INHIBITION OF CaMKII DISRUPTS THE FORMATION OF LONG-TERM ASSOCIATIVE OLFACTORY MEMORY

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by

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Abstract

Calcium signaling is essential for the formation of mammalian associative memory, yet once in the cell the exact course of signaling taken by Ca^{2+} remains vague. The kinase known as CaMKII is highly attuned to both the strength and frequency of Ca^{2+} influx and has been implicated in numerous memory paradigms. Moreover, its intrinsic autophosphorylating ability, which may be maintained up to an hour after Ca^{2+} influx, makes the kinase an extremely important molecule in the memory pathway.

In the present study, I investigated the effect of inhibiting this kinase on olfactory preference learning in a neonate rat model. Normally, learning may be achieved by pairing isoproterenol (2mg/kg) acting as an unconditioned stimulus with a conditioned stimulus odor, peppermint in this case, for 10 min. Here I found that infusion of KN-62 into the bulbs, a CaMKII inhibitor, 10 min before training, as well as 10 and 30 min after training could disrupt normal 24 hr memory. Furthermore, I also used a non-learning dose of Iso (6 mg/kg) paired with odor, and found that in this group KN-62 infusion could induce learning.

To confirm the role of CaMKII in this study we sacrificed animals at 5 min post-training to assay phosphorylated CaMKII levels and phosphorylated CREB levels. We also performed immunohistochemistry on animals sacrificed at 10 min post training to localize the effects of CaMKII and the distribution of the kinase in the olfactory bulb.

Western blot analysis revealed that KN-62 reduced phosphorylated CaMKII levels in non-learning Iso 2(mg/kg) animals as hypothesized. Immunohistochemistry findings revealed a high CaMKII presence in the olfactory bulb, especially in the external plexiform layer and glomerular layer. KN-62 infusion decreased phosphorylated CaMKII expression in all layers of the olfactory bulb.

In this study, we conclude that disruption of CaMKII during the early stages of memory consolidation, up to 30 min after training, in PND 6 rat pups may prevent the formation of long term memory.

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Abbreviations

5 HT-R	5-hydroxy tryptophan (serotonin receptor)
AC	Adenylyl Cyclase
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptor
ATP	Adeonosine Triphosphate
B1-R	Beta-1 Adrenoceptor
CaMKII	Calcium/Calmodulin Kinase II
cAMP	Cyclic Adenosine Monophosphate
CREB	cAMP Response Element Binding Protein
CS	Conditioned Stimulus
CaM	Calmodulin
CaM*	Calcium bound calmodulin
CaN	Calcineurin
CN1	Cranial Nerve 1 (Olfactory Nerve)
CREM	cAMP Response Element Modulator
CRE	cAMP response element
DMSO	Dimethyl sulfoxide
ECM	Extra cellular matrix
EPAC	Exchange protein activated by cAMP
Erk	Extra-cellular regulated kinase
GluR1-4	Glutamate receptor subunit-1 (of the AMPA receptor)
GTP	Guanine Triphosphate
I-1	Inhibitor 1
KN-62	Competitive inhibitor of CaMKII
LC	Locus Coeruleus
LTM	Long term memory
LTP	Long Term Potentiation
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MUPP1	Multi-PDZ domain protein 1
NE	Norepinephrine
NMDA	N-methyl-D-Aspartic Acid
NR1	NMDA receptor subunit 1
OB	Olfactory bulb
PKA	Protein kinase A
PDE4	Phosphodiesterase 4
PND	Postnatal day
PP1	Protein phosphatase 1
PSD	Post synaptic density
PSD-92	Post synaptic density 92
Ras	A GTPase in the Ras/MAPK pathway
Raf	Serine threonine specific kinase in the Ras/MAPK pathway
RNA	Ribonuclein Acid
siRNA	Small interfering RNA
SynGAP	Synaptic GTPase activating protein
UCS	Unconditioned Stimulus

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A graduate student must theorize and read research on the very frontiers of science relying fully on the work of thousands of scientists before their research began. Therefore, I find it of paramount importance to here recognize those scientists before me who laid the fundamental building blocks for this research.

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Chapter I: Introduction

1.1 Behavior& Olfaction in Rat Pups

It is generally accepted that memory, at the neuronal level, is, in the most superficial sense, a specific pattern of intricate connections between the neurons of the brain. The exact mechanism for how these patterns are established, weakened, or strengthened remains unclear. Within the individual neuron, it appears that the inhibition or disinhibition of nebulous, interconnected biochemical pathways is at the foundation of these processes. Although, to make the paradigm more complicated, at this neuronal level there is variance in how individual neurons react to their environment.

It is important to delineate a number of elements of memory. Firstly, the mechanism of encoding memory, wherein some sensory event has occurred and the brain feels the compulsion to retain information pertaining to this event. That is, something has happened to an individual, and the brain doesn't want to forget it; be it a conscious or subconscious appreciation. More indirectly, even without external sensory cues detected by the sensory array, people are able to form memories by associating various bits of information already possessed in memory. For instance, you may contemplate a pattern of events in your life, come to some conclusion about them, and remember the whole process without opening your eyes. In either case, information is presented along with an associated outcome and the paradigm is encoded into memory.

Secondly, the process of memory storage follows from initial encoding. Common among the various forms of memory is the fact that it must be stored and retrieved in some sense. So, how it is acquired, coded, and then retrieved becomes a point of great interest for someone seeking to characterize memory. Following information

presentation, the newly transduced neural signal must be transmitted to the appropriate regions of the brain via the anatomical substrate needed to deal with this data, and then must be stored. Then under normal circumstances some cue must result in retrieval of that memory, which has behavioral consequences; conscious or subconscious.

Lastly is retrieval itself, the process of recalling and bringing to mind a specific memory. It is now appreciated in neuroscience that memory can be thought of in five major ways. Among the most transient is immediate sensory memory, where recently sensed data is held in the networks of the sensory array. Another, working memory, is memory that is consciously attended to for immediate use. Declarative, explicit, episodic memories are stored in various regions of the brain and deal with memory of specific facts and events considered to be long-term memory. Non-declarative, implicit memory is another form of long-term memory. Implicit, also referred to as somatic memory, is primarily held in the basal ganglia or the cerebellum and deals with learned habits and skills. Finally, emotional memory most associated with the amygdala, deals with associations that elicit such emotions as fear, or sexual arousal, and is also a long-term form of memory. Priming the recall of these events is mostly contextual, and remains the subject of intense investigation.

The present study investigates a conditioning paradigm in which one conditioned stimulus, peppermint, is presented to the olfactory system along with an unconditioned stimulus, an element of maternal care for example. Thus, the initial sensory input is olfactory, and the region of initial encoding is along the olfactory pathway. The questions being asked herein are: What factors can modulate learning within the olfactory system? And, what effect can interfering with the olfactory system at various points have on the

natural learning process? In rats, it is difficult to know whether or not the learned association is conscious, and whether or not there are emotional aspects.

