	dominant negative CREB
DOI	2,5-dimethoxy-4-iodoamphetamine hydrochloride
DTT	dithoithreitaol
GABA	gamma-amino butyric acid
HSV	herpes simplex virus
I1	inhibitor 1
IP3	inositol 1,4,5-triphosphate
ISO	isoproterenol
LTF	long term facilitation
LTP	long-term potentiation
E-LTP	early phase LTP
L-LTP	late phase LTP
LOT	lateral olfactory tract
MOB	main olfactory bulb
NE	norepinephrine
Neo	neomycin resistance
NMDA	N-methyl-D-aspartate
ORN	olfactory receptor neuron
OSN	olfactory sensory neuron
pCREB	phosphorylated CREB
PBS	buffered saline
PDE	phosphodiesterase
PG	perigolmerular
PKA	cyclic AMP-dependent protein kinase A

PKC	protein kinase C
PND	post natal day
PP1	Protein phosphatase 1
PP2B	Protein phosphatase 2B (CN)
TTX	tetrodotoxin
US	unconditioned stimulus
Wt-CREB	wild type CREB

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Completing a Masters of Science degree has been a challenging and highly rewarding experience. In the process I have learned immensely about the growing field of neuroscience and have gained valuable research experience.

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TABLE OF CONTENTS

CHAPTER I INTRODUCTION

1 Background

Which neurotransmitters, molecules or proteins are involved in the transfer from short term to long term memory has been and continues to be extensively examined. To date several modulatory factors have been explored such as: phosphodiesterase inhibition (McLean et al., 2005), estrogen (Rhodes and Frye, 2006), colostrinin (Stewart and Banks, 2006), protein phosphatase PP1 (Zachariou et al., 2002) and calcineurin (CN) (Sanna et al., 2006) among others yet to be addressed.

There is evidence that in some circumstances life long memories are formed. However, most inputs are eventually forgotten after varying intervals, even if they were initially retained for 24 h. There are, for example at least two durations of long-term memory seen with different habituation training protocols in the *C. elegans* (Steidl et al., 2003). Such evidence suggests that long-term memory may itself occur in stages.

The following thesis explored the role of protein phosphatase 2B in the control and duration of memory. Specifically, the role of protein phosphatase 2B (calcineurin, CN) with respect to the initiation of learning, the transfer to long-term memory, and whether CN inhibition can modulate the signals which control the duration of long term memory in neonate conditioned olfactory learning.

2 Learning and Memory

Learning can be described as the process of acquiring information about the world, and memory the retention and storage of that information. Without learning and memory mammals would not be able to adapt from one situation to another, and would thus fail to survive in changing circumstances.

2.1 Memory duration

Memory has been classified based on the duration of its retention. The two primary types of recognized memory are: short-term and long-term memory.

2.1.1 Short-term memory

Short-term memory is a limited capacity storage system able to maintain an unrehearsed portion of information for a short time frame of minutes to seconds (Alvarez and Cavanagh, 2004). It is thought to be related to several forms of short-term synaptic plasticity, such as augmentation (a longer lasting form of short term synaptic enhancement) (Hempel et al., 2000), and facilitation (Zucker, 1989). Short-term memory can, however, become a long term memory by rehearsing and repetitively verbalizing the information over and over again (Romero et al., 2006). Experiments conducted by Miller demonstrated that short-term memory has the capacity to store 7±2 items. Short-term memory capacity can be increased by a phenomenon known as chunking, whereby items are combined into meaningful groups (Miller and Selfridge, 1950). Simon

showed the ideal size of a chunk was three letters or numbers (Zhang and Simon, 1985). Short-term memories are thought to be an acoustic type memory and are supported by transient patterns of neuronal connections which are mostly dependent on the dorsolateral prefrontal cortex, but can occur in many other areas within the brain, and can occur outside the cortex as well (Zhang and Simon, 1985).

2.1.2 Long-term memory

Unlike the brief storage capacity of short-term memory, long- term memory is potentially available for an unlimited amount of time (Thompson and Kim, 1996;Goda, 1995), and requires persistent functional and structural changes in the brain.

3 Two functional forms of memory

Recent research has shown that memory can be divided into two distinct forms or general categories, explicit or declarative memory, and implicit, or procedural memory (Bailey et al., 1996). Explicit memory is the conscious recall of people, places or things, and is a well developed skill in humans. In other vertebrates it is thought to represent the memory of a particular time and place, referred to as episodic memory, a memory best evidenced in birds (Skov-Rackette et al., 2006). Implicit memory or non-declarative memory is the unconscious recall of tasks and motor skills and includes simple associative forms of memory such as classical conditioning, and non-associative forms such as habituation and sensitization (Squire, 1992).

4 Explicit memory

4.1 Explicit memory storage in the brain

Studies conducted with both humans and animals suggest that explicit information is processed in the prefrontal, limbic, and parieto-occipital-temporal cortices, which synthesize information pertinent to visual, auditory, and somatic information. Next the information travels to the parahippocampal and perirhinal cortex, then to the entorhinal cortex, the dentate gyrus, and the subiculum, and then finally back to the entorhinal cortex. Then from the entorhinal cortex the information is sent back to the parahippocampus, the perirhinal cortex, and finally back to the initial polymodal association areas (Kandel et al., 2000;Verfaellie and Keane, 1997). Patients with certain types of amnesia (memory loss due to neural injury) are able to remember events from their past and the factual information they had gathered up to and before injury to the hippocampus occurred. This suggests that the hippocampus is involved in temporary memory storage, while the long-term storage of declarative memory takes place in the association areas of the cortex which were involved in the initial processing (Kandel et al., 2000;Barco et al., 2002).

4.2 Explicit memory stages: encoding, consolidation and retrieval

The formation of explicit memory consists of three stages: encoding, consolidation and retrieval (Daumas et al., 2005). During encoding, information is observed and attended to, and an attempt is made to integrate it with information already known (Kaakinen and Hyona, 2007).

Consolidation refers to modifications of the new and labile memory to make it a stable long-term memory. During consolidation gene expression and the production of new proteins occurs which appears to be required for long-term memory. Storage refers to maintaining the memory over time, and finally retrieval refers to the process of recalling the information which was encoded. Memory retrieval is a constructive process, and requires the information from several areas of the cortex to merge, as reviewed in the text, *Principles of Neuroscience* (Kandel et al., 2000). Implicit memory on the other hand requires the same sensorimotor paths or associative pathways which were used during the learning process (Squire, 1992). However, as reviewed in *Principles of Neuroscience* (Kandel et al., 2000), both forms of memory depend on similar molecular components. Both implicit and explicit memories are graded, whereby the duration of the memory is dependent on the number of trainings, and are divided into temporally distinct parts, short-term and long-term memories. Studies analyzing long-term memory in both implicit and explicit forms have determined that both forms have a consolidation period where the memory is fragile and easily disrupted. Additionally, in both forms, short-term memory requires modification of existing proteins, whereas long-term memory formation requires the growth of new synapses, involves cellular programmed changes in gene expression, and an increase in protein synthesis (Bailey et al., 1996).

Studies have also been done to suggest that long term changes in memory may involve protein synthesis at the dendritic level (Papa and Segal, 1996;Kelleher, III et al., 2004;Sutton and Schuman, 2006). Huber et al., (2000) conducted a study *in vitro* in which activation of group I metabotropic glutamate receptors, or paired-pulse low-frequency stimulation was able to induce a form of synaptic long-term depression (LTD) that required dendritic, but not somatic, protein

synthesis (Huber et al., 2000). Additionally, several recent studies have demonstrated that hippocampal dendritic fields can support protein synthesis dependent forms of synaptic long-term potentiation (LTP) (Cracco et al., 2005;Vickers et al., 2005). Furthermore, studies have also demonstrated that the application of dendritic protein synthesis inhibitors prevents hippocampal late phase LTP (Bradshaw et al., 2003). Clearly explicit memory is a functional form of memory which has received a great deal of attention. The following thesis will further examine memory, but will assess associative memory, a type of implicit memory.

5 Implicit memory

5.1 Implicit memory storage in the brain

Implicit memory does not rely on conscious processing. It is a type of memory which slowly builds through repetition and is expressed in behaviors rather than words. There are several forms of implicit memory and each type is stored in a different area of the central nervous system (CNS). Memory which is acquired through classical conditioning, habituation or sensitization involve changes in the motor and sensory systems which were involved in learning the task (Verfaellie and Keane, 1997). Long-term memory storage in implicit memory involves the cerebellum, amygdala, and the specific sensory modality which was recruited during the initial learning of the task.

5.2 Implicit memory can be associative or non-associative

5.3 Nonassociative learning

During nonassociative learning an animal is exposed to a stimulus once or repeatedly. The two most common forms of nonassociative learning are sensitization and habituation. Habituation is defined by a decrease in responsiveness to a stimulus after it is repeatedly presented (Glaser and Whittow, 1953). For example, a loud train which passes by your apartment every evening stops waking you from your sleep once you have lived there for a few weeks. Sensitization, on the other hand, is an enhanced response to a stimulus after repeated exposure. Sensitization occurs when a stimulus is presented immediately after a noxious stimulus; for example, an animal responds more vigorously to a mild foot shock; after experiencing one that was fairly intense (Hullett and Homzie, 1966;Kandel et al., 2000)

6 Associative learning and memory

In associative type learning tasks, an animal learns about the relationship between two stimuli, or learns about the relationship between a stimulus and its own behavior as reviewed in (Razran, 1955). There exist two types of associative learning: operant conditioning, and classical conditioning.

6.1 Operant conditioning

Discovered by Thorndike and intensely studied by Skinner and others, operant conditioning consists of response strengthening when it is paired with a positive reinforcer, and the weakening of a response when it is paired with a negatively reinforcing stimulus. Thus, when a behavior is rewarded, an animal tends to repeat that behavior, and when a behavior is related to an unpleasant experience, it is suppressed. For example, if a food reward is contingent on the performance of a behavior, such as a pressing a lever, the animal will learn to perform that behavior in order to receive its reward (Skinner, 1988).

6.2 Classical conditioning

Classical conditioning involves learning the association between two stimuli, known as the conditioned stimulus (CS) and the unconditioned stimulus (US). Classical conditioning was pioneered by the Russian physiologist Pavlov. In his famous experiment, a dog was trained to salivate to the sound of a bell. For several trials a bell (CS) was paired with food (US). Eventually after several pairings, the CS, which was normally unable to induce salivation by itself, began to act as a cue that food would be provided. Once the dog had successfully learned the association between the bell and the food, it began to salivate in response to the bell (conditioned response, CR) (Pavlov, 1927). The temporal pairing of the CS and US is crucial. The CS must be presented before the US in order to evoke a conditioned response. The type of US is also important, and determines the type of conditioned response which will ensue. If the US is positively reinforcing, such as food, the response will be to approach the CS, or cue.

However, if the US is noxious, such as a foul odor, or a shock, the conditioned response will usually be avoidance. The following thesis will further explore classical conditioning with a neonate olfactory learning model.

7 Memory modulators

A number of molecules have been implicated in memory potentiation (Araneda and Firestein, 2006;Mitchell and Neumaier, 2005;Stewart and Banks, 2006;Simpkins et al., 1997). A study conducted by Rhodes and Frye (2006) reported that estradiol has memory enhancing effects. Female rats given injections of 17beta-E2 to increase estradiol concentrations had shorter latencies to find a hidden platform in the Morris water maze. Phosphodiesterase-4 inhibition has also been found to prolong memory. McLean et al., (2005) demonstrated that injections of the PDE4 inhibitor cilomilast, to prevent the breakdown of cyclic adenosine monophosphate (cAMP), enhanced one trial conditioned olfactory memory by extending its duration from 24 to 48 hrs.

A study conducted by Stewart and Banks (2006) demonstrated that colostrinin (a biologically active proline-rich polypeptide) has a positive effect on memory retention. Previous work had shown that chicks would avoid pecking a red seed coated with 100% methylanthranilate when given a choice between two seeds 24 hrs later; coating the seed with a 10% methylanthranilate solution did not induce learning. However, when colostrinin was injected prior to training with the 10% solution, memory was potentiated, and long-term memory for the aversive taste was induced with the lower 10% solution.

Cholinergic innervation and/or acetycholine (ACh) has been implicated in memory improvements. Wise et al., (2007) asked if donepezil, a selective noncompetitive inhibitor of acetylcholinesterase (known to breakdown ACh) was able to improve spatial memory. Rats were trained in a two-phase radial-arm maze procedure which consisted of an acquisition and retrieval phase. When donepezil was administered before the acquisition phase there was a significant decrease in the number of errors committed during the retrieval test, suggesting that ACh is a memory modulator (Wise et al., 2007).

8 Associative learning: The conditioned olfactory preference learning paradigm in the neonate rat

Newborn rat pups are not able to see or hear easily, and are thus highly dependent on their sense of smell for survival. Regardless of the quality of care given by the mother, a pup must learn to depend on her, and must develop an attachment in order to survive (Sullivan et al., 2000). A conditioned approach behavior to an olfactory stimulus can thus be easily obtained in neonate rats. Olfactory preference learning, a type of classical conditioning, can be elicited in neonate rats when an odor is presented in conjunction with: tail pressure (Sullivan et al., 1986), warmth (Alberts and May, 1984), milk (Johanson and Hall, 1979), mild foot shock (Camp and Rudy, 1988), heat (Pedersen et al., 1982), maternal odor (Sullivan et al., 1986), tactile stimulation (Sullivan and Leon, 1997) or high humidity (Do et al., 1988). This acquired or learned preference for the odor is associative, and can therefore be described as a form of classical conditioning. The pairing of an odor (CS) with one of the many unconditioned stimuli

mentioned above results in a conditioned odor preference, whereby the odor itself is able to elicit the conditioned approach response. Sullivan and Leon (1987) trained postnatal day (PND) 6 rat pups to prefer an odor by pairing the odor with tactile stimulation. Pups were removed from the dam, and for 10 min, were exposed to an odor (CS) while being stroked on the back with a soft paint brush (US). Pups were tested 24 hrs later with a two odor choice test. After only one pairing of the training odor, peppermint, with tactile stimulation, pups displayed a significant preference for that odor when compared to control groups, which consisted of pups which only received stroking (US), pups which were only exposed to peppermint (CS), or pups which received backward pairing (stroked prior to their odor exposure) (Sullivan and Leon, 1986).

Work over the last several years in the McLean lab has focused on this early olfactory form of associative learning. In this learning model, peppermint odor acts as the CS and norepinephrine (NE) or isoproterenol (ISO, a β adrenoceptor agonist) acts as the US (Sullivan et al., 1989b) as a replacement for tactile stimulation or maternal care.

8.1 Isoproterenol (β adrenoceptor activation) can replace stroking as the US in conditioned olfactory learning

Early odor preference learning can be achieved by pairing a novel odor with tactile stimulation (stroking) (Sullivan and Leon, 1986), but can also be achieved with the non-selective β-adrenoceptor agonist ISO as the US (Langdon et al., 1997;Price et al., 1998;Yuan et al., 2003b). Systemic administration of ISO would have effects on other organs such as the cardiovascular (Trindade et al., 1992) and respiratory systems (Poderoso, 1995). However, intrabulbar

infusions of ISO also elicit conditioned olfactory learning (Sullivan et al., 2000). Upon tactile stimulation, NE is released into the olfactory bulb from nerve terminals originating in the locus coeruleus. This stimulus activates β-adrenoceptors to produce learning since intrabulbar infusions of propranolol, a non-selective β-adrenoceptor antagonist, prevents tactile stimulation induced conditioning. Subcutaneous injection of ISO, a β1-adrenoceptor agonist, which acts at NE receptors within the olfactory bulb, and when paired with odor also produces a conditioned odor preference (Sullivan et al., 1989a;Nakamura et al., 1987). When NE stimulates a β1-adrenoceptor, the G-protein coupled to its receptor becomes active, resulting in increased activation of adenylate cyclase (De Blasi, 1989). Adenylate cyclase then converts the energy molecule ATP into the second messenger cAMP. In this learning model, peppermint odor acts as the conditioned stimulus (CS) and NE or ISO (β adrenoceptor agonist) acts as the unconditioned stimulus (US) (Sullivan et al., 1989b) which, via adenylate cyclase, activates cAMP (Rosenberg and Li, 1995;Cui et al., 2007;Yuan et al., 2003b) that in turn activates PKA, resulting in the phosphorylation of CREB at serine 133 which leads to downstream CRE related genetic effects such as changes in protein structure, and the production of new proteins (Sun et al., 1992). The pathway described, and the resulting phospho CREB related changes in protein and RNA synthesis are thought to be what drives neonate olfactory learning and long term memory (Figure 1), (Yuan et al., 2003b)

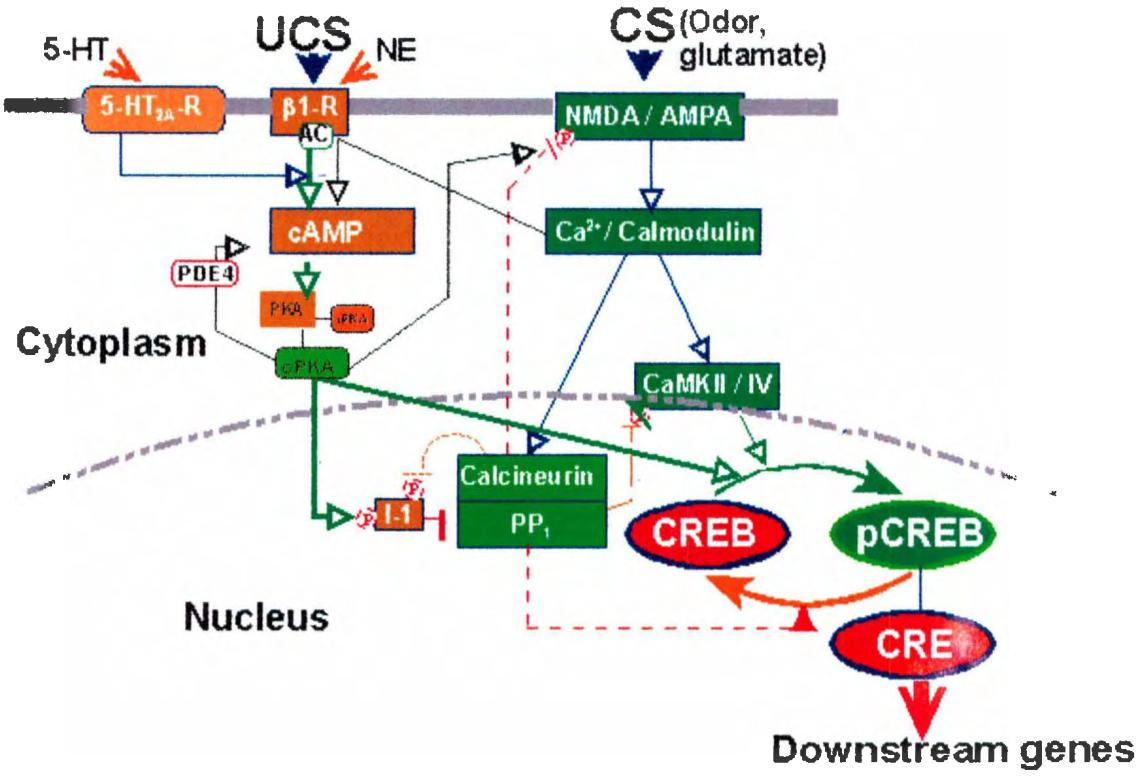

Figure 1: Olfactory learning model: When NE stimulates a β1-adrenoceptor, the G-protein coupled to its receptor becomes active, resulting in increased activation of adenylate cyclase. Adenylate cyclase then converts the energy molecule ATP into the second messenger cAMP. In this learning model, peppermint odor acts as the CS and NE or ISO (β adrenoceptor agonist) acts as the US which, via adenylate cyclase, activates cAMP that in turn activates PKA, resulting in the phosphorylation of CREB at serine 133 which leads to downstream CRE related genetic effects such as changes in protein structure, and the production of new proteins.