Returning to the idea that memory is based upon patterning in neural circuitry and the power of those connections, it is impossible not to discuss LTP. Improving a connection between two neurons involves many processes including increasing the number of axon terminals synapsing on the post-synaptic membrane, the density of specific proteins in the region of synaptic contact, and the structure of the post-synaptic membrane. LTP represents a change in synaptic efficacy following stimulation of nerve fibers. It is a model of plasticity and is believed to be a basis of learning and memory. Bliss and Lomo (1973) were among the first to investigate the idea that connections in the brain can be strengthened with patterned excitation. In their experiments, rabbits received electrical stimulation to the perforant path with the output monitored in the dentate area of the hippocampal formation. Stimulation of the perforant path produced a brief immediate electrical excitation of granule cells therein, followed by a brief depression. Thereafter, 15 of 18 rabbits demonstrated potentiation of electrical transmission between neurons for up to 10 hours following a conditioning (high frequency) stimulus, suggesting that LTP is the result of increased synaptic efficiency in the perforant path as well an increased excitability of granule cells of the dentate area.

Later the role of specific receptors in this pathway was investigated and the glutamate receptors were identified as likely candidates of the modulation of synaptic efficiency. Abraham and Mason (1988) examined the role of glutamatergic NMDA receptors in LTP by using two different NMDA antagonists; CPP and MK-801. Rats injected with a high dose of either drug demonstrated LTP deficits. Rats trained in a radial

arm maze task, a test of spatial memory, following injection of MK-801 or CPP demonstrated significant deficits. In a later study it was demonstrated that both CPP and MK-801 injected rats made more errors in the radial arm maze at doses greater than 1 mg/kg (Ward et al., 1990). Together, these studies indicated that NMDA receptors mediate LTP, and that LTP may be a necessary element in spatial memory.

The study of associative odor preference memory in neonate rats has particular relevance for understanding memory since many aspects of the system are conserved across species and brain regions. Common organizational and functional characteristics are seen in both invertebrate and vertebrate olfactory nervous systems (Lledo et al., 2006). It is known that many genes involved in learning and memory have conserved functions between phyla (e.g. rutabaga, protein kinase A (PKA), cAMP response element binding protein (CREB), neurofibromin (NF1), and neural cell adhesion molecules [NCAM), α-integrins, Notch] see Davis, 2005 for review) and that aspects of the biochemical pathways leading to memory storage are similar in many brain regions and memory paradigms (Malleret et al., 2001; Kandel, 2004; Barco et al., 2006).

In has been known for centuries that memory called up by smell may transcend the mere sense and evoke emotion and recollection of events in people in fascinating ways. It is clear from many of his novels, that Marcel Proust, in the 19th century, recognized that memories may be linked to the senses, which guide them into the conscious mind (Delacour, 2001). In people, such evoked memories may be described as long-term, episodic memories with precise spatial-temporal contexts (Delacour, 2001) sometimes with unfortunate consequences such as scent-induced drug addiction relapse or

incapacitating, scent-induced post-traumatic stress disorder (PTSD) episodes (Hinton et al., 2004).

In our lab, we study the phenomenon of odor memory in rat pups. Following their birth, rat pups are initially blind and nearly deaf to the world around them, primarily relying on olfaction to provide a sense of their surroundings. This sense remains important throughout the life of the rat with regards to things such as the perception of foods, cues regarding predators and prey, recognition of con-specifics, mediation of emotional responses, and the mediation of reproductive and maternal behaviors. As the dominant sense in neonatal rats, olfaction is directly involved with a number of behaviors essential for survival (Wilson et al., 2004).

Rodents, in general, are tuned to using olfaction for guiding behavior. Neonate rats rapidly acquire preferences for odors associated with maternal care (Johanson et al., 1984; Sullivan et al., 1989). Early olfactory preference learning in rat pups may occur when novel odors are paired with tactile stimulation, or other rewarding stimuli. In fact, past studies have demonstrated that repetitive pairing of a broad range of stimuli with olfactory cues could establish long-term memories that affect behavior, including a generally aversive shock pairing (Rangel and Leon, 1995; Sullivan et al., 2000). Furthermore, there is evidence that this sort of training performed in a repetitive learning paradigm can result in life long memory (Sullivan and Wilson, 1994).

1.2 Biochemical Pathways Specifically Involved with Olfactory Learning

The biochemical pathway of interest to the present study is that which is hypothesized to exist in the mitral cells of the olfactory bulb that deals with olfactory cues and olfactory conditioning in neonatal rats. Five main receptors related to olfaction and

learning in mitral cells have been described in a model of olfactory conditioning (McLean and Harley 2005; Hardy et al., 2005) the N-methyl-D-aspartate (NMDA) receptor, the a-amino-3-hydroxy-5-methy-4-lisoxazole-proprionic acid (AMPA) receptor, the tyrosine kinase receptor (Trk-R), the β1-adrenoceptor (β1R), and the serotonin 2A receptor subtype ($5\text{-}HT_{2A}$). Each of these aforementioned receptors has been demonstrated to mediate important elements with respect to olfactory conditioning. Also of note is a model of disinhibition from granular cells that innervate Mitral cells (Sullivan and Wilson, 1994) whereby noradrenaline (NA) and acetylcholine may modulate GABA input onto mitral cells, thus increasing the activity of mitral cells (Sullivan and Wilson, 1994; Linster and Hasselmo, 1997).

The olfactory bulb is the point of convergence for glutamatergic projections that deliver olfactory information from the olfactory epithelium to specific glomeruli, as well as noradrenergic, cholinergic, and serotonergic centrifugal inputs from other parts of the brain (*reviewed by* McLean and Harley, 2005). Integration of this information provides the basis for coincidence detection that may lead to long-term potentiation (LTP), an idea that underlies the theory of learning. Each of these categories of centrifugal projections modulates activity in the olfactory bulb and may deliver important information about the environment coinciding with specific odors. In a very basic sense, this represents an anatomical substrate capable of detecting the presence of both a conditioned stimulus (CS) via the olfactory nerve, and an unconditioned stimulus (UCS) via centrifugal inputs.

Merely scent or stimulation of one of several centrifugal inputs does not result in learned behavior, although learning can be achieved when they are paired. It has been shown that serotonin (5-HT) depletion (McLean et al., 1993) or inhibition of adrenergic

signaling prevents odor learning (Sullivan et al., 1991), deficits correctable using 5-HT2A/2C agonists and β1 agonists respectively (McLean et al., 1996; Harley et al., 2006). It is also important to note here that serotonergic input to the bulb does not appear to provide the UCS; however serotonin does facilitate conditioned olfactory learning induced by noradrenergic input in the neonate rat (Price et al., 1998).

A great deal has been learned about the effects that noradrenergic inputs have on olfactory learning. In rat pups, noradrenergic projections from the locus coeruleus (LC) deliver NE to the olfactory bulb when the animals experience certain stimuli such as stroking, maternal care, or a shock (Rangel and Leon, 1995; Sullivan at al., 2000), and NE is thought to potentiate the response to odor and initiate learning. The receptor subtype of greatest interest is the β1-R, a G-protein coupled receptor tied to cyclic-adenosine monophosphate (cAMP) signaling through adenylyl cyclase (AC) activation (Pfeuffer, 1977; Ross et al., 1978; Fu et al., 2007).

In the laboratory setting, stroking can simulate maternal care via activation of the LC (Nakamura et al., 1987). Local increases of NE release are seen in the olfactory bulb following such stimulation (Rangel and Leon, 1995) and pairing an odor with this stroking produces an odor preference 24 h later (Wilson et al., 1987; McLean et al, 1993). Infusion of a ß1-R agonist in the olfactory bulb or stimulation of the LC paired with odor may substitute for stroking and produces subsequent preference for that odor, an effect that can be blocked by a pre- or post-training injection of the ß-adrenergic antagonist propranolol (Sullivan et al., 2000).

It appears that serotonin potentiates the activity of AC (Castellucci et al., 1980; Kandel and Abel, 1995), and in the olfactory bulb facilitates learning (Yuan et al., 2000).

Serotonergic projections from the dorsal and median raphe nuclei deliver serotonin (5-HT) to the olfactory bulb (McLean and Shipley, 1987a; McLean and Shipley, 1987b), and 5-HT is thought to potentiate the response to odor and initiate learning (McLean at al., 1996; Price at al., 1998; Yuan at al., 2000). The 5-HT2A receptor located in the mitral cell is a G-protein coupled receptor (Frazer and Hensler, 1999) and should theoretically be able to activate AC (Tang et al., 1991; Yoshimura et al., 1996). Thus, it is very likely that this is the biochemical pathway by which it facilitates learning.

An odor is initially transduced into neural signaling at the olfactory epithelium in the nasal cavity, which, via the olfactory nerve (CN1), results in synaptic transmission at mitral cell dendrites around glomeruli in the OB (Ennis et al., 1996). The neurotransmitter at mitral cells released by CN1 is glutamate (Ennis and others, 1996), which acts on NMDA and AMPA receptors located within the mitral cell post synaptic density (PSD). At the outset, glutamate binds to its receptor, AMPA, which is an ionotropic transmembrane receptor that acts as a high sodium (Na+) and potassium (K+) conductance channel (Ward et al., 1990). Simultaneous glutamate binding also occurs at NMDA receptors, although these channels do not open directly due to the obstruction of the channel pore by magnesium (Mg2+)(Monyer et al., 1992). The Mg2+ must be repulsed into the extracellular matrix (ECM) before ions may pass through the NMDA receptor. With enough excitatory AMPA mediated postsynaptic potentials, cellular depolarization sufficient to expel the Mg2+ ion from the NMDA channel pore occurs (Ward et al., 1990). Once Mg2+ is repulsed in the presence of glutamate, NMDA receptors mediate a high Na+ and calcium (Ca2+) conductance. Thus, simultaneous postsynaptic and presynaptic activation potentiates the signal. Once in the cell, the cation

Ca2+ is associated with a broad range of intracellular functions, including LTP (Rasmussen and Rasmussen, 1990).

Interestingly, the apical dendritic tuft of the mitral cell, located in the olfactory bulb glomeruli, is integrated into a circuit that allows the cell, itself, to engage in self-excitation. The dendrites of mitral cells appear to be capable of releasing glutamate in order to excite nearby periglomerular cells for feedback and lateral inhibition, but also to perpetuate the cell's own excitation (Schoppa and Westbrook, 2002; Urban and Sakmann, 2002). Excitation of mitral cells results in a robust rise in Ca2+ in the excited dendrite, primarily in the more apical, dendritic tuft and to a lesser extent in lateral dendrites (Zhou et al., 2006; Yuan et al., 2007). It is well established that the mitral cell possesses excitatory synapses distributed in the distal glomerular tuft (Kasowski et al., 1999; Didier et al., 2001) and a high Na+ channel density along the dendrite allowing distal synaptic inputs to be integrated locally in the glomerular tuft and transformed into action potentials that propagate actively toward the mitral cell soma (Zhou et al., 2006).

Many proteins that respond to Ca2+ are directly associated with NMDA receptors in the PSD. One well explored protein that is activated by Ca2+ is calmodulin (CaM), which forms a complex, denoted henceforth with an asterisk (CaM*) with Ca2+ (Ghosh and Greenberg, 1995). Interestingly, NMDA receptors have been shown to be associated with CaMKII as well (Bayer et al., 2001), a kinase activated by CaM*. This special arrangement places the kinase in a region where it may quickly respond to changes in Ca2+ concentration. In the PSD, NMDA receptors are also closely associated with PSD-95, a protein thought to regulate its signaling and localization. PSD-95 mutant mice fail to

develop NMDA receptor-dependent sensitization, and show spatial learning and synaptic plasticity deficits (Migaud et al., 1998; Garry et al., 2003).

Ca2+ acts as a second messenger, binding, and thereby activating CaM, a ubiquitous, Ca2+-binding protein capable of binding to many more proteins and acting as a signal transducer in several biochemical pathways (Rasmussen and Rasmussen, 1990). In mitral cells, the CaM* complex may bind to and activate specific subtypes of the transmembrane enzyme AC, and the protein kinase Ca2+/calmodulin-dependent protein kinase II/IV (CaMKII/IV), proteins involved with further intracellular signaling (Harley et al. , 2006).