8.2 The inverted U-curve for early olfactory preference learning with β-adrenoceptor activation

The association of an odor with behavioral approach is dose dependent when β adrenoceptor (ISO) activation is used in place of stroking. There is an optimal dose of ISO which will induce a learned preference for the odor, however there exists sub and supra-optimal doses of ISO that cannot elicit the learned approach response (Sullivan et al., 1989b;Sullivan et al., 1991b;Langdon et al., 1997). An intermediate s.c. dose of 2 mg/kg ISO when paired with a 10 min exposure to odor elicits the learned approach response, however higher doses of 4 mg/kg or 6 mg/kg do not induce learning, nor does a low dose of 1 mg/kg (Sullivan et al., 1991b). It is possible that the balance between inhibition and disinhibition of olfactory bulb mitral cells may account for such a dose-dependent effect (Langdon et al., 1997). A second hypothesis suggests that higher doses of ISO induce an increase in phosphatase activity which may prevent the adequate phosphorylation of CREB.

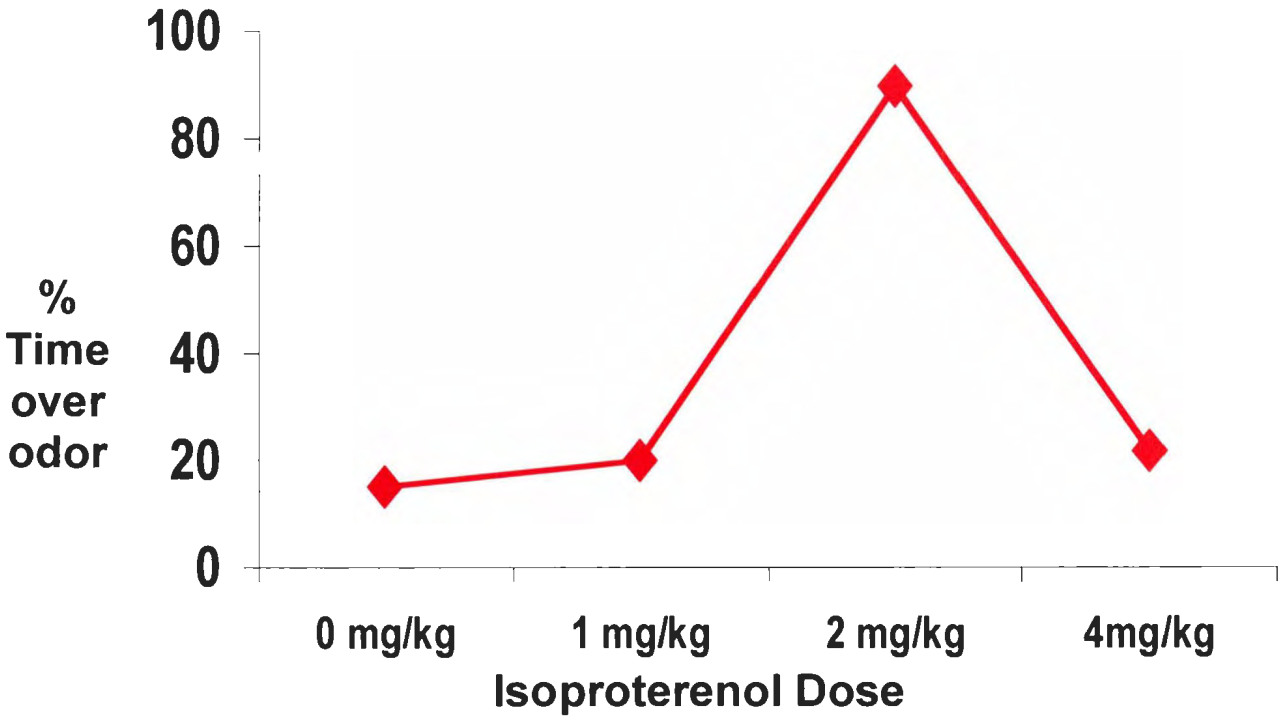

Figure 2: Animals that were given doses of 1 and 4 mg/kg ISO did not show a learned preference for peppermint 24 hrs after training.

8.2.1 Cilomilast combined with ineffective doses of ISO elicits a learned preference response

Previous work in the McLean lab has demonstrated that when the phosphodiesterase-4 (PDE4) inhibitor cilomilast is combined with a sub-optimal dose of ISO, conditioned olfactory learning and memory occurs (McLean, Darby-King & Harley, 2005). McLean et al., (2005) paired subcutaneous injections of the optimal 2 mg/kg dose of ISO or a sub-optimal lower dose of 1 mg/kg with a subcutaneous injection of one of several concentrations of cilomilast (0.001, 0.01, 0.1. 1.0, 2.0 or 3.0 mg/kg) to reduce the breakdown of cAMP. All doses of cilomilast higher than the lowest dose of 0.001mg/kg induced a learned odor preference 24 hrs after training, when combined with the low, learning ineffective 1 mg/kg dose of ISO. Furthermore, pups which received the 1 mg/kg sub-optimal dose of ISO and a dose of either 1 or 3 mg/kg of cilomilast displayed a conditioned odor preference 48 hrs after training. The higher dose of 3 mg/kg cilomilast also induced learning 96 hrs after training. This is in contrast to the usual 24 hrs memory seen after a single 10 min olfactory conditioning training trial. The findings of McLean et al (2005) suggest that there are specific requirements of cAMP activation associated with learning. Lower doses of ISO may not provide adequate cAMP production, and higher doses of ISO may provide too much activation leading to a critical imbalance in intracellular cascades in the olfactory bulb. This is supported by the observation that endogenous sources of NE released by stroking also sum with exogenous receptor stimulation to initiate (with mild stroking and weak ISO) or block (with stronger stroking and weak ISO) acquisition, suggesting again a dose dependence for effective intracellular cascades (Sullivan et al., 1989b).

The present thesis further investigates the mechanisms surrounding the inverted U-curve, and dose specific abilities of ISO to induce neonate olfactory learning. It is hypothesized that 1 mg/kg of ISO is a learning ineffective dose due to its inability to significantly elevate cAMP, thus providing insufficient stimulation to promote CREB phosphorylation (Antoni et al., 1998). On the other hand, a higher dose of ISO is hypothesized to induce a greater increase in phosphatase activity, which may subsequently dephosphorylate CREB (Groth et al., 2003), and prevent the down stream effects necessary for learning and plasticity. One objective of the present thesis was to assess whether or not inhibiting protein phosphatase 2B, also known as CN would produce memory when ineffective sub or supra optimal doses of ISO were administered.

8.3 The advantages of neonate olfactory preference learning

The olfactory bulb is an excellent structure for investigating the elusive mechanisms involved in learning and memory. Neonate olfactory learning is a relatively simple model where learning is localized in the olfactory bulb (Sullivan et al., 2000), and allows one to investigate modifications in metabolic (Wilson et al., 1987) and electrical activity (Wilson et al., 1987), in relation to the duration and saliency of memory. The olfactory preference learning paradigm allows direct manipulation of learning at the cellular level, and provides a fruitful mammalian model in the quest to discover the circuitry and molecular mechanisms involved in learning and memory.

9 The molecular and neural mechanisms of neonate olfactory learning

9.1 Olfactory bulb circuitry

Odor molecules are transformed by thousands of olfactory receptor neurons (ORNs) located in the olfactory epithelium. Subsequently, ORN axons project via the olfactory nerve into the olfactory bulb (Shipley et al., 1995). Conditioned odor preference learning takes place in the main olfactory bulb (MOB) (Wilson and Sullivan, 1994), a relatively simple cortical structure which receives sensory input from ORNs located in the olfactory epithelium.

The olfactory bulb in the rat has a ring-like structure made up of several different cellular layers. From the inside out they consist of the ependymal zone, the granule cell layer, the internal plexiform layer, the mitral cell layer, the external plexiform layer, the glomerular layer and the olfactory nerve layer. Odor-related input from the external environment arrives in the olfactory bulb from the sensory neurons of the olfactory epithelium, traveling along the olfactory sensory nerve to synapses in the glomerular layer. The glomerular layer consists of clusters of spherical round structures, called glomeruli. Glomeruli are the initial site of synaptic integration in the olfactory bulb, and are formed by the terminals of olfactory sensory axons and the dendritic branches of periglomerular, tufted, and mitral cells (Trombley and Shepherd, 1993;Shipley et al., 1995).

Olfactory receptor neuron axons form the olfactory nerve and project to the glomerular layer of the olfactory bulb, where they subsequently form excitatory synapses with terminal arborizations of the mitral cells (Yuan and Knopfel, 2006). Mitral cells are found in the mitral cell layer and are the primary output cells within the olfactory bulb. Each mitral cell has one apical dendrite which enters a glomerulus and is subsequently distributed among several sensory axons. The deepest layer in the olfactory bulb is the granule cell layer made up of small granule cells, which are arranged in tight groups of three or five in rows of somata (Shipley et al., 1996). Granule cells lack axons, but have a thick long apical dendrite which enters the external plexiform layer, and a thinner basal dendrite found in the granule cell layer. There is a considerable amount of synaptic connectivity between granule cells and secondary tufted mitral cells. Granule cell dendrites make inhibitory synapses with mitral cell dendrites (Davison et al., 2004), and mitral cell dendrites make excitatory synapses on granule cell dendrites, thus a reciprocal interaction exists involving dendro-dendritic synapses (Friedman and Strowbridge, 2000). Figure 3 below depicts the olfactory bulb cellular layers.

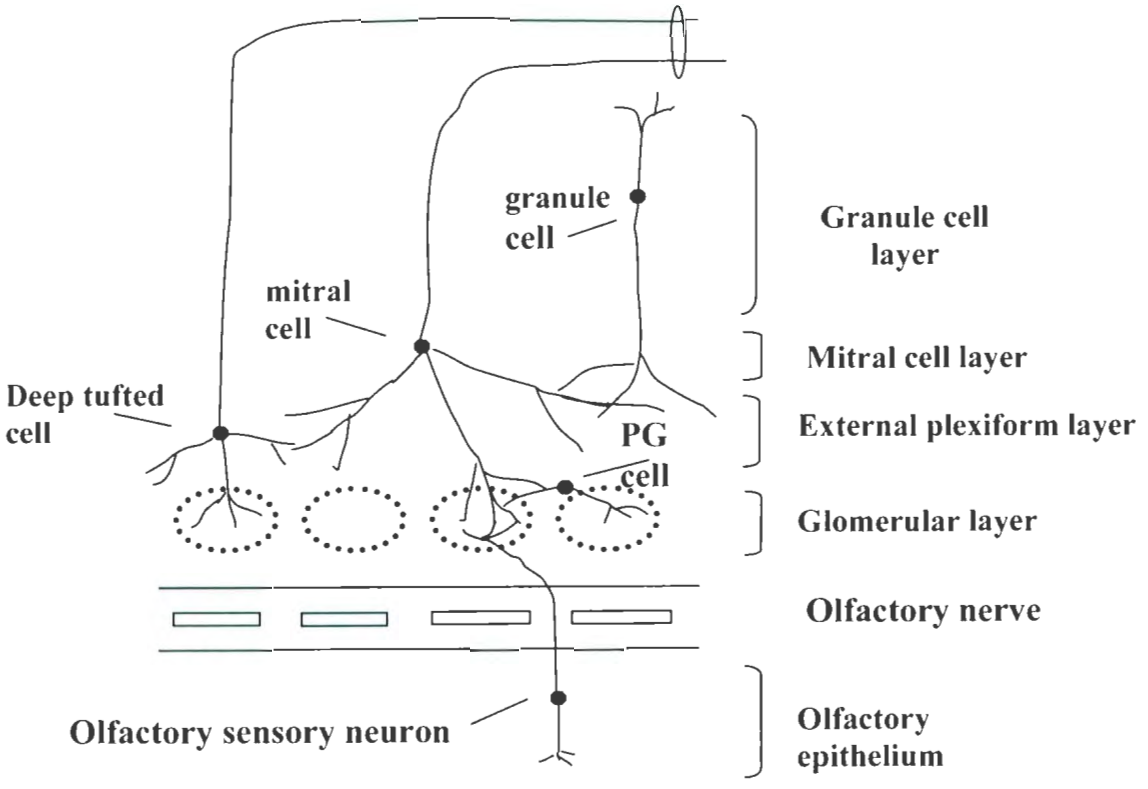

Figure 3: Olfactory bulb connectivity

Recent investigations have sought to assess the physiological processes involved in olfactory bulb signaling (Lowe, 2003;Lowe, 2002). Such studies have focused on the roles of both the mitral cell layer and the glomerular layer (Otsuka et al., 2001). Glutamatergic release from mitral cells requires a substantial amount of depolarization (Gilmor et al., 1996), and recently both imaging studies and dendritic recordings have suggested that the relationship between mitral and glomerular cells is shaped by both mitral cell, and granule cell excitability (Davison et al., 2003).

In the MOB, the release of glutamate from the lateral dendrites of mitral cells onto the dendrites of granule cells evokes recurrent and lateral inhibition of mitral cell activity (Moser et al., 1996). Furthermore, the primary dendrites of mitral cells have a high density of sodium channels which gives them the ability to easily propagate action potentials along their dendrites (Moser et al., 1996). Subsequently, mitral cell action potentials propagate into the lateral dendrites and evoke a transient increase in dendritic calcium concentration, which decreases as the distance from the soma increases (Margrie et al., 2001). *In vivo* and *in vitro* olfactory bulb whole cell voltage clamp recordings suggest that lateral and recurrent inhibition both control the duration, onset and number of odor evoked action potentials that fire within mitral cells (Fonseca et al., 1991). Therefore, the spread of action potentials from mitral cell to mitral cell can be influenced by both mitral cell activation and inhibitory input from mitral cells and granule cells (Nickell and Shipley, 1988a;Nickell and Shipley, 1988b).

Studies done to investigate granule cell inhibitory input have focused on the ascending dendrite, due to its dendrodendritic synaptic connections with mitral cell dendrites. Experiments have

determined that the granule cell resting membrane potential is rather hyperpolarized (Olianas and Onali, 1992) thus, spikes in granule cell activity tend to be suppressed (Le et al., 1996). However, Margrie et al., (2001) demonstrated that lateral inhibition between mitral cells and glomerular cells does not depend on granule cell action potentials, suggesting that granule cell depolarization is sufficient for granule cell-derived mitral cell lateral inhibition. Both mitral and granule cells are able to release neurotransmitters from their dendrites leading to a highly dynamic and variable pattern of connectivity. When an action potential propagates within mitral cell dendrites, it causes a release of glutamate at reciprocal synapses. Subsequently, glutamate activates granule cell spines which results in a release of GABA back onto the mitral cell dendrite (Margrie et al., 2001). Glutamate also activates NMDA autoreceptors on the mitral cell. These changes in voltage potential are then summed and together contribute to direct and indirect excitability via the activation of voltage gated channels on the mitral cell dendrite, such as potassium channels (Isaacson & Murphy, 2001).

The axons of both mitral and deep tufted cells in the MOB project to the olfactory cortex via the lateral olfactory tract (Schoenfeld et al., 1985;Scott, 1986). Rodent olfactory cortex consists of the transitional entorhinal cortex, the olfactory tubercule, the cortical and medial nuclei of the amygdala (important for the emotional aspects of smell), the piriform cortex, and the anterior olfactory nucleus. Information pertaining to olfaction is also relayed to the orbitofrontal cortex (thought to be involved in odor discrimination) and the thalamus via secondary and tertiary connections (Ruggiero et al., 1998). The olfactory bulb also receives input from centrifugal afferents which arise from the anterior olfactory nucleus, piriform cortex, entorhinal cortex,

periamygdaloid cortex, the amygdala, and the nucleus of the lateral olfactory tract. Subcortical centrifugal afferents originate in the basal forebrain and brainstem (Shipley et al., 1995).

9.2 Neural correlates of neonate olfactory learning

Neonate olfactory learning is a paradigm which can be achieved when an odor (CS) is combined with tactile stimulation (US) (Pedersen et al., 1982). The changes in the brain involved in conditioned odor preference learning have been extensively investigated (McLean et al., 1995;Wilson and Sullivan, 1994;Sullivan, 2003). The CNS in week old neonate rats pups is still quite immature, however, despite this, neonate olfactory learning is still dependent on several brain regions (Kucharski and Hall, 1988;Sullivan and Wilson, 1991b). The brain area that has received the greatest amount of attention is the olfactory bulb. Olfactory bulb activity occurs in clusters, meaning that groups of cells act together. Active mitral cells release glutamate onto granule cells, which in turn release GABA onto neighboring mitral cells, preventing them from becoming active through a process called lateral inhibition (Urban, 2002). Granule cells not only provide lateral and inhibitory feedback in the bulb, but are also a major target for centrifugal bulbar inputs, which consists of projections from the raphe nucleus (Andersen et al., 1983), the horizontal limb of the diagonal band (Luskin and Price, 1982) and the locus coeruleus (Shipley et al., 1985;Sullivan and Wilson, 1994) (Figure 4).

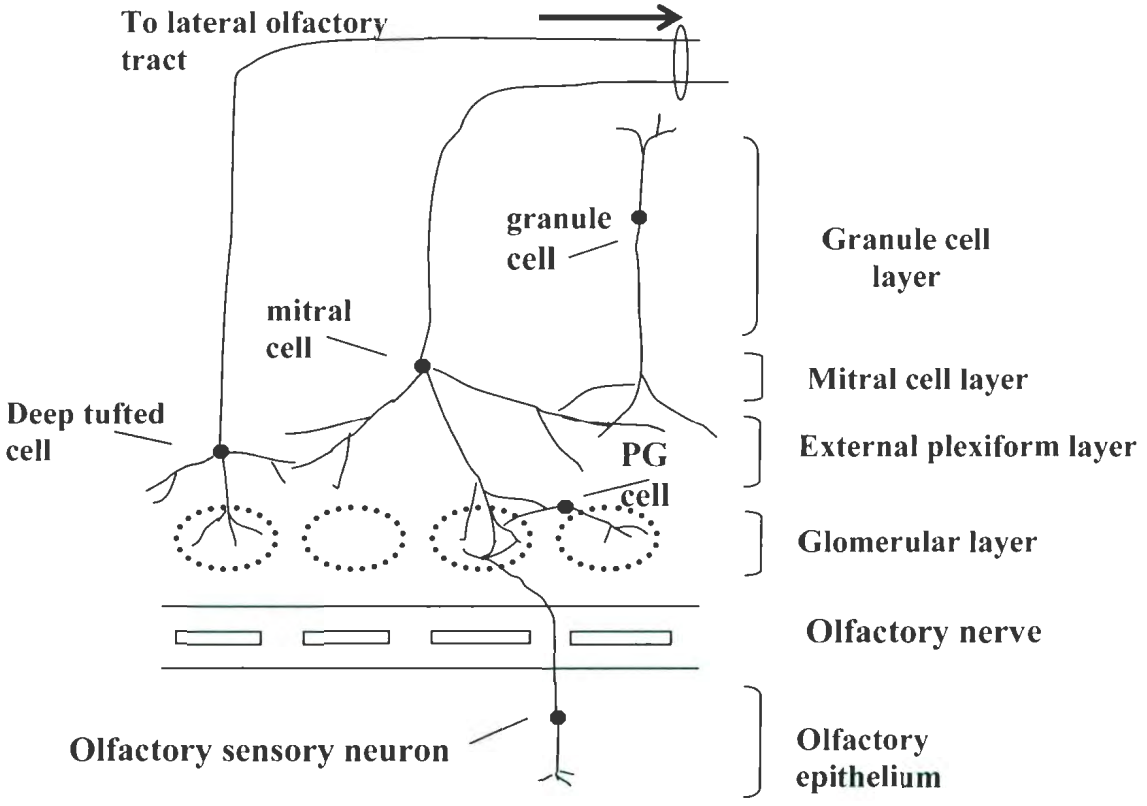

Figure 4: Olfactory bulb circuitry

9.3 Neural changes in response to olfactory learning as assessed by C^{14} 2-deoxyglucose autoradiography

The learned behavioral responses seen in neonate olfactory preference learning are associated with specific neural changes in the simple olfactory bulb cortical circuit. C^{14} 2-deoxyglucose (2-DG) autoradiography has been used to analyze the metabolic differences in activity within the olfactory bulb (Jourdan et al., 1980;Coopersmith and Leon, 1984;Sullivan and Leon, 1986). Within the olfactory bulb, 2-DG is enhanced with increases in olfactory bulb neural activity (Wilson and Leon, 1988b), and studies done with 2-DG have demonstrated that odor stimulation produces odor-specific spatial patterns of glomerular layer activity (Jourdan et al., 1980). After odor conditioning, a greater amount of 2-DG is observed in the glomerular foci of the olfactory bulb (Coopersmith and Leon, 1984). Specifically, pups which have a learned preference for an odor exhibit a statistically greater amount of 2-DG uptake in the odor specific foci of the glomerular layer, than pups that did not learn (Sullivan et al., 1989b). Additionally, training procedures which blocked learning acquisition also prevented enhanced olfactory bulb 2-DG responding (Sullivan et al., 1989b). These differences in 2-DG uptake were not due to modified respiratory responding, as there were no significant differences among treatment groups with respect to respiration rate (Sullivan et al., 1989b;Sullivan et al., 1988). Consistent with this, mitral and tufted cells in the same region show modifications in their single unit response pattern when presented with a conditioned odor (Wilson and Leon, 1988a). This response modification is characterized by an increase in suppressive responses towards the odor, and a decrease in excitatory responding (Coopersmith et al., 1986;Wilson et al., 1987). Mitral and tufted cells of pups trained with odor and ISO, or odor and tactile stimulation demonstrated more suppressive

responses than excitatory, whereas non learning pups displayed the opposite; more excitatory responses (Sullivan et al., 1989a). Clearly, neuronal response patterns within the olfactory bulb are modified by olfactory associative learning.