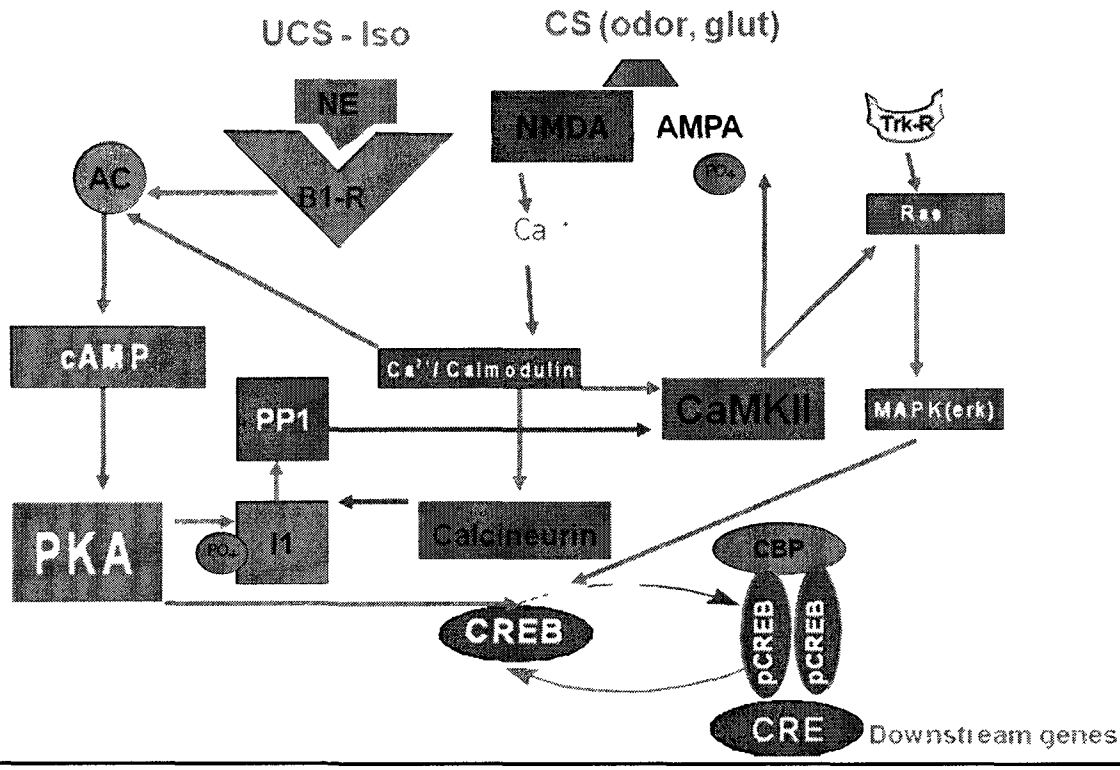


Figure 1: This schematic shows the proposed intracellular signaling pathways hypothesized to exist within the mitral cell in olfactory learning. Of primary interest to the present study are the cAMP pathway, calcium signaling, and the Erk pathway, each of which could feasibly have an effect on structural plasticity at the synapse and regulation of CREB in the nucleus. Red arrows in the schematic represent an inhibitory relationship, whereas grey arrows represent and excitatory relationship.

1.3 Adenylyl Cyclase/cAMP/PKA pathway

Once specific subtypes of AC are activated by CaM, the enzyme catalyses the conversion of adenosine triphosphate (ATP) to cAMP, increasing intracellular levels of cAMP (Antoni et al., 1998). Of primary interest to the present study, cAMP can activate cAMP-dependent protein kinase A (PKA), a kinase known to phosphorylate a broad range of intracellular proteins including the transcription factor, CREB (Gonzalez and Montminy, 1989; Montminy, 1997; Abel and Nguyen, 2008). This pathway is highly conserved among different species and is critical in learning and memory in nearly all organisms (Kandel and Squire, 2001). Interestingly, the rate at which cAMP is produced by this process may be augmented by serotonin acting at its 5-HT2A receptor in the mitral cell PSD (Abel et al., 1998), making AC a sort of coincidence detector for a number of neurotransmitters. In this lab, it was found in the rat pup olfactory bulb, that the timing of transient cAMP signaling is critical for the development of 24 hour odor preference learning (Cui et al., 2007) and supports the hypothesis that AC activation following pairing of Ca^{2+} influx and β1-R activation is essential in our learning model. It is also known that cAMP activates another protein, exchange protein activated by cAMP (EPAC), that may play a role in potentiating the Ras-Raf-ERK signal transduction cascade leading to CREB phosphorylation independent of PKA (Ma et al., ; Holz et al., 2008).

To date, at least nine AC subtypes have been described in mammals, each of which possesses its own distribution of expression in body tissues, substrate affinities and regulatory properties (Iwami et al., 1995; Guillou et al., 1999; Hanoune and Defer, 2001).

The spatial distribution of ACs in mice was described by Visel et al. (2006) and indicated that the primary ACs in the OB and the hippocampus were ACs 1, 2, 3, 5, 6, 8, & 9; each of which varies in density depending on layer and regional location in the respective tissues, and furthermore demonstrates developmental regulation.

ACs 1 & 8 are activated primarily by CaM* (Tang et al., 1991) AC3 is also activated primarily by CaM*, but only in the presence of other effectors (e.g. GppNHp or forskolin) in the cell (Choi et al., 1992). Together, AC1 and AC8 comprise the only CaM* activated ACs in the brain (Wong et al., 1999; Zachariou et al., 2008) and, with regards to their distribution in tissue associated with encoding memory and their responsiveness to increased intracellular calcium (Ca^{2+}), are likely candidates for being critical coincidence detectors in the learning biochemical pathway. There is extensive evidence that they are essential for hippocampus-based learning and synaptic plasticity because they couple glutamate-mediated increases in intracellular Ca^{2+} with the cAMP-PKA pathway (Wu et al., 1995; Wong and others, 1999; Wang and Storm, 2003). This molecular pathway is even seen in the honeybee where it appears coincident amine signaling and AC activity enhance cAMP signaling (Wachten et al., 2006). Although these molecules are present in the OB the relationship therein is yet to be confirmed.

Aside from the Ca^{2+} sensitive ACs, there is also a calcium insensitive group of ACs that is stimulated by $G\beta\gamma$ subunits of heterotrimeric G-proteins (Tang et al., 1991; Yoshimura et al., 1996), another group of ACs that are inhibited by calcium and $Gi\alpha$ subunits (AC 5, &6; (Yu et al., 1993); (Yoshimura et al., 1996) et al., 1996), and AC9, which is responsive to the CaM* stimulated phosphatase, calcineurin (Antoni et al., 1998). In fact, dephosphorylation of AC9 by calcineurin, or PP1 [which is activated as a

result of the calcineurin mediated dephosphorylation of inhibitor-1 (I-1)], causes cAMP increases (Chan et al., 2005).

In terms of the OB, there is a specific temporal and spatial expression pattern seen for AC. Visel et al. (2006) demonstrated that ACs 2, 3, 5, 6, 8 & 9 are detectable in mitral cells and the glomerular layer of adult mice. It was determined in their study that the expression pattern seen in the adult OB is established by post-natal day 7. Interestingly, it appears that in the mouse OB AC1 is the only AC that is developmentally down-regulated, perhaps due to a reduced dependency on olfactory learning as other senses are activated during development. Its continued presence, albeit at a lower concentration, demonstrates a pervasive requirement to detect food, predators, etc.

As previously noted, elevation of PKA is one of the primary outcomes of AC activation. A conformational change in PKA occurs with binding of cAMP that allows PKA to catalyze the phosphorylation of various proteins, including I-1 and phosphodiesterase 4 (PDE4)(Kim et al., 2007). The enzyme PDE4 is responsible for breaking of the cAMP-phosphodiester bond, thus completing a negative feedback cycle in this biochemical pathway. I-1 is a protein inhibitor of protein phosphatase 1 (PP1), a serine/threonine (Ser/Thr) phosphatase that, in its activated state, may dephosphorylate many proteins including NMDA receptors, CREB and CaMKII (El-Armouche et al., 2006). The threonine phosphorylation state of CaMKII can, thus, be regulated through PP1/PKA activity. The significance of various phosphorylation states of CaMKII shall be discussed in a later section. Outside of the post-synaptic density, PP1 dephosphorylates activated CaMKII at Thr286 and PKA prevents this dephosphorylation by activating I-1

(Makhinson et al., 1999), however it appears that the PSD itself may orient CaMKII in such a way that PP1 dephosphorylation is prevented.

1.4 Calcineurin

Working against PKA in this pathway is the CaM*-dependent protein phosphatase calcineurin (CaN). CaN is comprised of 4 subunits; a Ca2+ binding subunit, the calcineurin A (catalytic) subunit, the calcineurin B (regulatory) subunit, and the calmodulin-binding catalytic subunit. CaN, also known as protein phosphatase type 2B (PP2B), is one of four principal types of Ser/Thr-specific protein phosphatases present in mammalian tissues (Cohen, 1989). CaN is capable of indirectly dephosphorylating CaMKII, NMDA receptors, and CREB by deactivating I-1 and thus effectively potentiating PP1 activity (Sala et al., 2000). In other words, CaN couples Ca^{2+} signaling with PP1 through dephosphorylation of DARPP-32 or inhibitor-1. Baumgärtel et al. (2008) implicated CaN modulation as a major contributing factor in the development of highly robust and persistent aversive memory, noting its inhibition can markedly enhance these memories. In this lab, inhibition of CaN has been shown to markedly enhance odor preference memory, extending it from a 24 hour memory to a 96 hour memory (Christie-Fougere et al 2008).

CaN is rapidly activated following Ca^{2+} influx (Rusnak and Mertz, 2000) and appears widely distributed in the cell allowing it to act quickly on a broad range of cellular functions. In the PSD, CaN is localized near ligand-gated (Coghlan et al., 1995; Yakel, 1997) and voltage-gated ion channels (Smith et al., 2006; Oliveria et al., 2007). Multiple, diverse signaling molecules have, indeed, been shown to be modulated by CaN at the PSD, in the cytoplasm (Winder and Sweatt, 2001) and in the nucleus where it

appears that CaN activity is required to maintain CREB-dependent transcription in cultured mouse cortical neurons (Kingsbury et al., 2007). In the nucleus, CaN is also a known regulator of transcription factors (Hogan and Li, 2005) and transcriptional processes within the nucleus (Limback-Stokin et al., 2004). In the cytoplasm, CaN can be targeted to specific synapses by its interaction with such scaffolding proteins as AKAP79 (Oliveria et al., 2007). This, and a broad range of other data on this phosphatase, indicates that it is involved with a plethora of cellular functions depending on its location in the body and the cell.

1.5 Erk

It is known that early odor preference learning in rats is associated with increases in the phosphorylation of CREB (pCREB) in the OB (McLean et al., 1999). The cAMP/PKA-triggered phosphorylation of CREB and CaMKII-potentiated ERK associated phosphorylation of CREB have been implicated as mediators of learning and memory (Frank and Greenberg 1994; Steiner et al., 2007).

Initial activation of CREB occurs via phosphorylation at Ser133. In the biochemical pathway of specific relevance to the present study of olfactory learning, phosphorylation of CREB may occur via PKA, CaMKIV, or ERK (*see* McLean & Harley, 2004 *for review*). Activation of Erk occurs via a Trk receptor-Ras/Erk pathway (Miwa et al. 2005). Trk receptors dimerise, and phosphorylate one another in response to ligand binding, thereby creating a kinase domain capable of activating intracellular signaling molecules (Schlessinger, 2000). Following Trk dimerization, a Ras protein is phosphorylated which may activate mitogen-activated protein kinase kinase MAPKK, which in turn phosphorylates ERK (*also known as mitogen-activated protein kinase*

(MAPK)). Phosphorylated Erk may, in turn, activate a pathway leading to CREB phosphorylation. ERK has been previously implicated in mammalian associative learning and ensuing CREB activation (Atkins et al., 1998; Sweatt, 2001; Adams and Sweatt, 2002). Atkins et al. (1998) demonstrated that contextual and cued fear conditioning directly result in the activation of ERK in the hippocampus.

It has been demonstrated that ERK may be activated via NMDA-dependent Ca^{2+} influx (Sweatt, 2004). Within the post synaptic density, NMDA receptors modulate synaptic plasticity through their regulation of postsynaptic AMPA receptors and this has been shown to be dependent on the composition of the PSD and the NMDA receptor itself. The NMDA receptor is comprised of a complement of two NR1 subunits and two NR2 subunits of varying types (Monyer and others, 1992). It has been shown in mature cultured rat neurons that differing types of NR2 subunits confer different properties to the receptor. For instance, NR2A subunits have been found to support surface insertion of GluR1 AMPA receptor subunits and, conversely, the tonic activity of NR2B subunits is associated with reduction of GluR1 surface expression (Kim et al. 2005).

Coupling of the NR2B subunit to inhibition of the Ras-ERK pathway, a pathway that drives surface delivery of GluR1 (Krapivinsky et al., 2003; Kim et al., 2005), under basal cellular conditions may account for this property. The synaptic Ras GTPase activating protein (synGAP) is selectively associated with the NR2B subunit and is required for inhibition of NMDA-dependent ERK activation after a transient period of activation, and for depressing the ERK pathway under basal conditions (Kim and others, 2005). Coupling of NR2B to synGAP could explain the subtype-specific function of NR2B-NMDA receptors in inhibition of the Ras-ERK pathway (Kim et al. 2005). When

activated, synGAP acts to shorten the duration of activated Ras (RasGTP), by quickening GTPs conversion to GDP. NMDA receptor-dependent ERK activation requires RasGTP, which is stimulated by some guanine nucleotide exchange factors (GEF), such as RasGRF1, and inhibited by GTPase activating proteins, such as synGAP (Thomas and Huganir, 2004). Interestingly, RasGRF1 is reported to also bind directly to the NR2B subunit of NMDA receptors (Krapivinsky and others, 2003), placing it in close proximity to CaMKII, synGAP and many other PSD proteins involved with synaptic plasticity.

NMDA receptors are associated with synGAP through their interaction with MUPPI, a protein in the PSD-95 family of proteins, and the Ca^{2+}/calmodulin-dependent kinase CaMKII (Chen et al., 1998; Kim et al., 1998; Krapivinsky et al., 2004). In synapses of hippocampal neurons, synGAP and CaMKII are brought together by direct physical interaction with MUPP1, and in this complex, synGAP is phosphorylated by CaMKII at basal conditions (Krapivinsky and others, 2004). When the synapse is activated and CaM* binds to CaMKII, CaMKII dissociates from the MUPP1 complex and dephosphorylation of synGAP occurs as it is no longer in proximity to its kinase. With dephosphorylation of synGAP, there occurs a release of inhibition on the ERK pathway and subsequent potentiation of synaptic AMPA responses and an increase in the number of AMPAR-containing clusters in hippocampal neuron synapses takes place (Krapivinsky and others, 2004). This supports a previous finding indicating that the ERK signaling pathway can be activated by Ca^{2+} influx through NMDA receptors (Sweatt, 2001; Sweatt, 2004).