10 Neurotransmitters involved in neonate olfactory learning

As previously reviewed; information concerning the CS comes from odor receptors located in the olfactory epithelium and is then sent along the olfactory nerve to olfactory bulb glomeruli. Information regarding the US arrives via centrifugal inputs, such as noradrenergic input from the locus coeruleus. Several neurotransmitters are involved in both the CS and US which together act to modulate the olfactory bulb and induce a conditioned odor preference.

10.1 Norepinephrine is necessary for conditioned olfactory learning

The olfactory bulb receives a fairly significant noradrenergic input from the locus coeruleus (McLean et al., 1989;McLean and Shipley, 1991). To investigate NE targets within the olfactory bulb, immunofluorescence labeling for $ß_1$ adrenoceptors has been performed. Yuan et al. (2003b) revealed that $ß_1$ adrenoceptors are found predominantly in both mitral and tufted cells, in young and old animals.

Noradrenergic input is necessary for conditioned olfactory learning. Within the OB, the noradrenergic innervation from the locus coeruleus modulates the efficacy of the dendrodendritic synapses between mitral and granule cells (Okutani et al., 1998;Kaba and Keverne, 1988). The

acquisition of a learned odor response is blocked when a NE antagonist such as propranolol or timolol is administered (Sullivan et al., 2000;Sullivan and Wilson, 1991a). This blockade of conditioned olfactory learning is not due to any change in the detection of the odor, or the responsiveness to the US (Doty et al., 1988;Sullivan et al., 1991a). There are no NE neurons in the olfactory bulb, thus, noradrenergic input must come from centrifugal inputs. Sullivan et al., (1994) found that a bilateral 6-OHDA lesion to the locus coeruleus two days prior to conditioned olfactory training resulted in a 90% decrease in olfactory bulb NE, and prevented conditioned olfactory learning (Sullivan et al., 1994). NE is not however necessary for expression of the conditioned response if it has already been learned, suggesting that NE is involved in the acquisition of the conditioned olfactory response, but not its expression (Sullivan et al., 1994). Injection of propranolol 24 hrs after conditioned olfactory learning does not disrupt expression of the learned odor preference (Sullivan and Wilson, 1991a).

As previously mentioned, NE β-receptor activation produced via ISO can act as a US, replacing tactile stimulation, suggesting that NE is not only necessary, but is also sufficient in the induction of neonate conditioned olfactory learning (Sullivan et al., 1991a). Electrophysiologically locus coeruleus-induced activation depicts a biphasic paired-pulse inhibition of field potentials in the granule cell layer (Okutani et al., 1998). This has been related to granule cell disinhibition of the mitral cells, followed by an increased inhibition (Shepherd, 1972). Granule cell disinhibition of mitral cells, followed by a subsequent increase in inhibition has also been reported during rat pup olfactory conditioning (Wilson and Sullivan, 1994). These biphasic changes in olfactory bulb activity illustrate the dynamic plasticity of the dendrodendritic synapses in the olfactory bulb during olfactory learning.

It has been shown that mitral cell $ß_1$-adrenoceptor activation mediates neonate rat odor preference learning. Whether or not $ß_2$-, α_1-, and α_2-adrenoceptor agonists are involved in such learning was assessed by Harley et al., (2006). In their study, the ß1-adrenoceptor agonist, dobutamine, and the α_1-adrenoceptor agonist, phenylephrine, induced odor preference learning, and both exhibited the standard inverted U-curve dose-response relationship. In order to prevent indirect ß –adrenoceptor activation, phenylephrine was given in the presence of propranolol (Harley et al., 2006). The authors hypothesized that α1-adrenoceptor mediation could present a novel mechanism in the induction of learning. They suggested that α1-adrenoceptor activation may cause increases in cAMP within mitral cells via indirect activation of GABA(B) receptors (Harley et al., 2006). Clearly NE induced $ß_1$-adrenoceptor activation is necessary for conditioned olfactory learning. $ß_1$ adrenoceptors are found in the mitral cells of young and old animals, the primary output cell of the olfactory bulb, and the site where olfactory learning is thought to take place (Yuan et al., 2003b). The acquisition of a learned odor response is blocked when a NE antagonist such as propranolol or timolol is administered (Sullivan et al., 2000;Sullivan and Wilson, 1991a). α_1-adrenoceptor agonists may also be involved in odor preference learning via the indirect activation of GABA (B) receptors and a subsequent increase in cAMP (Harley et al., 2006).

10.1.1 Norepinephrine terminals in the bulb

The locus coeruleus contains noradrenergic neurons which project widely to the central nervous system, the olfactory bulb being one of its main targets. McLean et al., (1989) examined the laminar distribution of noradrenergic fibers from the locus coeruleus in both the main and

accessory olfactory bulb. The axons which leave the locus coeruleus were examined with injections of wheat germ agglutinin-horseradish peroxidase. The densest anterograde labeling observed within the accessory bulb was found in the external plexiform layer, granule cell layer, and the internal portion of the mitral cell layer respectively. Within the main olfactory bulb, labeled noradrenergic axons originating from the locus coeruleus were seen in the internal and external plexiform layers, the granule cell layer, occasionally in the mitral cell layer, and minutely in the glomerular layer. Subsequently, noradrenergic fibers in the olfactory bulb were identified using immunohistochemistry for dopamine-β-hydroxylase. The densities of innervation were determined with image analysis. Noradrenergic innervation to the accessory, and main olfactory bulb was from densest to least dense in: the inner portion of the mitral cell layer, the granule cell layer, the superficial portion of the mitral cell layer, and finally the external plexiform layer. There was no apparent noradrenergic innervation within the glomerular layer in the accessory olfactory bulb, nor the main olfactory bulb (McLean et al., 1989).

10.2 GABAergic disinhibition of mitral cells is necessary for conditioned olfactory learning

The excitability of the olfactory bulb is controlled by two layers of inhibitory interneurons: the dopaminergic and GABAergic periglomerular cells, and the GABAergic granule type cells. In the olfactory bulb mitral cell activity is inhibited by GABA which is released from the granule cell (Nicoll, 1971). Locus coeruleus activation is able to modify excitatory mitral cell output by adjusting granule cell GABAergic inhibition (Okutani et al., 1999). Okutani et al., (1999)

applied an aversive olfactory learning paradigm to PND 11 rats, and tested them for conditioned olfactory aversion on PND 12. When muscimol, a $GABA_A$ agonist was bilaterally infused into the olfactory bulbs prior to olfactory training, conditioned aversion learning did not occur (Okutani et al., 1999). On the other hand, infusion of bicuculline, a $GABA_A$ antagonist induced aversive responses to the trained odor, but did show odor nonspecificity. Pups displayed an aversion to all odors, rather than the trained odor only (Okutani et al., 1999). $GABA_B$ receptors also play an important role in conditioned olfactory learning (Okutani et al., 2003). Pups infused with baclofen, a $GABA_B$ agonist did not display conditioned odor aversion, whereas pups infused with saclofen, a $GABA_A$ antagonist displayed a non specific aversive response to all odors (Okutani et al., 2003).

10.3 Dopamine

The olfactory bulb is rich in neurons which contain dopamine (DA). Further, D1 and D2 type DA receptors are found in the same brain regions as GABAergic neurons, and have a complementary distribution pattern. As previously mentioned, GABAergic activity controls the inhibitory olfactory bulb processes. The fact that DA and GABA display similar distribution patterns suggests that DA may modulate inhibitory processes within the bulb. Recently, investigations with whole cell electrophysiology have demonstrated that dopamine is not only expressed in periglomerular cells but also exists in tufted cells (Davila et al., 2003). Studies utilizing DA agonists have revealed that DA inhibits excitatory neurotransmission between mitral/tufted cells and interneurons (Davila et al., 2003). Coopersmith et al., (1991) used *in vivo* microdialysis to assess dopaminergic activity in the olfactory bulb when odor was paired with

tactile stimulation. Clear air did not evoke any change in DA levels, whereas odorized air and stroking both induced an increase of 200%. When odor and tactile stimulation were combined, a 400% increase in DA levels was observed. It was thus hypothesized that large increases in DA activity may be related to the physiological changes which occur during olfactory preference learning (Coopersmith et al., 1991). DA agonism has also been found to suppress mitral cell activity (Duchamp-Viret et al., 1997). Duchamp-Viret, et al., (1997) found that when dopamine or its agonist apomorphine was applied, a drastic reduction in mitral cell spontaneous activity occurs, coupled with a decrease in firing rate to odor. DA can also act presynaptically on olfactory nerve terminals to inhibit their firing (Ennis et al., 2001).

Brunig et al. (1999) conducted a study which discovered that there are two distinct pathways by which DA receptors can either down or up modulate GABAergic function in the olfactory bulb, and therefore has a dual effect on learning. In their study, they demonstrated that olfactory bulb $GABA_A$ receptors are differentially modulated by dopamine in a cell specific manner. They observed that in granule cell interneurons, DA reduces the currents through GABA gated chloride channels. This action was mediated via D1 receptors and involved PKA phosphorylation of $GABA_A$ receptors. In contrast to these findings, they also observed an enhancement of GABA responding via dopamine. This response however, was mediated via D2 receptors and phosphorylation of $GABA_A$ receptors by PKC in the mitral cells. DA therefore has a dual action on the function of $GABA_A$ receptors in the olfactory bulb, which suggests that DA plays an instrumental role in odor detection, discrimination, and olfactory learning.

10.4 Glutamate

Olfactory learning is associated with several neural changes, and is, as shown thus far, influenced by multiple neurotransmitters. Glutamate is also highly involved in conditioned olfactory learning. Brennan et al. (1998) conditioned mice to form an association between a sugar reward and the odor that had been sprinkled over the wood shavings which covered the sugar. Animals were next exposed to the conditioned sprinkled odor, or a novel odor. Microdialysis was performed, and it was determined that presentation of the conditioned odor resulted in a significant increase in glutamate within the olfactory bulb. As previously mentioned, dendrodendritic synaptic activity between mitral and granule cells play a major role in odor learning. This dendrodendritic relationship forms the basis for mitral cell feedback inhibition, and the lateral inhibition necessary for odor discrimination (Brennan and Keverne, 1997). Glutamate has a large impact on the activity between mitral and granule cells. When glutamate is released from intracellular stores in response to odor, it activates postsynaptic receptors on the spines of granule cells, and activates presynaptic NMDA receptors within the mitral cell membrane. Recent studies have revealed that dendrodendritic olfactory bulb synapses, and granule cell activation from mitral cells are dependent on NMDA rather than AMPA type glutamate receptors (Aroniadou Anderjaska et al., 1999). Glutamate transmission and NMDA receptor activity are both necessary for olfactory learning (Lincoln et al., 1988). Staubli, et al., (1989) assessed the effect of intraventricular administration of D-amino-phosphono-valeric acid (AP5) an NMDA receptor antagonist on olfactory discrimination. Rats were trained to discriminate between two odors presented simultaneously with an infusion of 20 mM AP5 or vehicle into the olfactory bulb. Rats given the NMDA antagonist made a greater

amount of errors discriminating between two odors. NMDA receptor functioning is also necessary for olfactory preference learning. Systemic injections of AP5 blocks conditioned approach behavior, and prevents the enhanced olfactory bulb responsiveness which is usually observed (Lincoln et al., 1988).

10.5 Serotonin (5-HT)

Immediately postnatal, olfactory centrifugal innervation is almost exclusively serotonergic, and the density of 5-HT fibers increases from the first week of life into adulthood (McLean and Shipley, 1987). This serotonergic projection to the olfactory bulb originates in the raphe nucleus (McLean and Shipley, 1987). McLean and Shipley (1987) made injections of 1% wheat germ agglutin-horseradish peroxidase into the olfactory bulb, and with the combination of serotonin immunofluorescence and true blue retrograde fluorescence, determined that the majority of the raphe neurons projecting to the olfactory bulb contain serotonin.

Subsequently, McLean and Shipley (1987) injected WGA-HRP into the dorsal and median raphe. They observed dense anterograde labeling in the glomeruli, and a few labeled fibers in the external plexiform layer, internal plexiform layer, and granule cell layer. A large number of fibers originated in the raphe nuclei and entered the MOB via the olfactory nerve layer. Serotonergic fibers within the MOB were visualized by immunocytochemistry, and the distribution of specific 5-HT fibers was assessed. Serotonergic fibers in the olfactory bulb exhibited a specific morphology and laminar distribution. Specifically, the density of serotonergic innervation to the glomerular layer was two times greater than the innervation to

other layers within the MOB. Finally, McLean and Shipley (1987) discovered that electrolytic lesions of the dorsal and median raphe results in a total depletion of serotonin fiber staining within the bulb, which demonstrated that the sole source of serotonergic input to the main olfactory bulb originates in the raphe nuclei.

Other studies have been conducted to assess serotonin receptor distribution in the olfactory bulb and have localized 5-HT_{2A} receptors to mitral cells (McLean et al., 1995), and to a lesser extent granule cells (Hamada et al., 1998). 5-HT innervation in the glomerular cell layer of the MOB is extremely dense.

Given that 5-HT is present in the bulb at an early age, whether or not 5-HT plays a part in olfactory learning and memory has been investigated. McLean, et al. (1993) demonstrated that blocking serotonergic innervation prevents odor preference learning. Injection of 5, 7,-dihydroxytryptamine (5, 7-dHT) into the anterior olfactory nucleus (AON) selectively depleted olfactory bulb serotonin and prevented odor preference learning when stroking was combined with odor presentation.

Although conditioned olfactory learning is dependent on NE stimulation, intact serotonergic innervation is also required. 5-HT appears to have a modulatory role in cAMP activation, and therefore odor preference learning (Yuan et al., 2003b). A loss of 5-HT does not affect basal cAMP levels, but decreases the levels of cAMP seen after β-adrenoceptor activation. This suggests that NE and 5-HT act together to modify cAMP, where 5-HT modifies the cAMP levels which are driven by NE input (Rovescalli et al., 1993). This hypothesis is supported by the fact

that higher doses of ISO, which increase β-adrenoceptor activation, can produce learning when 5-HT is depleted (Langdon et al., 1997).

10.6 Cholinergic innervation from the diagonal band

Cholinergic innervation to the olfactory bulb, and the role ACh plays in olfactory learning and memory has also been examined (Levy et al., 1995;Thany and Gauthier, 2005). Le Jeune et al., (1995) assessed the distribution of cholinergic muscarinic and nicotinic receptor sub-types within the olfactory bulb by quantitative *in vitro* autoradiography. To visualize muscarinic M1-like receptors they applied [3H] pirenzepine, for M2-like sub-types [3H] AF-DX 384, for nicotinic alpha 4 beta 2-like receptors they applied [3H] cytosine, and for alpha 7- like nicotinic receptors they applied [125I] alpha-bungarotoxin (BTX). Additionally, labeling patterns for cholinergic nerve terminals were assessed with [3H] vesamicol (a marker for vesicular acetylcholine transport sites) and [3H] hemicholinium-3 (a high-affinity marker for choline uptake sites). Le Jeune and colleagues demonstrated that labeling for each cholinergic radioligand exhibits a specific laminar and regional pattern within the olfactory bulb (Le Jeune et al., 1995). Additionally, it was revealed that there is no overlap between cholinergic afferents and cholinergic receptors within the olfactory bulb. The distribution observed with the presynaptic markers ([3H] vesamicol and [3H] hemicholinium-3) demonstrated two strongly labeled bands corresponding to the glomerular layer and the mitral cell layer. Muscarinic M1-like and M2-like receptor sub-types were concentrated in the external plexiform layer; however intermediate binding densities were seen for M1-like and M2-like receptors throughout the deeper bulbar layers. In the glomerular layer, levels of muscarinic receptor subtypes were discovered to be

quite low, the level of M2-like sites being higher than M1. Both types of nicotinic receptor sub-types also displayed a very distinct pattern of distribution. Labeling for [125I] alpha-BTX (for alpha 7- like nicotinic receptors) binding was concentrated in the superficial bulbar layers, where as [3H] cytisine binding (alpha 4 beta 2-like nicotinic receptors) was primarily observed in the glomerular layer, as well as the mitral cell layer.

Studies have shown that cholinergic innervation is necessary for olfactory learning (Anglade et al., 1999;Levy et al., 1999). Ravel et al., (1992) administered systemic injections of the muscarinic antagonist scopolamine and assessed short term memory with a delayed match-to-sample test which was performed in a classic T maze, divided into two compartments. In the first compartment, rats were exposed to an odor then in the second compartment were trained to discriminate between two arms of the maze with different odors. To receive a food reward, the animal had to enter the arm which was signaled by the odor presented in the first part of the maze. Systemic injections of scopolamine, to antagonize muscarinic receptors, impaired performance, and rats did not accurately choose the arm scented with the trained odor. These results suggested that intact muscarinic transmission is required for olfactory memory (Ravel et al., 1992). Furthermore, an additional experiment was performed by Ravel et al., (1994) to assess if the observed olfactory memory impairment was due to a blockade of cholinergic transmission within the olfactory bulb. Drug was infused directly into both olfactory bulbs before each testing session. Intrabulbar infusion of scopolamine reproduced the behavioral deficits observed with systemic administration, suggesting that olfactory bulb cholinergic innervation at muscarinic receptors is necessary for olfactory memory (Ravel et al., 1992).

10.7 Summary of the neurotransmitters involved in olfactory learning

The above section described the neurotransmitters which are involved in conditioned olfactory preference learning. First, NE induced $ß_1$-adrenoceptor activation was clearly shown to be necessary in conditioned olfactory learning. $ß_1$ adrenoceptors were shown to be the primary output cell of the olfactory bulb, and are the site where olfactory learning is thought to take place (Yuan et al., 2003b). Furthermore, the acquisition of a learned odor response is blocked with NE antagonisms such as when propranolol or timolol is administered (Sullivan et al., 2000;Sullivan and Wilson, 1991a). Secondly, the excitability of the olfactory bulb is also controlled by GABAergic innervation to granule cells. In the olfactory bulb mitral cell activity is inhibited by GABA which is released from the granule cell (Nicoll, 1971). Locus coeruleus activation is able to modify excitatory mitral cell output by adjusting granule cell GABAergic inhibition (Okutani et al., 1999). The infusion of a $GABA_B$ agonist into the olfactory bulbs prevented olfactory learning (Okutani et al., 1999) suggesting that GABAergic innervation plays an important role in conditioned olfactory learning (Okutani et al., 2003). Thirdly, DA innervation was found to be involved in olfactory learning. DA and GABA were found to display a similar pattern of distribution which suggested that DA modulates inhibitory processes within the bulb. DA was found to have a dual action on the function of GABA receptors in the olfactory bulb. DA reduced the currents through GABA gated chloride channels via D1 receptors and enhanced GABA responding via D2 receptors which suggested that DA could play an instrumental role in odor detection, discrimination, and perhaps olfactory learning.