In addition to AMPA receptor regulation, ERK signaling has been suggested to be essential for Ca^{2+}-induced, CREB response element (CRE)-driven transcription in

hippocampal neurons. The full expression of late phase (L)-LTP–dependent CRE-mediated transcription reportedly requires ERK activation, supporting the hypothesis that the activation of CREB by ERK plays a critical role in neuronal plasticity (Impey et al., 1998a; Impey et al., 1998b). Consistent with this hypothesis, it is believed that NMDA receptor-dependent LTP is mediated by the surface insertion and synaptic delivery of GluR1, which is driven by CaMKII and the Ras-ERK pathway (Malinow and Malenka, 2002; Zhu et al., 2002; Sheng and Hyoung Lee, 2003). Thus, here we have a mechanism for delivering AMPA receptors to the appropriate active synapse and for making certain there are memory specific proteins produced to support the process.

1.6 CREB

The activation of CREB-dependent gene expression is thought to be critical for the formation of different types of long-term memory (LTM). CREB is a nuclear transcription factor that modulates the expression of genes with cAMP responsive elements (CRE) in their promoters and has been shown to be involved in LTM formation in such diverse learning behaviors as the siphon-gill reflex in Aplysia *californica* (Bartsch et al., 1995; Bartsch et al., 1998), odor associative memory in Drosophila (Yin et al., 1995) and spatial, contextual and cued memory in both mice and rats (Bourtchuladze et al., 1994; Silva et al., 1998). Furthermore, it is also known to be essential for plasticity at the physiological level of analysis such as late (L)-LTP (Barco et al., 2002). These data demonstrate the requirement of CREB in a broad range of LTM paradigms that rely on multiple distinct brain regions at the molecular, cellular, and behavioral level in a range of different species. It is now generally understood that CREB acts as a molecular switch that controls conversion from short term memory (STM) to LTM (Josselyn and Nguyen,

2005); Tully et al. 2003 *for review*). What remains to be established are the precise causal links between intra-neuronal molecular events, cellular connections, resultant behavior, and precise differences and commonalities between species.

Impaired L-LTP has been demonstrated in hippocampal slices taken from CREB knock-out mice with sparing of early (E)-LTP, a form of LTP that lasts one to three hours whose mechanism does not require new gene transcription or protein translation. The L-LTP phase requires *de novo* gene expression and its mechanisms are generally believed to resemble the molecular events of memory consolidation. With regards to behavior, these knock-out mice demonstrate short-term memory (STM) performance similar to controls, but significantly impaired LTM at 24 and 48 hours. These data indicate the importance of CREB at the molecular, cellular, and behavioral levels in this model of LTM. Furthermore, a transgenic mouse with a regulatable, constitutively active over-expression of CREB in hippocampal CA1 neurons has a lower threshold for eliciting a persistent L-LTP in the Schaffer collateral pathway (Barco and others, 2002). There are, indeed, several other studies that also indicate that the over-expression of activated forms of CREB result in the enhancement of LTM in Drosophila odor associative memory (Perazzona et al., 2004), in amygdala fear conditioning (Josselyn et al., 2001), and in social learning in rats (Jasnow et al., 2005).

Guzowski and McGaugh (1997) were the first to directly inhibit CREB by using an antisense oligo-deoxynucleotide (ODN) and demonstrated that CREB-mediated transcription in the rat dorsal hippocampus was a necessary component for learning and memory of a spatial task. Rats injected with CREB antisense ODN just prior to training had impaired retention at 48 hours. Similar results have been found in more recent papers

using the inducible expression of a dominant-negative CREB transgene in mice. Expressed in forebrain neurons, this dominant-negative transgene was shown to impair cued and contextual fear conditioning (Kida et al. 2002) and expressed in the dorsal hippocampus, was shown to impair spatial memory in the Morris water maze (Pittenger et al. 2002, supporting earlier findings by Guzowski and McGaugh 1997).

There, however, exists a great deal of debate surrounding the involvement of CREB in LTM. The original CREB knock-out model used to explore the protein's involvement in memory, known as the CREBαδ−/− mutant, had a neo gene inserted into exon 2 of the CREB gene. Although this mutation effectively blocks production of the CREBαδ isoforms, it fails to disrupt the CREBβ isoform, which appears to be up regulated in the knock-out animal (Blendy et al 1996). In addition to that, the levels of cAMP-response element modulatory protein (CREM) activator (τ) and repressor isoforms (α and β) are also increased in these mutants (Hummler et al 1994) and it is now suspected that the up-regulation of CREBβ and CREM isoforms may be compensating in part for the loss of the CREBαδ isoforms.

Mice with the CREBαδ−/− mutation show deficits in contextual fear conditioning and Morris water maze spatial memory when tested 24 hour and 1 hour but not 30 min after training (Bourtchuladze at al., 1994). Even one hour after training, memory for cued conditioning was still intact, even though memory for contextual conditioning was not (Bourtchuladze at al., 1994), indicating that the two tasks may be mediated by distinct neural processes. However, a more recent study using both CREBαδ−/− and conditional CREB knock-out mice suggested that performance abnormalities could contribute to the

putative memory deficits previously attributed to CREBαδ−/− mice (Balschun et al., 2003).

Balschun et al. (2003) used mutants with a conditional disruption of all CREB isoforms in the hippocampal CA1 sub-region and other forebrain regions and reported that CREB knock-out is not sufficient to impair hippocampal LTP and LTD, or contextual fear conditioning, and causes only a modest impairment in the early stages of hippocampus-dependent water maze learning. Many mutants performed normally in Morris water maze spatial memory probe trials. Mutants were, however, significantly impaired in conditioned taste aversion.

Aside from the findings regarding conditioned taste aversion, the other findings remain in conflict. This study and similar ones that have detected conflicting findings regarding hippocampus-dependent memory have generally conceded that their results could have been confounded by genetic background, subtle developmental abnormalities that could have affected the anatomical substrate for memory, or molecular compensatory mechanisms such as CREBβ and CREM isoforms (Balschun et al. 2003; Gass et al. 1998, *see* Josselyn & Nguyen 2005 *for review*).

The amygdala-dependent conditioned taste aversion (CTA) memory depends less on potentially performance issues that many other memory paradigms rely upon and therefore likely avoids the interference that reducing CREB may have on performance. Using CTA and a tamoxifen (TAM)-regulated conditional CREB knock-out, known as the $CREB^{IR}$ mouse line, which avoids the potential confound of chronically up-regulating other proteins such as CREM, Josselyn et al. (2004) found that CREB activation appears to be critical for CTA memory in mice.

More recently, the involvement of CREB with contextual and cued memory was addressed by Peters et al. (2009). Here, investigators used small interfering RNAs (siRNAs) injected directly into the hippocampus of wild-type mice to knock-down the expression of CREB. It was found that CREB siRNA injection impaired LTM, but not STM after contextual fear conditioning and impaired LTM in trace cued fear conditioning. Moreover, this study also explored the effects of using siRNAs targeting CREB's negative regulator, PP1. PP1 is known to dephosphorylate CaMKII, AMPA receptors (Lisman and Zhabotinsky, 2001), and CREB (Lonze & Ginty 2002). Investigators found that by knocking down PP1 in the hippocampus they could significantly enhance memory retention after contextual and trace fear conditioning. The authors stressed the importance of being able to use very specific siRNAs to enhance or to disrupt memory as important evidence that the method alone does not disrupt memory.

In the model of olfactory preference learning in neonatal rat pups used in this lab, it has been shown that learning results in increases in the phosphorylation of CREB in mitral cells of the olfactory bulb (McLean et al., 1999), and that optimal CREB phosphorylation is essential for long term preference learning (Yuan et al., 2003). Results demonstrated that: 1) injection of HSV-mCREB viral vectors which effectively reduce CREB's normal activity results in memory deficits and that 2) HSV-CREB viral vectors that result in over-expression of CREB also disrupt normal olfactory memory. An interesting add on to this experiment was the finding that increasing activation of β1-adrenoceptors could overcome deficits in the former case and sub-optimal activation of β1-adrenoceptors may result in learning in the latter case. In other words, an inverse U-

curve for CREB activation was described, similar to that seen for B1-R activation, and CaMKII activation mentioned previously in this introduction.

Several recent papers looking at the effects of over-expression of CREB further add to the controversy over where, when and if CREB is required in specific learning models. Transgenic mice that express a regulated and constitutively active form of CREB (VP16-CREB) that is restricted in expression to the forebrain have been recently used to investigate the effects of chronic elevation of CREB on learning and memory. These mice have demonstrated enhanced neuronal excitability (Lopez de Armentia et al. 2007) and a reduced threshold for L-LTP in the hippocampus and amygdala (Barco et al., 2002; Viosca and de Armentia, 2009; Viosca et al., 2009). However, with regards to behavior, investigators found that chronic enhancement of CREB activity delayed the acquisition of hippocampal-dependent spatial memory in the Morris water maze (Viosca et al., 2009). These results support the notion that activity in the biochemical pathway leading to memory formation follows an inverted-U curve, and that optimal learning requires accurate regulation of this pathway.

These same investigators then explored the consequences of over-expression of CREB on the consolidation of fear conditioning and found that the expression of VP16-CREB does not disrupt contextual and cued fear memory (Viosca and de Armentia, 2009). In fact similar findings have also been reported elsewhere demonstrating that CREB over-expression may facilitate fear memory formation (Josselyn et al., 2001; Han et al., 2008). In addition to this, (Viosca and de Armentia, 2009) also found that VP16-CREB mice overcame LTM memory deficits that result from an immediate post training injection of the protein translation inhibitor, anisomysin. These results suggest two

possibilities: 1) high levels of CREB resulted in high levels of prerequisite mRNA transcription which overcame anisomycin inhibition and sufficient protein translation occurred to support memory formation, or 2) pre-existing plasticity factors required for the consolidation of contextual and cued fear memory that normally must be induced were present in the basal state in VP16-CREB-expressing neurons. This latter interpretation supports a model where VP16-CREB activates the transcription of CRE-driven genes and leads to a cell-wide distribution of proteins which are then available to be translocated to activated synapses. With a higher availability of such proteins it is conceivable that the cell could more easily strengthen synapses. Translocation of RNA or proteins may then prime synapses for subsequent synapse-specific strengthening and therefore learning via synapse specific translocation of CaMKII, or the synapse-specific trafficking of AMPA receptors. In this sense, over-expressing CREB may result in a generally higher availability in the cytoplasm of proteins needed for learning.

Knowing the activation profile of CREB is a necessary step in establishing its role among other proteins known to be involved with memory. Presumably it would demonstrate activation sometime after the activation of membrane receptors on neurons known to be involved with memory. It would also be expected to follow sometime after the intracellular second messengers known to be involved with memory. The activity of CREB can be gleaned from an assay of its activity, determining the magnitude of phosphorylation at Ser133 (Montminy et al. 1990), or by looking at levels of CREB mediated transcription. All of the aforementioned should follow from activation of kinases known to activate CREB that are also implicated in LTM, such as Erk and PKA.

Using a step-down version of inhibitory avoidance (IA) learning, Bernabeau et al. (1997) found a learning-specific pCREB increase in the hippocampus with a biphasic profile. Their immunohistochemical analysis revealed two peaks of activation in CA1 hippocampal neurons, one between 0 and 30 minutes, and the other between 3 and 6 hours post-training. It wasn't until 9 hours after training that investigators found pCREB returned to levels observed in naïve animals. Stanciu et al. (2001) reported a similar biphasic pattern of activation in the hippocampus, parietal cortex and amygdala of mice trained in contextual fear conditioning. Alternatively, quantitative Western blot analyses in the rat hippocampus, revealed a significant increase in pCREB extending temporally from immediately after inhibitory avoidance training until 20 hours after training as compared to untrained controls (measured at 0, 3, 6, 9, and 20 hours). The pCREB increase was found to be learning specific and was the result of phosphorylation of pre-existing CREB (Taubenfeld et al., 1999; Taubenfeld et al., 2001).

Physiological investigation of the activity of CREB in hippocampus also reveals a similar temporal pattern. Schulz et al. (1999) found an initial peak in CREB phosphorylation at 30 min following *in vivo* tetanic stimulation in the perforant pathway and a second long-lasting peak began at 2 hours that lasted for at least 24 hr. This activation was blocked by an NMDA receptor antagonist. Further examination by Ahmed and Frey (2005) supports this biphasic pattern. Their studies of CREB activity during E-LTP and L-LTP revealed a delayed onset of pCREB increase with two peaks of phosphorylation at 45 min and 6 hours, returning to baseline levels at 8 hours after LTP induction. This study also found LTP to be dependent upon NMDA receptors, and furthermore that L-LTP and the late enhancement of pCREB was translation dependent.

The temporal discrepancy between these studies may be accounted for by the latter having been performed *in vitro* and the former *in vivo*.

1.7 CaMKII

The kinase, CaMKII is a protein ubiquitous in the body, but especially abundant in the brain, comprising nearly 2% of its total protein content. Therein, it serves as a serine/threonine protein kinase that is capable, in its activated state, of phosphorylating in excess of 28 proteins in the PSD (Yoshiyuiki et al. 2000).