Fourthly, Glutamate was shown to have a large impact on the activity between mitral and granule cells. Glutamate transmission and NMDA receptor activity were both found to be necessary for olfactory learning (Lincoln et al., 1988). Staubli, et al., (1989) discovered that the intraventricular administration of an NMDA receptor antagonist hindered olfactory discrimination learning. Fifthly, 5-HT was found to be involved in olfactory learning. McLean, et al. (1993) demonstrated that blocking serotonergic innervation prevents odor preference learning. Injection of 5, 7,-dihydroxytryptamine (5, 7-dHT) into the anterior olfactory nucleus (AON) selectively depleted olfactory bulb serotonin and prevented odor preference learning when stroking was combined with odor presentation. Furthermore, it was suggested that 5-HT has a modulatory role in cAMP activation, and therefore odor preference learning (Yuan et al., 2003b). A loss of 5-HT did not affect basal cAMP levels, but decreased the levels of cAMP seen after β-adrenoceptor activation. This suggested that NE and 5-HT act together to modify cAMP, where 5-HT modifies the cAMP levels which are driven by NE input (Rovescalli et al., 1993). Finally, cholinergic innervation to the olfactory bulb, and its role in olfactory learning and memory was examined with systemic injections of scopolamine, to antagonize muscarinic receptors; which prevented olfactory memory (Ravel et al., 1992).

11 Cellular mechanisms behind conditioned olfactory learning

11.1 Disinhibition model

Sullivan and Wilson (1994) suggested that conditioned odor preference learning results from the disinhibition of mitral cells, allowing NMDA channel activation, which promotes long-term

changes in the olfactory granule cell to mitral cell connection. In this model, noradrenergic input from the locus coeruleus to the olfactory bulb acts as the US, and inhibits granule cell interneurons, producing disinhibition.

11.2 The cAMP, PKA and pCREB model for olfactory learning

Another line of thought suggests that the US action occurs directly on the mitral cells, rather than indirectly through intermediate granule cells. This line of reasoning stems from the fact that β-adrenoreceptor agonists elicit weak granule cell responses (Trombley, 1992;Trombley, 1994). In this model, the calcium signal from the odor input via the olfactory nerve, together with a cAMP signal from noradrenergic stimulation act synergistically with 5-HT input within the mitral cells to elevate the amount of phosphorylated CREB to produce learning (McLean et al., 1999). When stroking or ISO is combined with odor, a learned preference is formed in conjunction with a marked increase in cAMP and phosphorylated CREB (Yuan et al., 2000). Increases in pCREB are specific to the quadrant of the bulb which perceives the particular odor used (McLean et al., 1999) and results in an increase in glutamatergic input to mitral cells (Yuan et al., 2000). In light of these findings, a new learning model for olfactory memory suggests that the critical event is in fact the production of cAMP in the mitral cells. In this model, CREB phosphorylation occurs when US derived cAMP merges with glutamatergic input from the CS (odor). This notion is evidenced by the fact that confocal imaging has shown that β1-adrenoceptors and 5-HT2a/c receptors are co-localized together on mitral cells. Electrophysiological results provide further evidence which is consistent with a mitral cell centered hypothesis. Research has shown that when a learning effective dose of ISO is paired with olfactory nerve input, it enhances the field

olfactory nerve excitatory postsynaptic potential in mitral cells, and this enhancement shows an inverted U curve profile with higher ISO doses (Yuan et al., 2003b). This newer olfactory preference learning model focuses on a convergence of noradrenergic stimulation and 5-HT on mitral cells to produce an increase in phosphorylated CREB (Figure 5). The US provides NE stimulation at β-adrenoceptors, β-adrenoceptor activation leads to cAMP activation, and subsequently activates the cAMP-dependent protein kinase A (PKA) pathway. The CS, mediated by glutamate (odor) activates both AMPA and NMDA receptors, which recruit the Ca^{2+}/ CaM pathway (Isaacson and Murphy, 2001). These two pathways converge to stimulate CREB phosphorylation. Finally, CREB phosphorylation triggers the structural and functional changes necessary for long term memory (Figure 6)

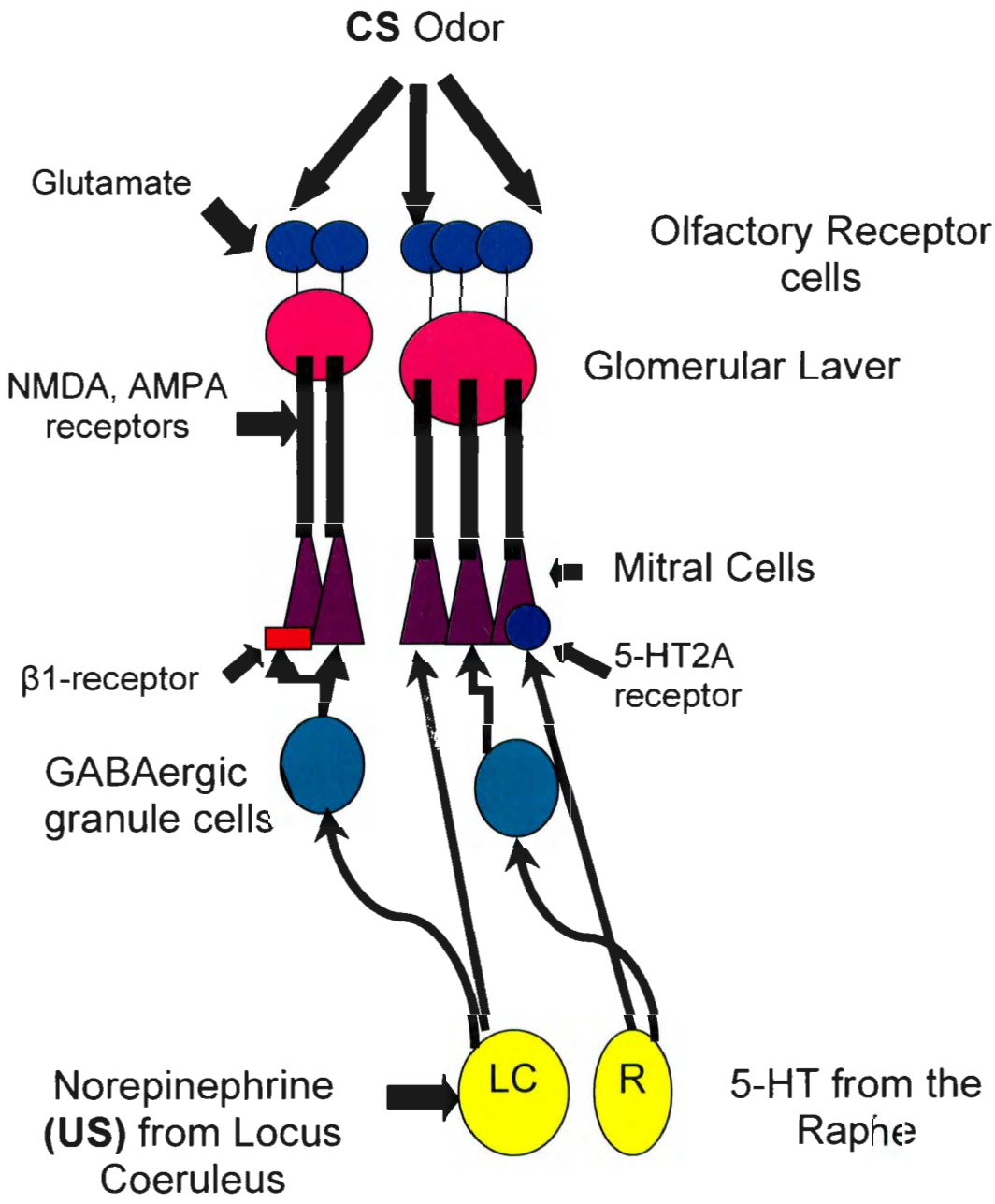

Figure 5: The convergence of noradrenergic, serotonergic and glutamatergic input at the mitral cell

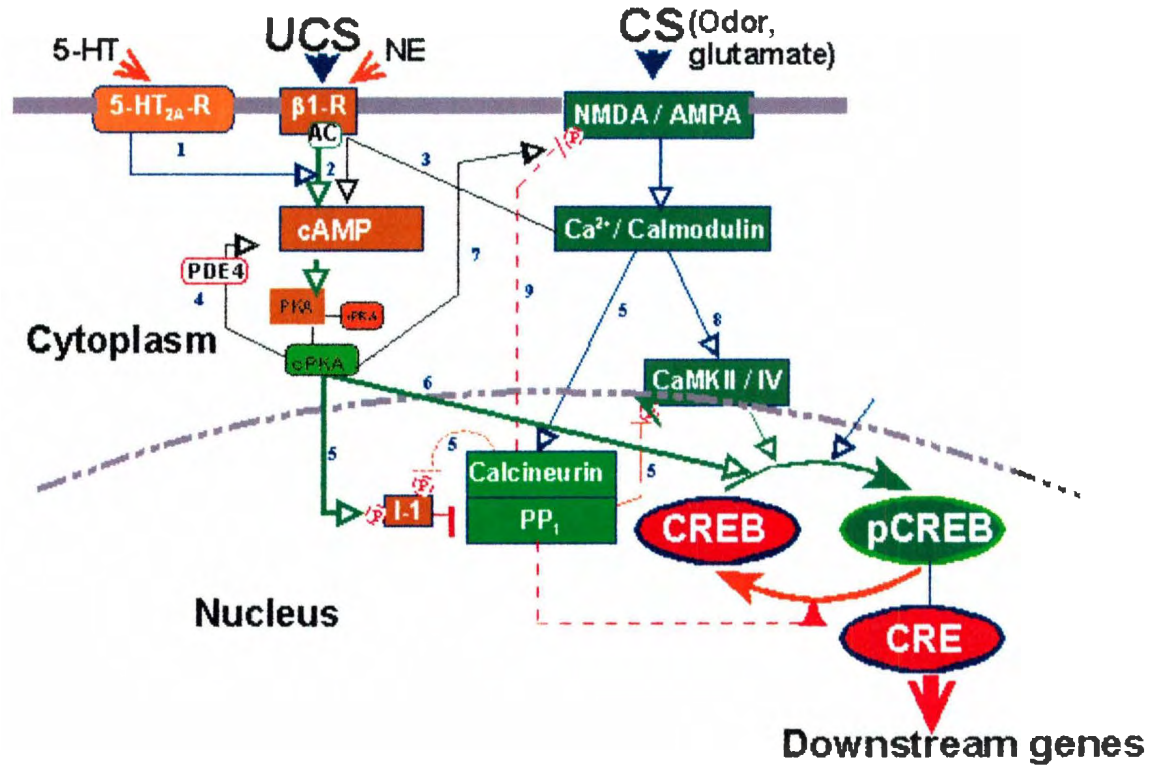

Figure 6: Proposed intracellular and intercellular pathway for olfactory learning in the olfactory bulb which focuses on a convergence of noradrenergic stimulation and 5-HT on mitral cells to produce an increase in phosphorylated CREB. The US provides NE stimulation at β-adrenoceptors, which leads to cAMP activation, and subsequently activates the cAMP-dependent PKA pathway. The CS, mediated by glutamate activates both AMPA and NMDA receptors, which recruit the Ca^{2+}/ CaM pathway. These two pathways converge to stimulate CREB phosphorylation

12 Cyclic AMP response element binding protein (CREB)

12.1 CREB and transcription

CREB is a member of a large family of transcription factors which all share the same basic structure, and bind to cAMP response element (CRE) promoter sites (Takemori, 2007). One well known pathway for CREB phosphorylation involves the activation of adenylate cyclase via transmembrane receptors and a subsequent increase in cAMP, which in turn dissociates the PKA regulatory subunit. Once PKA is activated, its catalytic subunit is then able to translocate into the nucleus and phosphorylate CREB at serine 133 (Karkoulias et al., 2007;Bacskai et al., 1993;Hagiwara et al., 1993;Lee et al., 2006)

12.2 The phosphorylation of CREB is necessary for learning and memory.

12.2.1 Electrophysiological studies with *Aplysia*

The first studies to suggest that CREB phosphorylation is required for the formation of memory were done with Aplysia cultured neurons (Dash et al., 1990), and since then much work has been done to determine the exact mechanism by which CREB mediates learning and plasticity (Bacskai et al., 1993;Bailey and Kandel, 1993;Bernier et al., 1982;Brunelli et al., 1976;Byrne and Kandel, 1996;Castellucci et al., 1982;Kaang et al., 1992;Martin et al., 1997;Montarolo et al., 1986). In Aplysia, tactile or electrical stimulation of the siphon results in a defensive withdrawal

response of the siphon and gill. Repeated stimulation of the siphon or tail can lead to sensitization of the defensive response, and can last for minutes or hours. Sensitization of the withdrawal response results from a facilitation between the sensory synapses (responding to the sensitizing stimulus) and the motor neurons, which mediate the response (Byrne, 1982;Frost and Kandel, 1995). The use of cultured motor and sensory neurons has made it remarkably simple to analyze the electrophysiological and neuroanatomical cellular events involved in long-term facilitation (LTF) of the withdrawal reflex. A stable enhancement in synaptic function is observed between the sensory and motor neurons. The production of new proteins is necessary for LTF, evidenced by the fact that protein production blockers prevent facilitation (Montarolo et al., 1986). Long term facilitation of conditioned withdrawal is dependent on the cAMP/PKA second messenger pathway (Byrne et al., 1993). When levels of cAMP increase, the catalytic subunit of PKA translocates to the nucleus of the sensory neuron where it activates the transcription of CREB dependent genes, such as the lacZ reporter gene (Kaang et al., 1993), required for LTF.

CREB dependent genes play a causal role in LTF (Dash et al., 1990). When cultured sensory neurons are injected with CRE sequenced oligonucleotides (which renders a portion of the CRE dimmer inactive), LTF does not occur. Blocking CRE binding proteins, in particular, a 43 kilo Dalton (kD) CREB related protein prevented facilitated responding which suggested that CREB related proteins are necessary for transcriptional activation of the genes which are involved in LTF (Kaang et al., 1993).

12.2.2 Olfactory memory in *Drosophila*

Like the studies with *Aplysia*, long-term sensitization in *Drosophila* also requires the cAMP/PKA pathway (Tully, 1991). *Drosophila* are able to show extremely robust olfactory learning and memory (Tully, 1991). When presented with two odors, they will learn to avoid an odor which was paired with mild shock. Studies done where one odor was paired with a shock showed that *Drosophila* chose the non-paired odor significantly more than the odor paired with foot shock in a simple t-maze. When trained pairings of the odor and shock are done with many trials spaced over time, memory for the odor can last for several days (Tully, 1991). Several studies have determined that CREB has a causal role in *Drosophila* learning. In *Drosophila*, CREB function can be disrupted with transgenic expression of a dominant negative form of CREB protein (Yin et al., 1994). Induction of this CREB repressor binding protein prior to training blocked the conditioned avoidance response normally seen when the odor was paired with shock (Tully et al., 1994).

Memory in the *Drosophila* can be of different durations. An intermediate form of memory called middle term memory has also been observed. Several of the mutant strains identified have shown learning of the classically conditioned odor avoidance response, but without short term memory. This intermediate-memory stage was determined by studying mutant strains that demonstrate normal long term learning and short term memory but fail to exhibit middle term memory. A mutant strain known as *amnesiac* shows this memory deficiency (Quinn et al., 1979) and have a defect in the cAMP cascade. *Amnesiac* flies have a mutation that prevents production of a peptide that regulates the activity of adenylyl cyclase, which catalyzes the hydrolysis of ATP

to cAMP, resulting in lower levels of cAMP (Feany & Quinn, 1995). These flies show memory immediately after training and normal retention 7 hrs after training, but they show problems in retention between these time points. Genetic analyses of lasting memory in Drosophila have led to the isolation of two interesting stages of long term memory: anesthesia resistant memory and normal long-term memory (Tully et al., 1994). Furthermore, these two stages of memory function together to produce memory for classical conditioning that lasts for days to weeks. Long-term memory lasts much longer than anesthesia resistant memory, is produced by a distributed training protocol, and is protein synthesis dependent. Anesthesia resistant memory on the other hand is not protein synthesis dependent and is produced by a massed training protocol (Folkers et al., 1993).

12.2.3 CREB function and memory in mutant mice.

The necessity of CREB related genes in mouse memory has been assessed with targeted mutations of the CREB gene. CREB mutant mice were developed by inserting a promoterless Neo gene, in-frame, into the second exon of the CREB gene in embryonic cells (Hummler et al., 1994). Insertion of the neo mutation in the CREB gene resulted in a loss of α and Δ CREB isoforms. However, the CREB $^{\alpha\backslash}$ mutants did not show any developmental difficulties, were not ataxic, and did not show deficits in their generalized nervous system (Bourtchuladze et al., 1994). Three different types of memory tasks; the Morris water maze, contextual fear testing, and the social transmission of food preferences have been utilized to determine if CREB $^{\alpha\backslash}$ mice exhibit memory deficits (Bourtchuladze et al., 1994). Control mice can be trained with a single foot shock to be fearful of the context in which the shock occurred. CREB $^{\alpha\backslash}$ mice show contextual memory deficits when tested at 24hrs, but not at 30 min, suggesting that CREB is

required for long-term memory, but not short term (Abel et al., 1997). Consistent with this, CREB $^{\alpha\Delta}$ mutant mice also display a deficit in long-term transmission of social food preference. Rodents tend to develop a natural preference for a food that they smell on the breath of another rodent (Galef, Jr. et al., 1988). Normal mice show this preference up to 24 hrs later. CREB $^{\alpha\Delta}$ mutant mice, however when given a brief 5 min exposure to a socially transmitted odorant, do not show a preference for the food when tested 24 hrs later (Winocur et al., 1990).

The Morris water maze has also been utilized to examine the causal role of CREB with respect to memory. This task requires that mice learn to find a submerged platform in a pool of water over multiple trials (Morris, 1984). CREB $^{\alpha\Delta}$ mutant mice show a deficit in this task, which suggests that CREB is necessary for spatial forms of learning (Hebda-Bauer et al., 2007).

12.2.4 Studies in the rat

Whether or not acute changes in CREB function can affect memory has been addressed in rats with oligonucleotides designed to prevent mRNA translation. When CREB antisense oligonucleotides were infused into rat hippocampus prior to training, problems with long-term spatial memory were observed (Guzowski and McGaugh, 1997).

Hippocampus-dependent social transmission of food preference significantly increased CREB phosphorylation in trained rats compared to controls (Countryman et al., 2005). Brightwell et al., (2005) assessed if CREB phosphorylation is necessary for long term social transmission of food preference. Rats received intrahippocampal infusions of HSV-mCREB (Ser133 replaced

with Ala), HSV-LacZ or saline. Rats were subsequently trained and then tested for food preference. Long-term memory tests revealed that HSV-mCREB animals ate significantly less of the trained preferred food when compared to controls, demonstrating that hippocampal pCREB is necessary for long term social transmission of food preference learning (Brightwell et al., 2005). Activation of the PKA/CREB pathway also occurs during spatial memory formation in the rat (Mizuno et al., 2002).

CREB is clearly involved in learning and memory. Previous research has demonstrated it has a role in *Aplysia* LTF, long-term sensitization in *Drosophila* and rodent memory related tasks. CREB is also however crucially involved in conditioned neonate olfactory memory, the learning which will be explored in the present thesis.

12.2.5 Neonate olfactory preference learning

Previous research has shown that there is a significant increase in CREB phosphorylation 10 min after associative odor training (McLean et al., 1999). Western blot analysis revealed that the olfactory bulbs of pups which were exposed to odor for 10 min in combination with stroking stimulation had a greater amount of pCREB in the olfactory bulbs 10 min after training than controls that received odor only or stroking only. CREB phosphorylation has also been examined with NE induced odor preference learning. Pups that were given 2mg/kg of ISO, combined with a 10 min exposure to odor demonstrated increases in pCREB levels 10 min after training and learned a preference, however pups with serotonin depletion did not learn and did not show pCREB increases (Yuan et al., 2000)

To further assess the role of CREB and pCREB in early olfactory preference learning, the olfactory bulb was infected with 2 forms of herpes simplex virus (HSV), one with an over expression of wild type CREB (HSV-wt-CREB) and another expressing a dominant negative mutant (HSV-dn-CREB) (Yuan et al., 2003a). Injection of the HSV vector, which resulted in the expression of a mutant form of olfactory bulb CREB, prevented olfactory learning, whereas pups injected with the control HSV (expressing LacZ) demonstrated a learned preference for the conditioned odor. Furthermore, when the mutant form of CREB was expressed, a change was seen in the inverted U dose-response curve obtained when ISO is used as the US. The 2 mg/kg dose of ISO normally able to induce learning was insufficient, but the higher learning ineffective dose of 6 mg/kg ISO induced a learned preference (Yuan et al., 2003a). Given that the pups that expressed the mutant form of CREB were unable to form a learned preference to peppermint, Yuan et al., (2003a) concluded that CREB and pCREB both have a causal role in neonate odor preference learning.

12.2.6 CREB and long-term potentiation

LTP is a memory mechanism which has been extensively studied due to its relevance to synaptic plasticity (Madison and Schuman, 1991;Bliss and Collingridge, 1993). The term LTP refers to long lasting enhancements in synaptic efficacy. LTP has several properties which are relatable to learning and memory, such as the fact that it is long lasting, demonstrates associability and is reversible (Malinow, 1994). Several studies have, in fact, suggested that LTP is directly involved in memory formation (Maren and Baudry, 1995;Barnes, 1995). There are several forms

of LTP which have different time courses and underlying biochemical properties. NMDA-dependent LTP is however the form which has been examined the most. Early NMDA-dependent LTP (E-LTP) lasts for approximately 1-2 hrs, and is sensitive to CaMK inhibitors but is not affected by protein synthesis inhibitors (Frey et al., 1988;Huang et al., 1994;Frey et al., 1993). Late NMDA-dependent LTP (L-LTP) lasts for a significantly greater amount of time, as much as 7 hrs *in vitro*, and requires new protein synthesis and PKA activation (Ahmed and Frey, 2005). Genetic manipulations which target PKA or CREB result in severe problems in LTP, for example; late-LTP is impaired in the area CA1 of the hippocampus in CREB $^{\alpha\backslash}$ mice (Bourtchuladze et al., 1994;Abel et al., 1997). Studies assessing LTP have found that an increase in phosphorylated CREB is concordant with LTP induction (Alzoubi and Alkadhi, 2007). Additionally, a study done by Ahmed and Frey (2005) to assess LTP induction and maintenance revealed a delayed onset of continuous CREB phosphorylation, at two times, with two separate peaks; one 45 min after LTP induction and another at time 6 hrs, before pCREB levels decayed back to baseline (Ahmed and Frey, 2005).

12.3 CREB phosphorylation and protein synthesis

The phosphorylation of CREB is necessary for the transcriptional production of new proteins, which is required for the formation of most long term memory and learning (Walton and Dragunow, 2000). As mentioned, the post-translational modification of CREB critically influence memories for olfactory avoidance conditioning in fruit flies (Yin et al., 1995;Tully, 1996) defensive withdrawal conditioning in *Aplysia* (Abel and Kandel, 1998;Bartsch et al., 1998;Michael et al., 1998) avoidance conditioning, spatial escape learning, social preference

learning in rodents (Silva et al., 1998), and neonate rat olfactory associative learning (Yuan et al., 2003a). Once phosphorylated, CREB activates the transcription of immediate early genes. These transcription factors then go on to activate the transcription of late response genes (Tao et al., 1998) and these late response genes are responsible for changes in neurotransmitters, neurotrophins, adhesion molecules, cytoskeletal proteins, and ion channels, among several other cellular changes which constitute the necessary components for long-term plasticity and learning (Brunelli et al., 1976;Dale et al., 1987;Dash et al., 1990;Montarolo et al., 1986;Yovell et al., 1987).

13 The relationship between protein phosphatases and CREB

13.1 Protein phosphorylation and de-phosphorylation

Protein phosphorylation is a crucial and diffuse post-translational modifier of cellular signaling, and drives several activity-dependent signal transducers. Phosphorylation activates many targets such as membrane receptors, cytoskeleton proteins and enzymes which regulate protein interactions, trafficking, and the many processes necessary for synaptic modulation and even apoptosis (Munton et al., 2004). Phosphorylation is controlled by a dynamic interplay between kinases and phosphatases. Phosphatases are often considered secondary to their kinase counterparts as protein kinases execute protein phosphorylation, whereas phosphatases are responsible for dephosphorylation (Mansuy and Shenolikar, 2006). It is, however, the balance between the two that determines molecular activation, making them both crucial and of equal importance.

14 Protein phosphatases

There are several protein phosphatases in the central nervous system, which together act to coordinate the dephosphorylation of both threonine and serine phosphorylated residues. They are protein phosphatase 1, 2A, 2B, 2C, 4 and 5. They can be found distributed throughout the entire CNS and together act to control synaptic plasticity, memory and the removal of phosphate from multiple cellular targets (Cohen, 1989).

14.1 PP1

PP1 is involved in glycogen metabolism, cell division, transcription, translation and apoptosis (Munton et al., 2004). PP1 activity is, for the most part, controlled by interacting proteins which act as regulatory subunits in order to modulate its activity and is also controlled by the phosphorylation of inhibitor proteins, such as Inhibitor 1 (I1) (Sakagami et al., 1994;Munton et al., 2004).

PP1 is highly involved in postsynaptic transmission and has a significant effect on glutamatergic synaptic activity (Hu et al., 2007). Glutamate has a major excitatory role in the CNS, which is mediated through AMPA receptors or NMDA receptors. Both of these ionotropic type receptors bind glutamate, which triggers calcium (Ca^{2+}) to enter the cell. PP1 is able to modulate both AMPA and NMDA receptors. PP1 directly dephosphorylates NMDA receptors, which has been shown to decrease the probability of channel opening (Flores-Hernandez et al., 2002). AMPA receptor activation is maintained by phosphorylated DARPP-32, which is a DA and cAMP

regulated phosphoprotein. DARPP-32 activation inhibits PP1. When dephosphorylated DARPP-32 is applied, PP1 levels increase, and subsequently AMPA receptor activity diminishes (Snyder et al., 2003).

PP1 has also been implicated in long-term synaptic changes. Changes in the excitability of neurons, and strengthening or weakening of synapses is termed synaptic plasticity (Lisman et al., 2002). One form of synaptic plasticity consists of NMDA channel activation, followed by calcium entry, and an increase in kinase activity.

14.1.1 PP1 with respect to learning and memory

Research done to investigate the role PP1 plays in learning and memory related tasks has revealed that PP1 inhibition enhances learning, and that genetic inhibition of PP1 improves learning and memory. Genoux et al., (2002) discovered that PP1 inhibition improves repetition learning. Repetition learning is a prerequisite for the formation of accurate and long-lasting memory. Furthermore, practice is most effective when widely distributed over time, rather than when closely spaced or massed. The molecular mechanisms of such time dependent constraints on learning and memory were unknown. Genoux et al. (2002) demonstrated that PP1 determines the efficacy of learning and memory. They discovered that short intervals between training episodes are sufficient for optimal performance in the novel object recognition task when PP1 is inhibited (Genoux et al., 2002). These observed improvements in learning and memory were related to an increase in CaMKII and CREB phosphorylation (Genoux et al., 2002).

PP1 inhibition also improves memory abilities in aged animals. Genoux et al. (2002) found that inhibition of PP1 after training resulted in memory retention in older animals. Studies have also been done to assess the effect of increased PP1 activity with respect to learning and plasticity. Mice lacking inhibitor 1, which display an increase in PP1 activity had deficits in conditioned place preference, exhibiting a decreased preference for a context which had been associated with cocaine (rewarding drug) (Zachariou et al., 2002).

15 Calcineurin (PP2B)

Calcineurin, or CN, named for its affinity for calcium, and its abundance in the nervous system is a calcium/calmodulin dependent serine/threonine phosphatase selectively enriched in the central nervous system (Sanna et al., 2006;Mansuy et al., 1998). Activation of CN requires calcium and calmodulin bound to its catalytic subunit, and once activated, it dephosphorylates inhibitor 1 (Morishita et al., 2001), causing an increase in protein phosphatase 1 activity (O'Dell and Kandel, 1994), and subsequently the dephosphorylation of CREB (Figure 7). CREB activation is regulated by the phosphorylation of several residues, ser-133 in particular. Given that there are a multitude of kinases able to induce CREB phosphorylation, random signaling events could potentially lead to CREB phosphorylation. In light of this, a mechanism to filter activation in order to prevent excessive CREB stimulation must be in place. CN acts as this filter, in order to guarantee that only robust CREB stimulation results in CRE induced changes in cellular functioning (Groth et al., 2003).

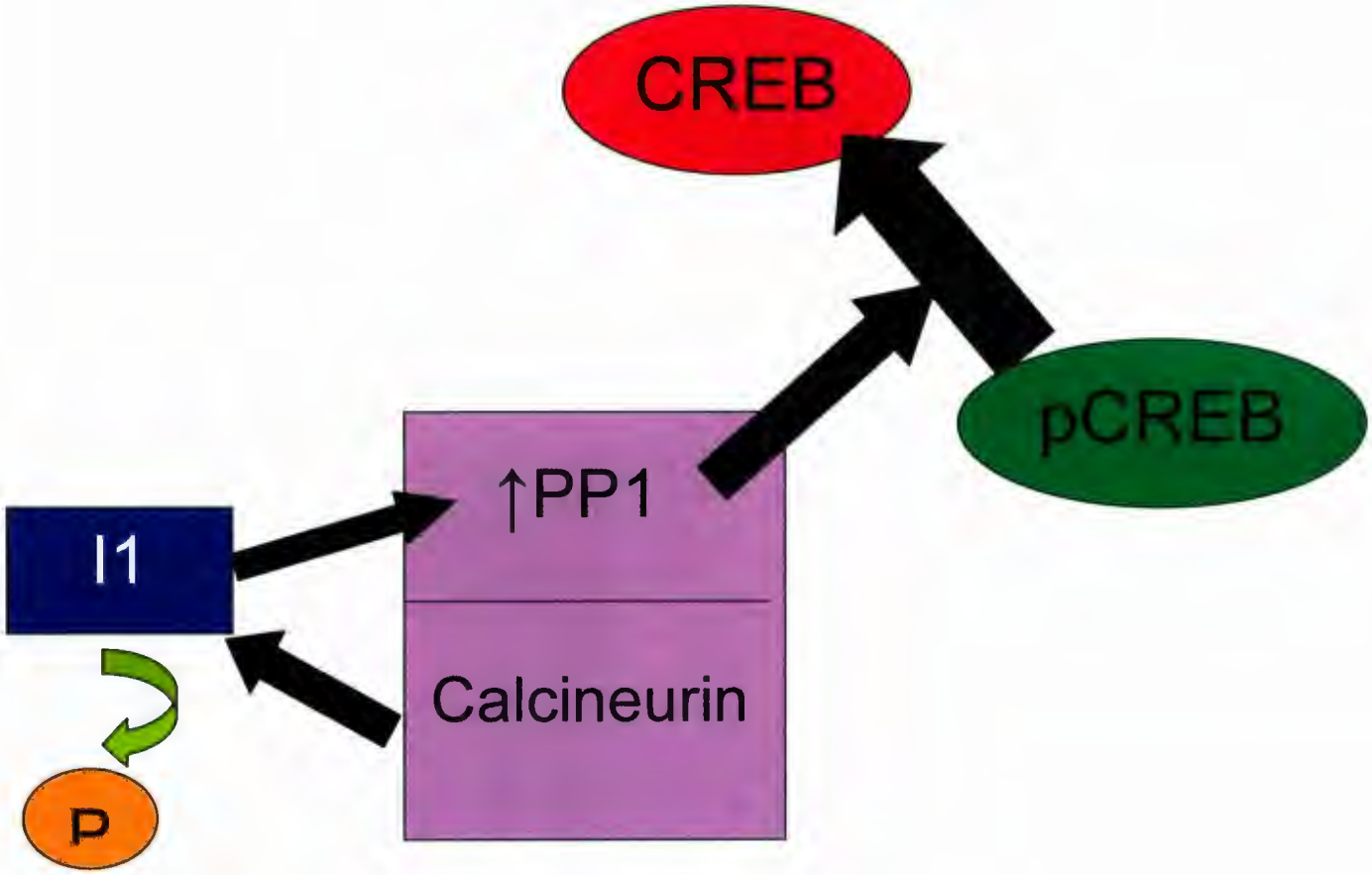

Figure 7: CN activation dephosphorylates inhibitor 1, which results in an increase in PP1 activity, which subsequently leads to the dephosphorylation of CREB.

15.1 CN in the CNS

15.2 Presynaptic, postsynaptic and cytoplasmic CN

Presynaptic terminals are full of CN, which act to regulate most of the proteins which are involved with neurotransmission. CN impacts both the endo and exocytotic portions of neurotransmitter release from synaptic vesicles (Greengard et al., 1993). CN is strategically placed in the postsynaptic densities of postsynaptic neurons where its main role is to negatively regulate signaling pathways downstream (Husi et al., 2000). It is also able to directly dephosphorylate synapsin I which prevents vesicles from joining the readily releasable pool, which results in a reduction of neurotransmitter release (King et al., 1984).

CN is very involved in neuronal cytoplasmic activity; it controls calcium homeostasis and, therefore, plays a large role in the modulation of neurons. CN is one of the first enzymes on the postsynaptic membrane to be activated by calcium ion influx (Klee et al., 1979). CN acts to weaken calcium influx, and downregulates postsynaptic calcium signaling (Armstrong, 1989). It also has an effect on voltage gated ion channels by acting on sodium ion activity (Chen et al., 1995). CN affects calcium by inhibiting its release from intracellular endoplasmatic reticulum stores. Release of calcium occurs via a process that requires IP3 receptors and ryanodine receptors. CN prevents excessive calcium discharge by reducing the sensitivity of both IP3 and ryanodine receptors (Cameron et al., 1995;Cameron, 1997). The control CN exerts on calcium-regulated negative feedback is crucial for learning, memory, neurotoxicity and neurodegeneration (Mansuy, 2003).

15.3 CN and long-term potentiation

Synaptic plasticity is defined by modification of synaptic transmission efficiency. Specifically, the connection between one neuron and another is either strengthened or weakened. It is this change in the efficacy of synaptic transmission which is believed to be responsible for learning and memory (HEBB and KONZETT, 1949).

LTP is widely studied because of its relevance to synaptic plasticity. As mentioned, LTP is characterized by an increase in excitatory neurotransmission after high frequency stimulation, and most forms of LTP are initiated by NMDA receptor activation. CN has a negative regulatory role in LTP. Inhibition of CN leads to enhanced LTP, and lowers the threshold of activation needed for LTP induction (Wang and Kelly, 1996). Consistent with this, transgenic expression of CN, doubling its activity, impairs LTP in the hippocampus area CA1 (Winder et al., 1998). On the other hand, when CN activity is blocked with antisense oligodeoxnucleotides, LTP in hippocampal slices is facilitated and enhanced (Dawson et al., 1993;Wang and Kelly, 1996).

15.4 CN's role in invertebrate learning

CN is involved in several sensory evoked behaviors in the nematode (worm) *C. elegans*, such as osmotic avoidance, chemotaxis, olfactory avoidance and thermotaxis (Kuhara et al., 2002). When a worm is grown at a specific temperature, it will seek that temperature when placed on a thermal gradient (Hedgecock and Russell, 1975). Mutated worms with a loss of CN function

seek temperatures which are warmer than the temperature they usually seek, and are most familiar with (Kuhara et al., 2002). Worms are able to sense several odors, and display chemotaxis towards them (Dusenbery et al., 1975). When pre-exposed to an odor, they display an adaptation to that odor, and upon subsequent exposure, will not chemotax towards the odor. Loss of CN function in these animals results in a faster adaptation to the odor, lending support to the notion that CN is a negative regulator of signaling in sensory neurons responsible for behavior modulation (Kuhara et al., 2002).

CN inhibition has also been examined in *Aplysia*. As previously mentioned, memory can be assessed in *Aplysia* with tail pinch-elicited siphon withdrawal behavior. After only a single shock to the tail, sensitization to tail stimulation will occur, and can last up to 30 min. However, application of FK506 to inhibit CN results in an intermittent 90 min sensitization, and a long-term sensitization of 18 hrs in response to two spaced shocks, compared to control animals which require at least five tail stimulations to exhibit a similar time course of sensitization (Sharma et al., 2003)

15.5 The role of CN in vertebrate learning and memory

CN mediates rapid and enduring changes in neuronal activity and excitability, thus it seems appropriate to expect that it is able to modify behavior. Deciphering the functional role of CN in learning and memory is challenging given that it has multiple targets in the brain. However, the general consensus based on pharmacological, genetic, behavioral and electrophysiological studies is that CN is a negative regulator of learning and memory (Yakel, 1997). CN directly

dephosphorylates CREB which, as mentioned, has a causal role in learning and memory. It has therefore been suggested that overexpression of CN or its up regulation can lead to memory deficits and problems with long-term learning (Genoux et al., 2002). This is, in fact, the case; mice genetically manipulated to over-express CN exhibit memory impairments on the spatial version of the Barne's maze (Mansuy et al., 1998). On the other hand, diminishing CN within the CNS has a positive effect on learning and memory (Malleret et al., 2001) . Suppressing CN activity resulted in longer lasting object recognition (3 days and even one week, vs. 24 hrs for controls), as measured by longer exploration of a novel object relative to a familiar object (Malleret et al., 2001). Infusion of FK506, a calcineurin inhibitor, into the hippocampus after training, enhanced conditioned place preference. Animals that were exposed to a specific context, which was paired with amphetamine, preferred the conditioned context for twice as long when CN was inhibited (Gerdjikov and Beninger, 2005). Interestingly, in one study, mice with an over-expressed, truncated form of CN exhibited spatial memory problems relative to control mice, however, spatial memory was still intact in those animals since when the number of behavioral trials was increased, mutant animals performed on par with controls (Mansuy et al., 1998). Mansuy et al. (1998) provide evidence for the notion that CN is critical for setting the threshold of stimulation necessary for memory. Concordant with the observed behavioral effects, inhibiting CN in cultured hippocampal neurons slows the kinetics of pCREB decay, delaying its return to baseline levels (Lee et al., 2005). This suggests that the kinetics of increases in pCREB is likely to control learning thresholds and/or memory durations.

16 Rationale and hypothesis

Previous work in our lab has focused on the cellular events underlying memory formation in the olfactory bulb. Pairing odor and β-adrenoceptor activation results in increases in cAMP (Yuan et al., 2003b; Cui et al., 2007) and increased CREB phosphorylation in olfactory bulb mitral cells (McLean et al., 1999).

The present thesis sought to expand on the growing link between CN and memory by investigating its effect on CREB in the neonate odor preference learning model. The transcription factor CREB is of crucial importance to this learning paradigm, its phosphorylation is both causal, and necessary in neonate rat odor preference learning and when CREB phosphorylation is disrupted, learning no longer occurs (Yuan et al., 2003a).

Consistent with the memory deficits that ensue when CN activity is upregulated, CN's ability to dephosphorylate CREB, and the causal role CREB phosphorylation plays in the formation of a long term olfactory memory, it was hypothesized that bilateral infusion of a CN inhibitor into the olfactory bulb of neonate rats would delay the dephosphorylation of CREB, and extend the duration of the conditioned odor preference. Further, it was predicted that CN inhibition would have the greatest effect immediately following training, based on previous work that demonstrated CREB phosphorylation peaked 10 min after training, and was back to baseline levels 60 min after training (McLean et al., 1999).

It was also hypothesized that CN inhibition would modulate the inverted U-curve obtained when β-adrenoceptor activation via ISO is used as the US, in place of stroking. It was predicted that FK506 would produce learning when both the low and high learning ineffective doses of ISO are combined with odor. The present thesis suggested that higher doses of ISO do not induce conditioned olfactory learning due to excessive phosphatase activity, and hypothesized that low doses are ineffective due to insufficient cAMP activation.

17 Objectives

The main objectives of the present thesis were:

1. to assess if a bilateral intrabulbar infusion of FK506 extends the duration of conditioned olfactory memory in neonate rats
2. to determine whether CN inhibition with FK506 is able to modify the inverted ISO dose response curve obtained when ISO is used as the US
3. to assess if CN inhibition prolongs the duration of CREB phosphorylation

CHAPTER II METHODS

1 Animals

A total of 252 Sprague–Dawley rat pups from 34 litters culled to 12 pups on PND 0 or 1 were used. All dams were housed in polycarbonate cages containing hardwood chips on a 12 hr light/dark cycle at 21°C in the animal care facility at the Health Sciences Centre of Memorial University of Newfoundland. Prolab RMH 3000 rat diet (Brentwood, MD) and water were available *ad libitum*. All procedures were approved by the Memorial University Institutional Animal Care Committee and conformed to the standards set by the Canadian Council on Animal Care.

2 Guide Cannulae

CN inhibition was performed via a direct intrabulbar infusion of FK506. Cannulae were made from dental acrylic fashioned in clay putty (4x4mm). Subsequently, two guide cannulae (Small Parts Inc. Florida, 23 gauge tubing cut to 6mm) were placed into the molded square, 2 mm apart from one another, extending 1mm below and above the imprinted square. Once the guide cannulae were in place, dental acrylic (Lang Dental mfg. Co. Wheeling, IL and Lang Fast Curing Jet Acrylic resin) was poured into the imprint. When the acrylic had hardened, excess acrylic was trimmed off to make the cannulae assembly as small as possible. Insect pins (size 00 Indigo Instruments) were placed into each guide cannula to act as stylets and prevent the ascension of blood and cerebrospinal fluid into the guide cannulae. The insect pins were trimmed and sanded

to sit exactly even with the guide cannulae at the intracranial side, and were crimped slightly to prevent their removal by the dam.

3 Infusion Cannulae

Infusion cannulae were made with a piece of 30 gauge stainless steel tubing cut to the length of 10 mm. The ends of the 30 gauge tubing were sanded to create an opening, and the infusion cannulae were inserted into a piece of PE20 polypropylene tubing. The attached infusion cannula and tubing were measured and trimmed to assure that the end of the tubing sat at the very tip of the top portion of each 23 gauge guide cannula, and extended exactly 1mm below the bottom portion.

4 Surgery

On PND 5, each pup was anesthetized by hypothermia, (approximately 5-6 min) and once unresponsive to tail pinching, surgery was begun. The pup was placed into a stereotaxic holder on ice, and its head was secured with ear clamps. A number 15 scalpel blade was used to make a sagittally-oriented incision along the skin of the pup's skull. The skull was cleaned and dried - with a long-tipped cotton swab. Next a hole was drilled through the skull over the centre of each olfactory bulb. A clean hole was created, just piercing the dura, but without harming the brain. In some cases it was necessary to remove tiny pieces of bone with fine forceps. A small plastic screw (Small Parts: Florida, MN0440-02F-C) was glued to the skull posterior to the drilled holes to act as an anchor. Next the molded cannula assembly was lowered to a level where the paired

guide cannulae sat just above the drilled holes. Dental acrylic was placed all around the cannulae (cannulae were held in place until the acrylic hardened with a modified electric clip and stereotaxic apparatus). Once the dental acrylic had hardened and the cannulae had been secured to the skull, 4-0 suture was used to close the skin on the head of the animal, protecting the acrylic and implanted cannulae. Finally, the cannulae and sutured area were covered with Bitter Orange (Gourmet Pet, Georgia) to prevent the mother from chewing and licking the pups in the region of the cannulae. Surgery typically lasted 15-17 min, and pups were rubbed by hand and re-warmed on a heating pad. Most pups began to breathe regularly after being warmed for 2-3 min, and were returned to the dam when 4 surgeries had been completed.

5 Olfactory learning, drug injection, and sample collection

On the afternoon of PND 6, each pup was removed from the dam and given a s.c. injection of ISO. Pups were then placed back with the dam, until 30 min had passed, at which time they were once again removed and placed on clean bedding for 10 min prior to the commencement of training. Subsequently, each pup was placed on peppermint scented bedding for 10 min (0.3 ml of peppermint extract per 500 ml of bedding). Immediately following training each pup received an intra-bulbar 1 µl infusion of the CN inhibitor FK506 (Alexis Biochemicals, Switzerland) dissolved in DMSO to a concentration of 5mM, or vehicle. All infusions were 1 min in duration. Pups were then replaced with the dam and were subsequently tested for conditioned odor preference on PND 7, or were sacrificed for pCREB immunohistochemistry. The concentrations of infused FK506 (5, 10 and 20 mM) were selected based on previous research (Lin et al., 2003; Nakazawa et al., 1995; Gerdijikov et al., 2005). For example, Lin et al., (2003) infused 7.77mM

of FK506 into the amygdala, Nakazawa et al. (1995) infused 1.99mM FK506 into the accessory olfactory bulb and Gerdijikov et al. (2005) infused 2.49 and 12.43 mM FK506 into the nucleus accumbens.

6 Testing

On PND 7, each pup was given a test for odor preference. The tester was blind in all cases to which experimental procedure the pup had been subjected. The testing apparatus consisted of a stainless steel testing box (36 x 20 x 18 cm) with a mesh bottom (1 cm x 1 cm openings) centered over two trays, which were placed 2 cm apart from one another creating a neutral zone. One tray contained 500 ml of fresh bedding, and the other contained peppermint scented bedding, prepared by the same method outlined in training. A small polypropylene mesh grid (1000 µm, Small Parts, Inc. Florida) was placed on the floor of the box to allow easy movement of the pup about the box, and to prevent pups from falling between the gaps of the mesh bottom.

Each pup underwent five one-min trials. A trial was begun with the pup in the neutral zone, the direction the pup faced was alternated between trials, and pups were given 30 seconds of rest between trials. If the pup's nose and one paw moved from the neutral zone into either the control or peppermint zone, the timer for that side was started. Summation of the time spent over peppermint was divided by the total activity time (time spent out of neutral zone) to give the percent time over peppermint odor.

7 Collection of sample for immunohistochemistry, and quantification with relative optical density

Infusions were performed immediately after peppermint exposure. Each pup was given an infusion of vehicle into one bulb, and FK506 into the other (the bulb receiving FK506 was randomly assigned among pups). Forty min after infusion, pups were removed from the dam, and were decapitated using surgical scissors. An incision was made into the skin caudal to the snout, and the skin was peeled back to expose the skull. The cannulae and skull were next removed using bone rongeurs, paying careful attention not to pull the dural covering of the olfactory bulb. The underlying attachments between the brain and skull were removed with fine forceps, and the entire brain was transferred into a vial containing 4% paraformaldehyde fixative in a 0.1 M phosphate buffer. Brains were left in fixative overnight and then were transferred into a 20% sucrose solution in 0.1 M phosphate buffer. Brains were kept in the sucrose solution at 4 °C, until they were cut 24 hrs later.

Brains were mounted on a cryostat stage with cryomatrix frozen specimen embedding medium, and were quick frozen in powdered dry ice for approximately 5 min. Brains were subsequently moved to the cryostat, which was maintained at -20 °C. Coronal sections through the olfactory bulb and anterior olfactory nucleus were cut at 30 µm, keeping one out of every 7 sections. Sections were placed directly onto frozen chrom alum-gelatin subbed slides. Subsequently slides were incubated overnight with a 1/500 dilution of pCREB antibody made in 0.1 M buffered saline (PBS) with 2% normal goat serum, and 0.2% Triton X-100. The next day, following 3 rinses of the slides in PBS at room temperature, the sections were incubated in biotinylated

secondary antibody for one hour, rinsed and incubated in an avidin-biotin peroxidase solution for an hour, and, finally developed with diaminobenzodine tetrahydrochloride (activated by 0.01% hydrogen peroxidase). Sections were next dehydrated with a graded series of alcohols followed by xylene and were covered with permount and a cover slip.

Two middle (rostral to caudal) sections were selected per pup, and to avoid subjectivity, slides were coded so that the analyzer was blind to which bulb was infused with FK506. Image analysis (Bioquant, R&M Biometrics, Inc.) was performed by tracing the dorsal medial, ventral medial, dorsal lateral and ventral lateral quadrants of the glomerular, mitral and granule cell layers. An area of the olfactory nerve layer was captured digitally, and was assigned as the background level for optical density. Subsequently cells labeled with pCREB were captured digitally from the mitral, granule and glomerular layers. The relative optical density of each cellular layer was determined by subtracting the background level from the density of the pCREB labeled area of interest, and the difference was divided by the olfactory nerve background level, as described previously (Yuan et al., 2003b).

8 Statistical analysis

In experiment I, a one-way analysis of variance (ANOVA) was used to compare the statistical difference in time spent over peppermint between pups given 2 mg/kg of ISO and dimethyl sulfoxide (DMSO), or one of three concentrations of FK506 (5, 10, or 20 mM).

Also in experiment 1, a one-way ANOVA was used to assess the statistical difference in time spent over peppermint between pups given 2 mg/kg ISO with odor and 5mM FK506, 2 mg/kg ISO with odor and DMSO, saline with odor and FK506, or saline with odor and DMSO. The Bonferroni Multiple Comparison post hoc test was used to compare whether the time spent over peppermint was statistically different between all possible combinations of treatment pairs.

In experiment 2, a one-way ANOVA was used to determine if at 48 hrs there was a significant difference in preference for peppermint between pups given 2 mg/kg ISO, odor and CN inhibition or 2 mg/kg ISO, odor and DMSO. The Dunnett post hoc test was used to assess if pups that received 5, 10 or 20 mM of FK506 showed a significant difference for the conditioned peppermint odor compared to pups infused with vehicle control.

In the second portion of experiment 2, a one-way ANOVA was used to assess the time spent over peppermint between pups given 2 mg/kg ISO, odor and CN inhibition or 2mg/kg ISO, odor and DMSO. The Bonferroni Multiple Comparison post hoc test was used to compare the two treatment groups 24 hrs, 72 hrs, 96 hrs and a week after training.

In experiment 3, a one-way ANOVA was used to assess the relative optical density between bulbs which were infused with FK506 and bulbs infused with vehicle. The Bonferroni Multiple Comparison test was used for post-hoc comparisons.

In experiment 4, a one-way ANOVA was used to assess the time spent over peppermint 24 hrs after training between pups given 0, 1, 2, or 6 mg/kg ISO and CN inhibition. The Dunnett

multiple comparison post hoc test was used to assess if pups given 1, 2 or 6 mg/kg of ISO were statistically difference from control pups given 0 mg/kg ISO.

CHAPTER III RESULTS

1 EXPERIMENT 1

1.1 Surgery, intrabulbar infusion, and CN inhibition do not affect the memory normally seen 24 hrs after training.

In order to assess whether surgery and/or intrabulbar infusion affected the learning normally observed when odor is combined with 2 mg/kg ISO, various concentrations of FK506 were bilaterally infused, and conditioned odor preference was assessed 24 hrs after training. On PND 6 training was begun as described above. Each pup was given a subcutaneous injection of 2 mg/kg ISO, and following a 10 min exposure to peppermint was given a bilateral 1µl intrabulbar infusion of vehicle (DMSO) or one of three concentrations of FK506 (5, 10 or 20 mM). Pups were then placed back with the dam, and were tested for olfactory learning the next morning. A one way analysis of variance (ANOVA) revealed no significant difference among treatment groups, suggesting that infusion and surgery did not hinder conditioned odor preference learning (Figure 8). Pups that received 2 mg/kg of ISO, despite surgery and FK506 infusion acquired a preference for peppermint, spent greater than 50% of their time over the CS, and were not significantly different from the learning controls.

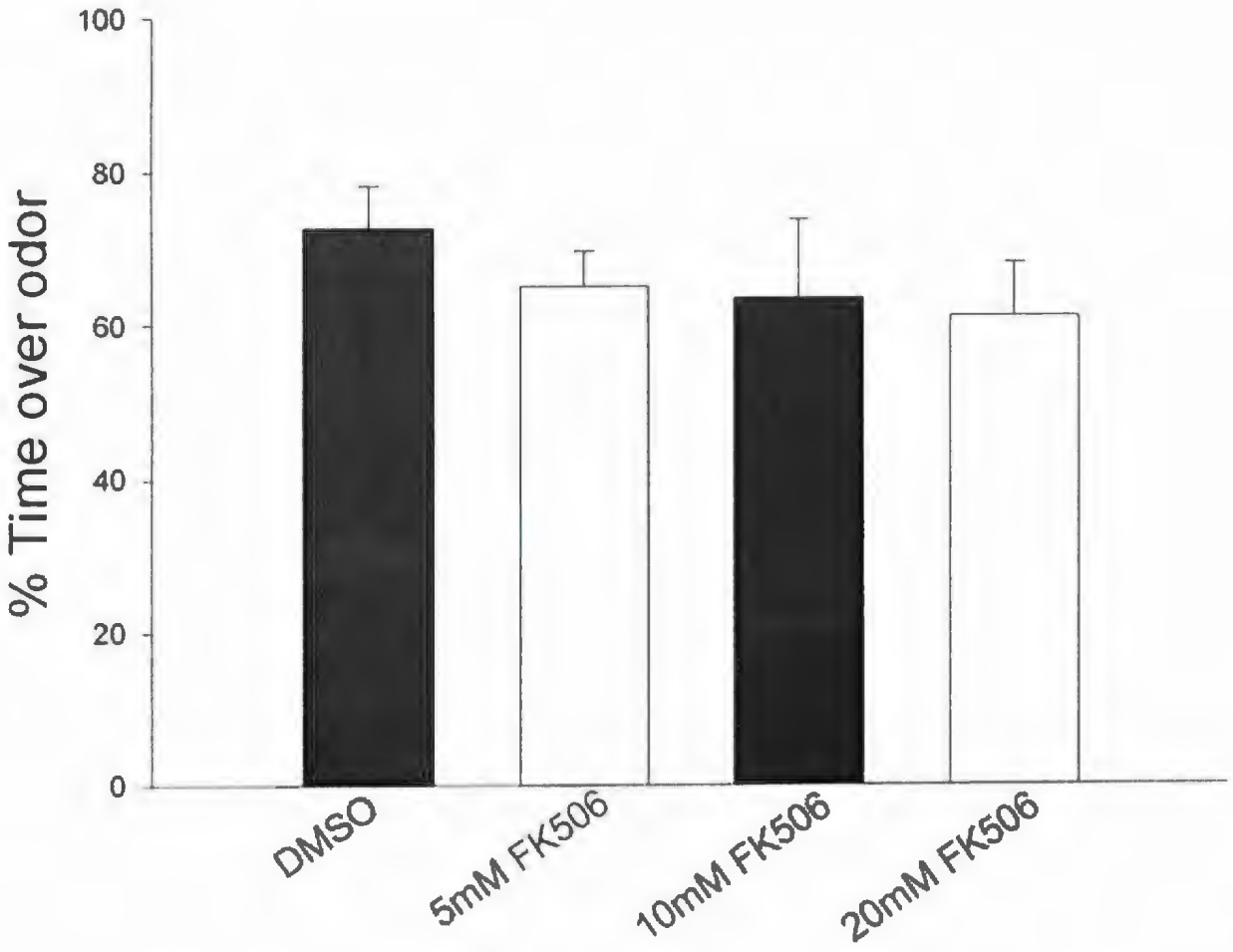

Figure 8: The combination of 2 mg/kg Isoproterenol (ISO) with DMSO or various concentrations of FK506 (5, 10 and 20 mM) did not hinder the normal learning observed at 24 hrs. N= 5 pups/group ($F_{(3, 16)} = 0.4846$; $p= 0.698$).

1.2 CN inhibition on its own does not produce olfactory memory

To better assess whether or not normal learning occurs when pups are exposed to surgery and an intrabulbar infusion, an additional control experiment was performed. On PND 6 paired litter mates were given a subcutaneous injection of saline, or 2 mg/kg of ISO. Peppermint exposure occurred 40 min later, and immediately thereafter litter mates were given either a bilateral intrabulbar infusion of 5 mM FK506 or vehicle. As can be seen in Figure 9, pups that received a subcutaneous injection of saline paired with odor did not show a learned preference for peppermint whether given an infusion of FK506 or vehicle. Pups that received a subcutaneous injection of ISO did however show normal olfactory learning when given both an infusion of FK506 and vehicle. These results confirm that 2 mg/kg of ISO combined with infusion results in a learned preference for peppermint. However, when ISO is replaced with saline learning does not occur with infusion of FK506 or DMSO alone.

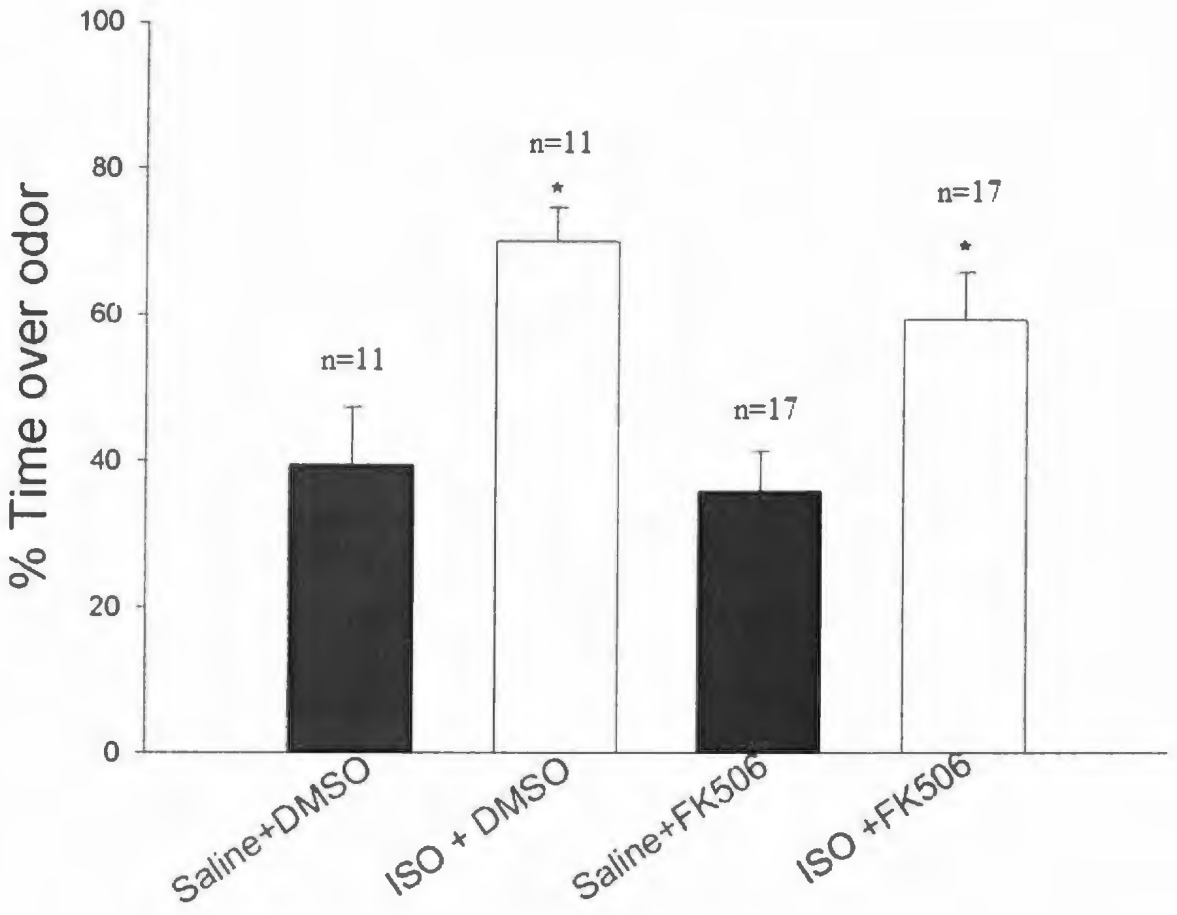

Figure 9: CN inhibition on its own with 5 mM FK506 was not able to induce a learned odor preference. Pups that received a subcutaneous injection of saline paired with odor did not show a learned preference for peppermint whether given an infusion of FK506 or vehicle. Pups that received a subcutaneous injection of ISO did, however, show normal olfactory learning when given both an infusion of FK506 and vehicle. These results confirm that 2 mg/kg of ISO combined with infusion results in a learned preference for peppermint. However, when ISO is replaced with saline, learning does not occur with infusion of FK506 or DMSO alone [$F_{(3, 52)}$ =6.223; $p < 0.05$, *Post-hoc (*Bonferroni Multiple Comparison test) $p<0.05$].

2 EXPERIMENT 2

2.1 Inhibition of CN with FK506 extends the duration of an olfactory memory

Whether or not a bilateral infusion of FK506 is able to extend the duration of a conditioned olfactory preference was assessed next. On PND 6, pups were again removed from the dam and given a subcutaneous injection of 2 mg/kg of ISO. As previously described, they were exposed to the peppermint odor for 10 min 40 min after receiving ISO. Immediately following odor presentation, pups were given a bilateral intra-bulbar 1 µl infusion of vehicle or one of three concentrations of FK506 (5, 10 or 20mM). Pups were then placed back with the dam and were tested for conditioned odor preference 48 hrs later. One way ANOVA revealed a significant difference between treatment groups ($F_{(3, 31)}$= 4.212; p<0.05) and a post-hoc Dunnet test revealed that pups that received 5, 10 or 20 mM of FK506 all showed a significantly higher preference for the conditioned peppermint odor when compared to pups infused with vehicle (p<0.05). These results confirm that pups given 2 mg/kg if ISO when combined with odor forget their preference for peppermint 48 hrs after training (having remembered at 24 hrs), while all three concentrations of CN inhibitor facilitated extended memory (Figure 10).

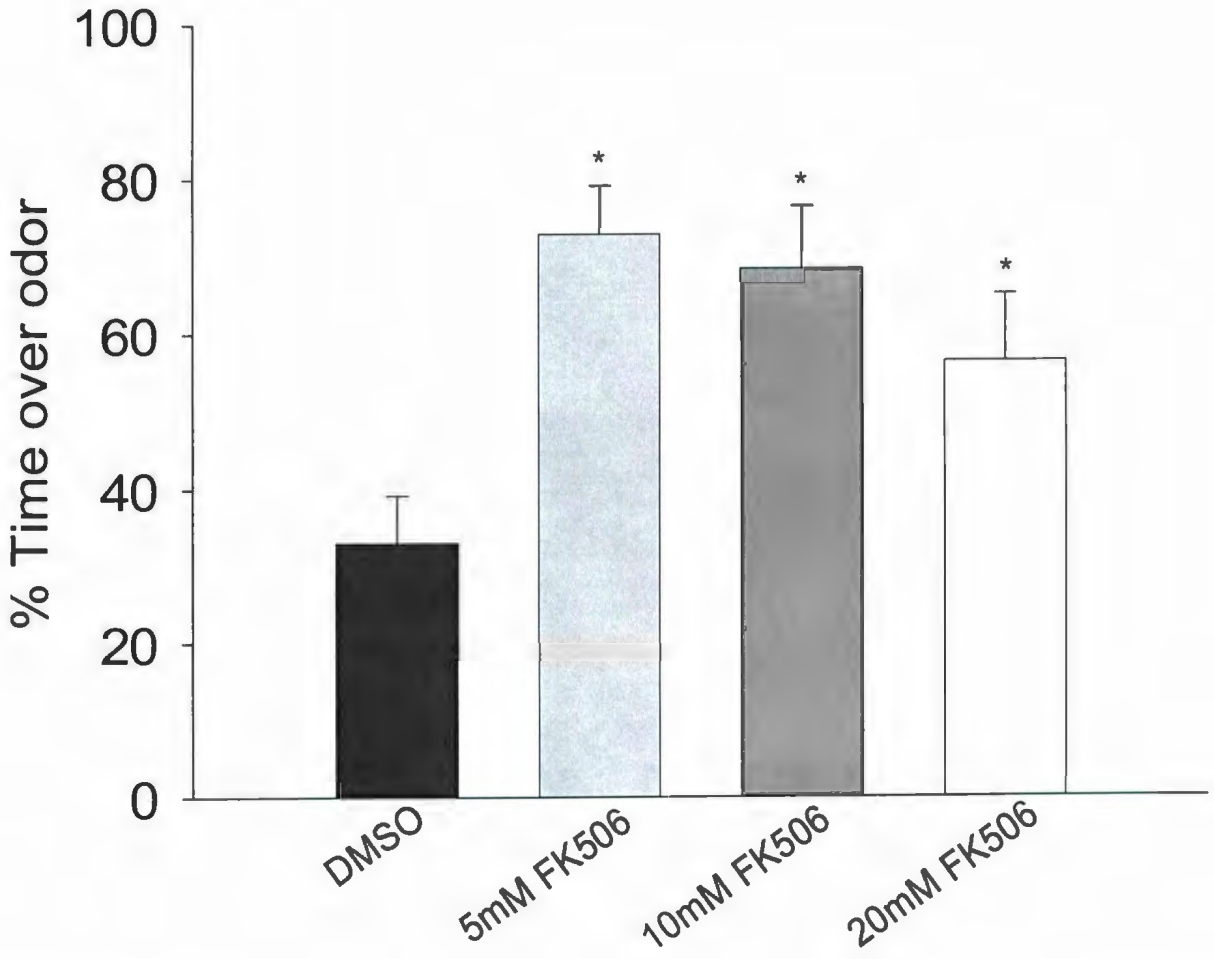

Figure 10: CN inhibition with infusion of FK506 extended memory of earlier training with 2 mg/kg ISO (n=9/group). Conditioned odor preference was seen at 48 hrs.

Once it was determined that CN inhibition with FK506 does, in fact, extend the duration of conditioned odor preference, longer durations of memory extension were investigated. On PND 6 pups received a subcutaneous injection of saline or 2 mg/kg ISO, and an intra-bulbar bilateral 1 µl infusion of 5 mM FK506. Paired litter mates were subsequently tested for odor preference 24 hours, 72 hours, 96 hours, or one week after training (pups were subjected to repeat testing, each pup was trained on peppermint and tested for odor preference one time). A one way ANOVA and post-hoc Bonferroni Multiple Comparison Tests revealed a significant difference in preference for peppermint between pups infusion with FK506 or vehicle 24, 72 and 96 hours after training.

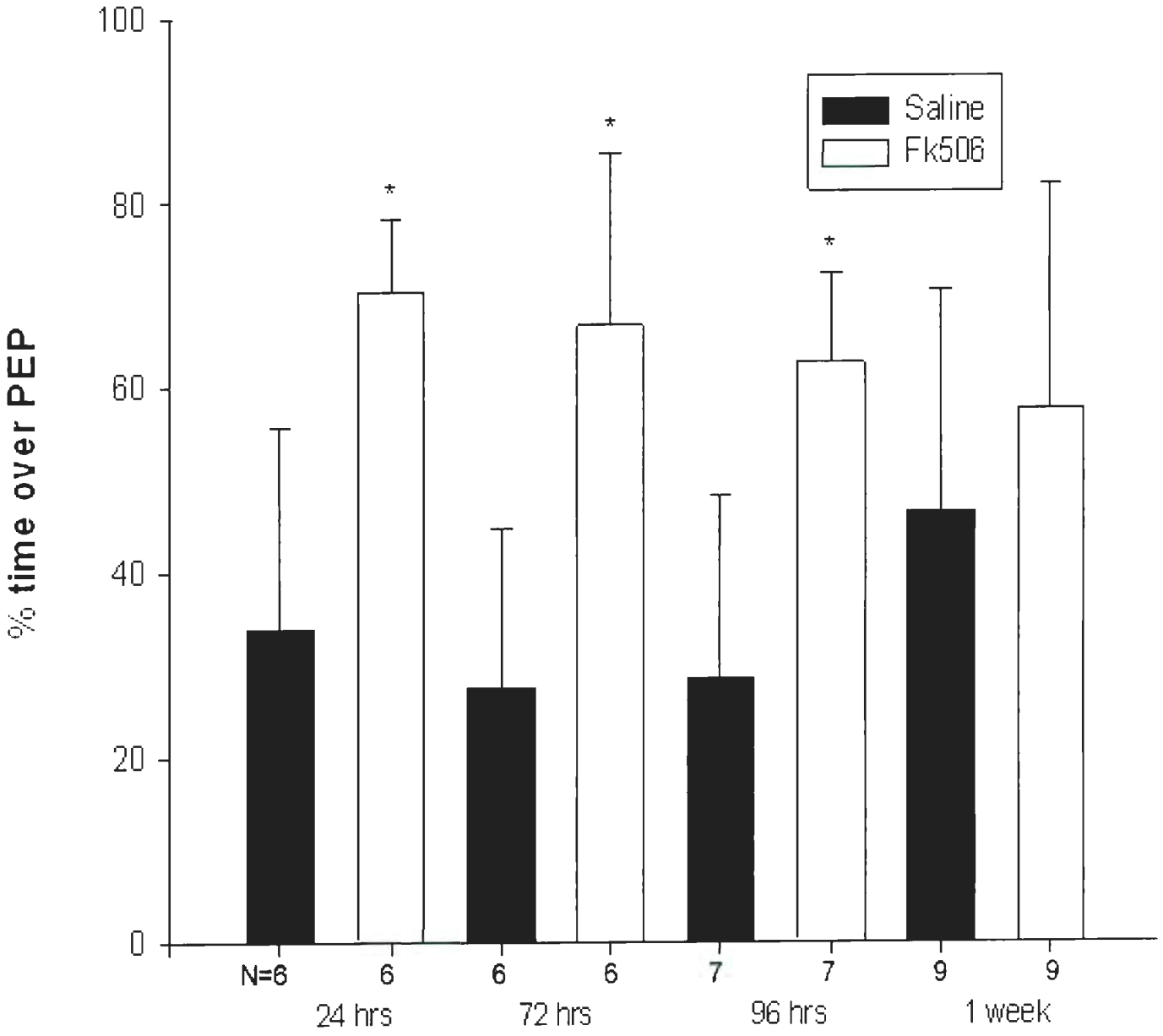

Figure 11: Preference for peppermint was observed 24, 72, and 96 hrs after training when CN was inhibited.

3 EXPERIMENT 3

3.1 Immunohistochemistry reveals that CN inhibition results in a greater concentration of olfactory bulb pCREB 40 mins after training.

Once it was determined that CN inhibition had a clear behavioral effect, and was able to extend the duration of olfactory memory, the hypothesis that prolonged CREB phosphorylation is responsible for memory extension was evaluated.

On the afternoon of PND 6, each individual pup was removed from the dam and given a subcutaneous 2 mg/kg injection of ISO. Immediately after peppermint exposure, pups were given a 1 µl unilateral infusion of FK506, and a unilateral 1 µl infusion of DMSO. The side given each infusate varied from pup to pup. In neonate olfactory learning, CREB phosphorylation at Ser 133 returns to baseline level 30 min after training (McLean et al., 1999). Thus in the present experiment, pups were killed 40 min after training to investigate whether or not bulbs infused with FK506 would show a greater number of phosphorylated-CREB positive nuclei in comparison to bulbs infused with vehicle. Slices were first observed under a microscope for visible differences in pCREB staining. As depicted in Figure 12, there were noticeable differences in the number of nuclei stained positively for pCREB in the granule, mitral and glomerular cell layers.

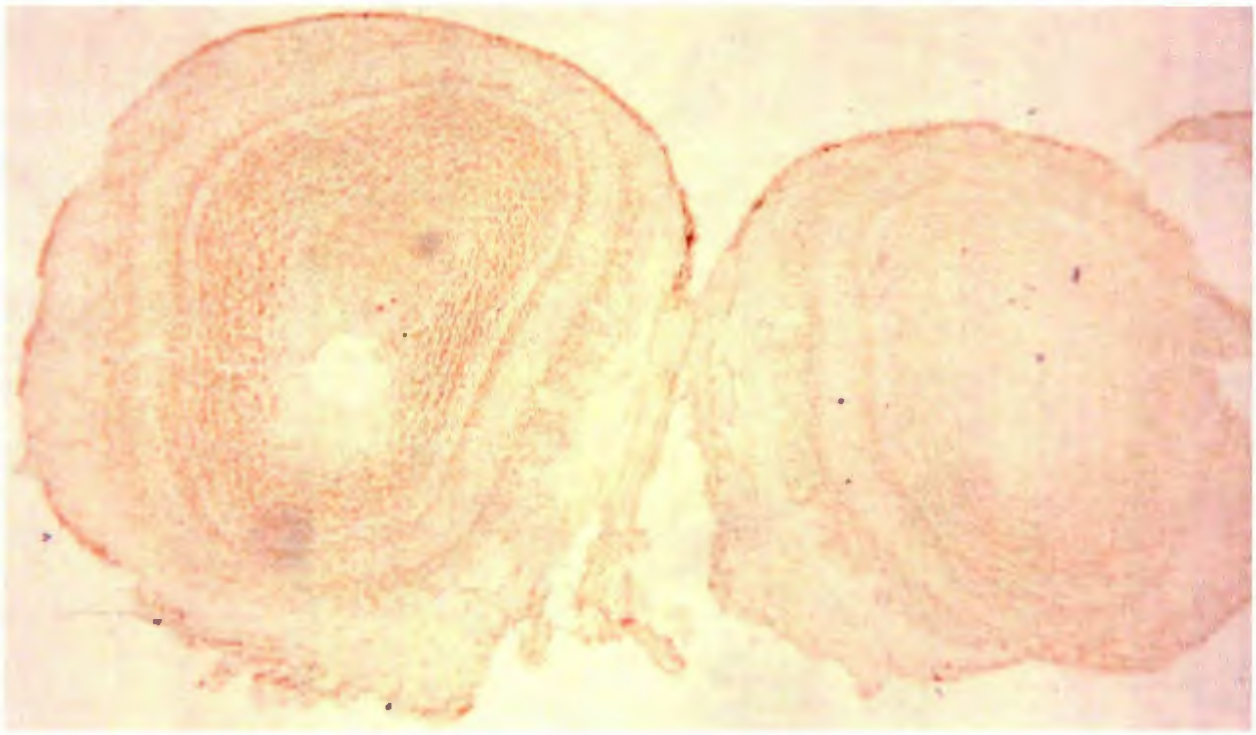

Figure 12: Odor plus 2mg/kg of ISO causes a generalized increase in CREB phosphorylation throughout the olfactory bulb. When FK506 was infused into the left bulb immediately following training, a greater number of nuclei stained positively for pCREB, compared to the right bulb, which was infused with vehicle. Pups were killed 40 min after training.

3.2 Quantification of immunohistochemistry with relative optical density

Subsequently, four brains were quantified for relative optical density of pCREB expression. Two middle (rostral to caudal) sections were selected per pup, and image analysis was performed by tracing the dorsal medial (DM), ventral medial (VM), dorsal lateral (DL) and ventral lateral (VL) quadrants of the glomerular, mitral and granule cell layers. Labeled cells were captured digitally using a background subtraction method. An area of the olfactory nerve layer was traced, and captured digitally to represent the background optical density level. Relative optical density was determined by subtracting the background level from the density of pCREB labeled cells in the area of interest, and dividing that difference by the olfactory nerve background level. The relative optical density between the bulb infused with FK506, and the bulb which received vehicle was significantly different in the mitral, granule and glomerular cell layers when all four quadrants (DM, VM, DL and VL) were combined (Figure 13). The relative optical density between the olfactory bulb infused with FK506, and the olfactory bulb which received vehicle was significantly different in the mitral, granule and glomerular cell layers when all four quadrants (DM, VM, DL and VL) were combined, n=4 for each group; [$F_{(5, 12)} = 4.443$; $p<0.05$, Figure 13]. *Post Hoc* Bonferroni Multiple comparison tests revealed that the number of nuclei stained positively for pCREB was significantly different in the glomerular layer ($p<0.01$), mitral cell layer ($p<0.05$) and the granule cell layer ($p<0.01$). Differences were also seen between bulbs in 3 of the 4 quandrants examined. A one way ANOVA revealed that the number of nuclei stained positively for pCREB significantly differed between bulbs in the ventral lateral quadrant of the olfactory bulb ($p<0.05$). *Post hoc* analysis with the Bonferroni Multiple comparison test determined that in the ventral lateral quadrant the number of nuclei stained positively for pCREB

was only significantly different in the glomerular layer. A second one way ANOVA revealed that the number of nuclei stained positively for pCREB differed between bulbs in the dorsal lateral quadrant of the olfactory bulb ($p<0.01$). *Post hoc* analysis with the Bonferroni Multiple comparison test determined that in the dorsal lateral quadrant, the number of nuclei stained positively for pCREB was significantly different in the mitral cell layer ($p<0.01$) and the granule cell layer ($p<0.05$). A third one way ANOVA revealed that the number of nuclei stained positively for pCREB was not significantly different between bulbs in the ventral medial quadrant of the olfactory bulb ($p=0.082$). A final fourth ANOVA revealed that the number of nuclei stained positively for pCREB significantly differed between bulbs in the dorsal medial quadrant of the olfactory bulb ($p<0.01$). *Post hoc* analysis with the Bonferroni Multiple comparison test determined that in the ventral lateral quadrant, the number of nuclei stained positively for pCREB was significantly different in the mitral cell layer ($p<0.01$) and the granule cell layer ($p<0.05$).

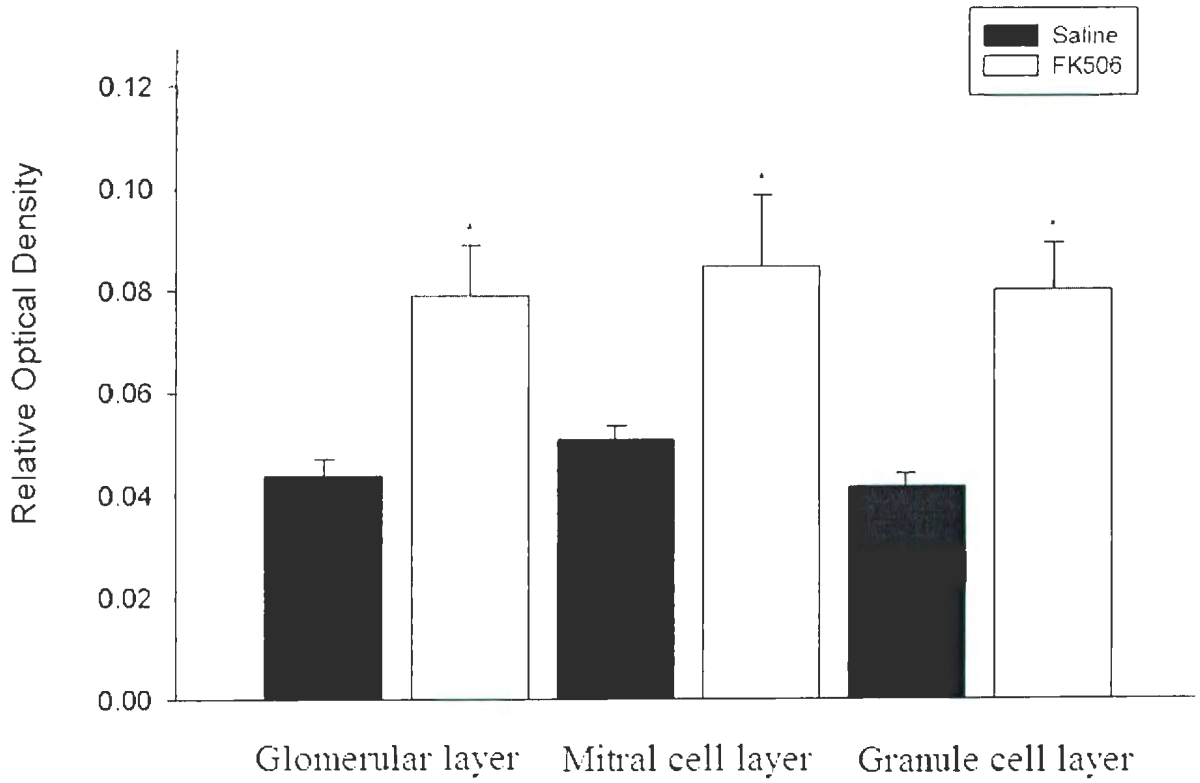

Figure 13: The relative optical density between the olfactory bulb infused with FK506, and the olfactory bulb which received vehicle was significantly different in the mitral, granule and glomerular cell layers when all four quadrants (DM, VM, DL and VL) were combined, n=4 for each group.

Experiment 4 Modification of the ISO inverted U-curve with CN inhibition

On PND 5 cannula placement surgery was performed, and the following day olfactory training was begun. Pups were given a subcutaneous injection of saline, or of one of three concentrations of ISO (1mg/kg, 2 mg/kg or 6mg/kg). After odor exposure, pups were given a bilateral intrabulbar infusion of 5 mM FK506. Subsequently on PND 7 pups were tested for olfactory preference learning.

A one-way ANOVA revealed that pups infused with FK506, and given ISO in the concentration of 1, 2 or 6 mg/kg all showed a significant preference for peppermint when tested 24 hrs after training when compared to control pups which received infusion of FK506 and saline ($F_{(3, 32)} = 4.212$; $p<0.05$). *Post hoc* analysis with the Dunnet multiple comparison test revealed that all three concentrations of ISO led to a greater preference for peppermint when compared to control pups.

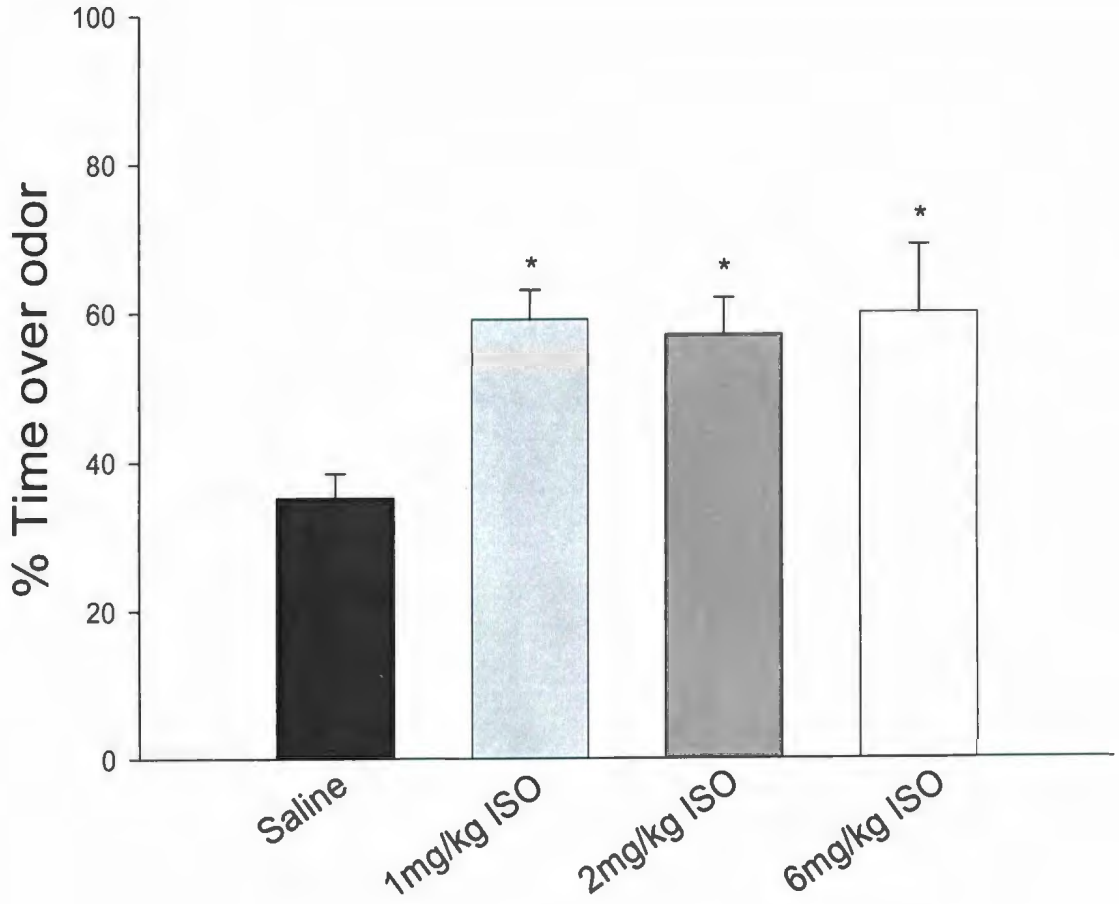

Figure 14: CN inhibition modified the inverted U-curve normally seen when 1, 2 and 6 mg/kg of ISO are administered as the US. Conditioned odor preference was seen 24 hrs after training in pups that received 1, 2 and 6 mg/kg of ISO when CN was inhibited with 5mM FK506, n=9 for each group.

CHAPTER IV DISCUSSION

1 Summary of findings

The present project extended the duration of associative olfactory memory from 24 hrs to 96 hrs with FK506, a CN inhibitor. The memory abilities of FK506 at 24 hrs were not dose dependent at the range tested. Three different concentrations of FK506 (5, 10 and 20mM) all led to a significant odor preference 48 hrs after training, whereas control animals without CN inhibition produced memory at 24 hrs only. These are exciting and novel findings since CN inhibition in the olfactory bulb has not previously been examined, and olfactory bulb memory has not been extended using a CN inhibitor. It is suggested that the mechanism of memory extension is prolonged CREB phosphorylation. We show that CN inhibition is able to delay the dephosphorylation of CREB, and enhance memory consistent with the proposed causal role of CREB phosphorylation in plasticity and memory. Further, these findings suggest that it may not be the quantity of pCREB, but the duration of its activation that determines the duration and saliency of a memory. This latter hypothesis will now need more detailed exploration.

2 Reports and experiments that support the hypothesis that CN inhibition enhances learning and memory

The role of the protein phosphatase CN in learning memory has previously been examined (Bennett et al., 2003;Lin et al., 2003;Mansuy et al., 1998). The present thesis, consistent with

previous work, proposes that CN inhibition has an enhancing effect on long-term learning and plasticity. As reviewed earlier, Ikegami et al. (2000) infused antisense oligodeoxynucleotides against CN into adult rats, and found that LTP was enhanced in area CA1 of the hippocampus. They also assessed hippocampal-related learning tasks and discovered that CN inhibition in rats with the antisense oligodeoxynucleotide facilitated contextual fear learning, whereby CN inhibited rats displayed a greater number of conditioned freeze responses. Mansuy et al. (1998) conducted a study which demonstrated that over-expression of CN produces memory deficits, lending further evidence to the suggested memory-enhancing role of CN inhibition. In their study, transgenic mice with an over-expressed truncated form of CN were deficient in the spatial version of the Barnes maze. Mice with increased expression of CN made a significantly greater number of errors when trying to locate a tunnel in order to exit a brightly lit and aversive maze. Gerdijikov et al (2005) assessed conditioned place preference in rats, and discovered that rats given an infusion of FK506 to inhibit CN continued to show a preference for the conditioned context 3 days after training, compared to a memory that lasted only 24 hrs in control animals. This latter result is similar to the present pattern in demonstrating extension of memory duration. Finally, Malleret et al. (2001) assessed spatial and non-spatial learning in a mouse where CN activity was decreased via the expression of a genetic inhibitor. They assessed the discrimination ratio between a novel and familiar object. Animals which underwent temporary CN inhibition continued to prefer the novel object 3 days and even 1 week after training, compared to control animals which began to show an equal ratio between the novel and familiar object after only 24 hrs. The present thesis suggests that CN might promote the conversion of short term memory to long term memory. The experiments which were performed in the present thesis and earlier studies suggest that CN also has a significant role in determining the duration

of long-term memory. There is evidence that in some circumstances life long memories are created, however most inputs are eventually forgotten after varying intervals, even if they were initially retained for 24 h. There are at least two forms of long-term memory seen with different habituation training protocols in the *C. elegans*. One form of memory lasting 24 hrs is mediated by glutamate, dependent on protein synthesis and is a long term memory produced by spaced and distributed training. A second form, lasting at least 12 hrs, but less than 24, is protein synthesis independent produced by massed training (Steidl et al., 2003). This evidence supports the hypothesis that long-term memory also occurs in stages. This suggests that in the present thesis, CN inhibition acted in a similar manner, and modulated the signals which control the duration of long term memory.

3 CN inhibition as a human memory enhancer

We have demonstrated that a bilateral intrabulbar infusion of FK506, to inhibit CN, is able to extend the duration of neonate olfactory associative memory. Several studies have solidified the hypothesis that there exists a relationship between CN and learning/memory. Improved hippocampal LTP (Malleret et al., 2001) enhanced contextual fear conditioning (Ikegami and Inokuchi, 2000) and heightened condition place preference learning (Gerdjikov and Beninger, 2005) as previously mentioned have all been observed with CN inhibition. In light of these findings, there is the potential to consider FK506 as a cognitive enhancer in humans. FK506 has been used safely in human populations as an immunosuppressant (Staatz and Tett, 2005) and readily crosses the blood brain barrier (Yokogawa et al., 1999). The fact that FK506 has been used safely in human populations suggests that memory enhancement, induced by FK506 in

human populations could be investigated. Administration of FK506 in humans could possibly lead to improvements in cognitive abilities, and may even ameliorate aging-related memory deficits.

4 CN inhibition for dementia and ageing

In this regard previous papers have suggested that CN inhibition could play a contributing factor in the alleviation of the learning and memory deficits present in the normal aging population and in those with dementias such as Alzheimer's disease (Agbas et al., 2005;Foster et al., 2001;Mayford and Kandel, 1999;Monti et al., 2005). Blocking CN activity has been shown to prevent beta amyloid induced LTP deficits in hippocampal cells (Yang et al., 2005;Chen et al., 2001;Wang and Kelly, 1997). Chen et al. (2001) inhibited the induction of late phase LTP in the dentate gyrus medial perforant path of hippocampus by causing an increase in beta-amyloid peptides with $A\beta_{1-42}$. When the cells were bathed in a CN inhibitor prior to $A\beta_{1-42}$ application, Aβ induced deficits in the induction and maintenance of L-LTP were prevented. This suggested that inhibiting CN could potentially ameliorate hippocampal-type learning deficits associated with Alzheimer's disease.

CN inhibition may also be effective in alleviating learning and memory deficits present in aged populations. Hippocampal CN activity is increased in aged rats, and consistent with an aging related increase in hippocampal CN activity, the phosphorylated form of CREB is significantly reduced in these aged animals, which suggests that chronically active CN might inhibit and prevent the phosphorylation of CREB, leading to poorer memory outcomes (Foster et al., 2001).

Another study conducted by Mayford and Kandel (1999), demonstrated that over expression of CN is correlated with an increase in forgetting and impaired consolidation in young adults. Such studies suggest that CN not only plays a role in learning and plasticity, but may also play a part in the deficits which ensue during normal aging and Alzheimer's disease.

5 A delay in the decay of pCREB levels may be responsible for memory extension

McLean, Harley, Darby-King and Yuan (1999) qualitatively demonstrated that pCREB levels in the olfactory bulb return to baseline levels 30 min after olfactory training. Furthermore, Yuan et al. (2000) quantitatively determined with immunocytochemistry that pCREB levels are significantly higher when ISO is combined with odor 10 min after training, but that no significant difference exists between rats given saline or ISO 1 hrs and 2 hrs after training (Yuan et al., 2000).

To assess if delayed CREB dephosphorylation is responsible for memory extension, pups were trained with ISO and peppermint, given an infusion of FK506, and sacrificed 40 min after infusion. In support of our hypothesis that prolonged CREB phosphorylation is behind our memory enhancement, is the finding that there is a significantly greater amount of phosphorylated CREB 40 min after CN inhibition, while normally pCREB levels are back to baseline at this time with 24 hrs limited memory. These findings together with the earlier observation that excessively high levels of pCREB accompany nonlearning (Yuan et al., 2003a), suggest that it is not the absolute level of phosphorylated CREB, but rather the duration of CREB

phosphorylation which determines the saliency and duration of memory. Lee et al., (2005) related the duration of pCREB activation to CN activity. They found that pCREB duration in neuronal cultures from embryonic day 20 rats was affected by CN inhibition. 5uM of NMDA was used for 15 min to induce expression of pCREB, which peaked immediately and returned to baseline 15 min later. They observed that pre-incubation of the neuronal cultures with FK506 resulted in a sustained increased in pCREB for 180 min (Lee et al., 2005). Consistent with this finding, Bito et al, (1996) found that incubating hippocampal neurons with FK506 also delayed the return of pCREB levels to baseline. They applied a short stimulus train (18s at 50 Hz) and found that in the presence of FK506, pCREB remained elevated at 45 min, whereas control slice levels of pCREB had returned to baseline level by that time. Based on the results of these culture experiments, it is predicted that prolonged pCREB activation is also responsible for the memory extension observed in the present thesis with FK506.

Future research should further assess pCREB levels when CN is inhibited. The mechanism responsible for memory extension requires further investigation. How does prolonged CREB activation lead to memory at 72 and 96 hrs? At what point in time do pCREB levels return to baseline? What cellular changes occur in response to prolonged CREB phosphorylation?

6 CN inhibition modified the inverted ISO U-curve, sub and supra-optimal doses led to a learned preference.

Memory-related effects with CN inhibition were non-dose specific. Three differing concentrations of FK506 were able to extend the duration of conditioned olfactory memory.

Previous work in our lab has demonstrated that β-adrenoceptor activation with ISO is dose dependent. Learning occurs with a medium 2mg/kg dose of ISO, but higher doses of 6mg/kg or lower doses of 1mg/kg do not induce a learned odor preference (Langdon et al., 1997). Given the permissive, dose independent properties of FK506 on memory extension, we assessed whether CN inhibition with FK506 has an effect on the dose response curve typically seen when ISO is used as the US in conditioned olfactory learning.

Phosphatase activity is a known negative regulator of memory-related processes (Sun et al., 2003;Waddell, 2003;Genoux et al., 2002). More specifically, the balance between phosphatase and kinase activity is crucial for the molecular changes necessary for long-term learning and memory (Hsu et al., 2002;Norris et al., 1998;Wang and Kelly, 1996). It therefore seems plausible that a high concentration of ISO may not induce conditioned olfactory learning due to excessive phosphatase activity. The present thesis supports this hypothesis. Inhibiting the protein phosphatase CN resulted in a learned preference when a high, non-optimal dose of ISO was utilized.

Interestingly, in one study mice with an over expressed, truncated form of CN which changes it's functional ability, exhibited spatial memory problems relative to control mice. When the number of behavioral trials was increased, mutant animals performed on par with controls (Mansuy et al., 1998). This provides evidence for the notion that CN is critical for setting the threshold of stimulation necessary for memory, which suggests that when blocking CN regulation, learning could occur with both low and high ineffective doses of ISO.

As mentioned in the introduction, McLean et al. (2005) demonstrated that when the PDE-4 inhibitor cilomilast is combined with a sub-optimal dose of ISO, conditioned olfactory learning and memory occurs (McLean, Darby-King & Harley, 2005). Subcutaneous injections of the optimal 2 mg/kg dose of ISO or the sub-optimal lower dose of 1 mg/kg was paired with a subcutaneous injection of one of several concentrations of cilomilast (0.001, 0.01, 0.1. 1.0, 2.0 or 3.0 mg/kg) to reduce the breakdown of cAMP. All doses of cilomilast higher than the lowest dose of 0.001mg/kg induced a learned odor preference 24 hrs after training, when combined with the low, learning ineffective 1 mg/kg dose of ISO. Furthermore, pups which received the 1 mg/kg sub-optimal dose of ISO and a dose of either 1 or 3 mg/kg of cilomilast displayed a conditioned odor preference 48 hrs after training. The higher dose of 3 mg/kg cilomilast also induced learning 96 hrs after training. This is in contrast to the usual 24 hrs memory seen after a single 10 min olfactory conditioning training trial. The findings of McLean et al (2005) suggest that there are specific requirements of cAMP activation associated with learning. Lower doses of ISO may not provide adequate cAMP production, and higher doses of ISO may provide too much activation leading to a critical imbalance in intracellular cascades in the olfactory bulb. This is supported by the observation that endogenous sources of NE released by stroking also sum with exogenous receptor stimulation to initiate (with mild stroking and weak ISO) or block (with stronger stroking and weak ISO) acquisition, suggesting again a dose dependence for effective intracellular cascades (Sullivan et al., 1989b).

The present thesis further investigated the mechanisms surrounding the inverted U-curve, and dose specific abilities of ISO to induce neonate olfactory learning and hypothesized that 1 mg/kg of ISO is a learning ineffective dose due to its inability to significantly elevate cAMP, thus

providing insufficient stimulation to promote CREB phosphorylation (Antoni et al., 1998). On the other hand, a higher dose of ISO was hypothesized to induce an increase in phosphatase activity, which led to subsequent dephosphorylation of CREB (Groth et al., 2003). A high dose of ISO would induce high sustained levels of cAMP which could change the balance of kinase and phosphatase activity in favor of phosphatase, this would make sense given that this change in the balance between kinase and phosphatase activity could act as a protective mechanism for the deleterious consequences of cAMP dysregulation. The present thesis demonstrates that with CN inhibition, cAMP induced learning effects can occur with high doses of ISO.

CN inhibition also produced memory when a low dose of ISO, normally insufficient for memory induction, was administered. Previous work has found that when a low learning ineffective dose of ISO is combined with the PDE4 inhibitor cilomilast, learning occurs. Cilomilast is a phosphodiesterase inhibitor and, thus, prevents the breakdown of cAMP, allowing sufficient PKA activation for CREB phosphorylation (McLean et al., 2005). CN inhibition could also affect cAMP production. cAMP synthesis is controlled by several molecules, including, but not limited to several members of the adenylate cyclase family. Molecular cloning of adenylate cyclase has determined that there exist nine different isoforms. Broadly, they are classified as Ca^{2+} stimulated cyclases or Ca^{2+} inhibited cyclases, based on their regulation by intracellular Ca^{2+} (Antoni et al., 1998). The Ca^{2+} stimulated cyclases play crucial roles in plasticity. One in particular, adenylate cyclase 1 (AC1) is involved in cAMP production. AC9, a Ca^{2+} inhibited cyclase also plays a crucial role in cAMP activation, and therefore learning and memory. AC9 positively regulates cAMP, but is inhibited by CN (Antoni et al., 1998). It is, thus, possible that infusion of FK506 into the olfactory bulb to inhibit CN resulted in increased cAMP production.

Future studies should assay cAMP levels when CN is inhibited with both optimal and low doses of ISO.

Memory formation was not dependent on the dose of ISO when CN was inhibited. Both low and high learning ineffective doses of ISO led to the conditioned approach response 24 hrs after training. This highlights the advantage of CN inhibition for memory extension over phosphodiesterase inhibition. When PDE4 is administered to inhibit the breakdown of cAMP, and extend memory, an ISO dose dependent effect is observed. The low learning ineffective dose of 1 mg/kg ISO produced learning at 24 and 48 hrs, whereas the optimal, 2mg/kg, and high, learning ineffective doses of 6mg/kg, did not induce conditioned olfactory learning and memory (McLean et al., 2005).

7 Future directions

The present findings and earlier evidence provide compelling evidence that CN inhibition plays an important role in learning and memory. It is suggested that CN inhibition could act as a cognitive enhancer in human populations, or as a treatment option for dementia and ageing related memory deficits. Future studies should assess the effect of CN inhibition on olfactory memory in aged animals, and on learning and memory in genetic mouse models of Alzheimer's disease. If possible, learning and memory should also be examined in non-human primates in order to assess the utility of FK506 in models closer to humans. The possibility that FK506 could play a therapeutic role in memory improvement, or relieve the deficits which emerge with dementia and ageing is exciting. Future research should further explore such possibilities, and

confirm the relationship between CN inhibition and improved memory abilities. The modulatory role CN played in the inverted ISO U-curve also requires further investigation. Do pups that receive low or high ineffective doses of ISO, coupled with FK506 generate similar levels of phosphorylated CREB 10 min after training when levels of pCREB are normally at their maximum? Is memory extension also possible with learning ineffective doses of ISO when they are combined with FK506? Finally the variation in long term memory that CN appears to control, and the hypothesis that pCREB duration determines memory duration are both original proposals that should be examined more extensively. Clearly CN inhibition has a robust and multivariate effect on learning and memory. Research in the future will surely continue to highlight its innovative and exciting role.

8 Reference List

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