Initial activation of CaMKII occurs via the Ca2+/calmodulin complex (CaM*). Following calcium associated activation, this kinase may then reach a state of calcium independent autophosphorylation at threonine 286 (T286), a site separate from the CaM* binding site (Li et al., 2000). In its autonomous state the molecule specifically binds to the proteins in the PSD (Strack et al., 1997). The kinase possesses the capacity to self-perpetuate its own activity, thus, CaMKII may continue to operate in the cell after calcium is removed, reducing potentially toxic effects of chronic Ca2+ exposure. CaMKII is involved with functions including the regulation of gene expression, modulation of neurotransmitter release, biosynthesis of neurotransmitters, regulation of cellular ion concentrations, and the regulation of various receptors and cytoskeletal proteins (Bronstien et al., 1993; Lengyel et al., 2000).

The CaMKII holoenzyme is comprised of 12 subunits of 4 different types; alpha, beta, gamma and delta, each of which is encoded by separate genes. Each of these 4 types of subunit genes may, through alternative splicing, generate multiple isoforms of their specific subunit (Wang et al., 2000). The genes themselves show a high degree of conservation across mammalian species (Li et al. 2002). This is not only indicative of

high evolutionary constraint, but also suggests studying the gene's mechanism in rats may translate into valuable findings applicable to human biochemistry. The subunits appear to arrange as a dodecamer, having two rings of 6 subunits side by side. Each individual subunit is comprised of three main parts; a regulatory domain, a catalytic domain, and the association domain (Rosenberg, et al. 2005).

The most predominant CaMKII subunits found in the brain are CaMKIIα and CaMKIIβ (Tombes and Krystal, 1997). With regards to differences between these two dominant subunits, Li et al. (2000) reported no amino acid changes within the ATP-binding site, the CaM*-binding domain, or the autophosphorylation site. Activity-dependent differential regulation among subunit levels has, however, been reported. For example, with heightened activity in rat pup hippocampal neurons, αCaMKII levels appear to increase relative to βCaMKII levels, whereas βCaMKII levels appear to increase with activity suppression (Thiagarajan et al., 2002).

A great deal of activity in the hippocampus is mediated by the neurotransmitter glutamate, and another fundamental difference between α and β subunits is their inverse regulation in response to NMDA, and AMPA activation. That is, regulation of αCaMKII transcription appears to be strongly influenced by NMDA receptor activity, which has no influence on βCaMKII activity. Alternatively, βCaMKII transcription levels are strongly influence by AMPA receptor activity (Thiagarajan et al. 2002). PSD-bound CaMKII is nearly immobile during glutamate stimulation (Shen and Meyer, 1999). It is thought that the cell fine-tunes its response to changing Ca2+ signaling levels by regulating the relative amount of αCaMKII to βCaMKII. The α to β ratio has been shown to change as

much as 5-fold after 24 hours as a response to changing intensities of Ca2+ signaling (Thiagarajan et al. 2002).

Prior to a post synaptic excitatory event, βCaMKII is bound to F-actin (Lisman et al. 2002) that, among other things, is important in maintaining the mature appearing dendritic spine. A great deal of evidence suggests that memory may be encoded by passing through a destabilizing phase to a new, stronger stabilized phase and CaMKII would presumably play a critical role in this process based on its role in the postsynaptic density. The association domain of βCaMKII is capable of binding to F-actin in its non-activated form and serves a role in bundling actin in the dendritic spine structure. The βCaMKII subunit slows down the actin dynamics indicative of an immature or labile state. Reduction of the βCaMKII expression results in spines appearing more immature; this can be reverted to the control state by restoring βCaMKII expression (Okamoto et al., 2007). These associations demonstrate that CaMKII is in a perfect arrangement to be directly involved with dendritic spine remodeling following Ca++ influx.

It is in the regulatory domain where CaM* binds individual subunits of the CaMKII holoenzyme. The regulatory domain is located on the C-terminal side of the intrinsically active catalytic domain, and exerts an inhibitory effect when not bound to CaM*. The binding of CaM* induces a conformation change in the protein resulting in removal of the auto inhibitory effects of the regulatory subunit, thereby exposing the intrinsically active catalytic domain. At this stage, binding of ATP and specific substrates to the catalytic domain can occur (Mayans et al., 1998; Rosenberg et al., 2005).

Another site in the regulatory domain, the T286 regulatory residue, becomes accessible following CaM* binding. This site may become phosphorylated by the

catalytic domain of another adjacent subunit preventing the regulatory domain from rebinding with the catalytic domain (Rosenberg et al., 2005). It is important to note both subunits must be bound to CaM* to initiate autonomous activity (Hunter and Schulman, 2005). The kinase is capable of autophosphorylation when two or more sub-units are activated by CaM*, and this may be sustained after CaM* dissociation, thus sustaining kinase activity (Shen et al., 2000).

Adjacent to the regulatory domain in the direction of the C-terminal is the association domain which is thought to be involved with linking subunits into the holoenzyme structure (Colbran, 2004). As a holoenzyme, the efficiency of autophosphorylation in this kinase increases as more subunits become active. Phosphorylation at T286 also seems to increase affinity for further CaM* binding by as much as 13,000-fold (Meyer et al., 1992). With high intracellular Ca^{2+} concentrations, a high rate of T286 spreading is observed, whereas with low Ca2+ concentrations T286 is dephosphorylated before significant spreading can occur (Mullasseril et al., 2007). Spreading is the process whereby CaMKII subunits autophosphoylate neighboring subunits.

A very intriguing property of CaMKII is its Ca^{2+} pulse frequency activation dichotomy. Kinase activity is reasonably proportional to Ca^{2+} concentration at low pulse frequency, yet with high frequency the trend is adjusted by the enzyme's constitutive, Ca^{2+} independent, activity (De Koninck and Schulman, 1998), a property that enhances the graded difference between different frequencies of stimulation. In other words, high frequencies of excitatory activity have a more profound impact on intracellular activity

than would be expect by a simple linear relationship. This switch is an intriguing property of the enzyme that appears to have both physiological and behavioral correlates.

With regards to memory models dealing with CaMKII, it is known that when a synaptic site is activated and Ca^{2+} influx occurs, the kinase translocates to that synapse. CaMKII translocation requires Ca^{2+} entry through NMDA receptors and it appears to be the NR1 subunit of NMDA receptors whose affinity attracts CaMKII (Thalhammer, et al. 2006). It is of note that NMDA receptor associated elevation in Ca^{2+} in the proximity of the NMDA receptor pore is required for CaMKII trapping at the PSD (Thalhammer, et al. 2006). At the PSD, activated CaMKII can also bind to NR2B subunits. Here, within this configuration of proteins at the PSD, autophosphorylation facilitates and strengthens the binding of CaMKII to PSD components (Bayer et al., 2001). Moreover, enhancement of autophosphorylation has been demonstrated to prolong retention times of the kinase at the PSD (Shen et al., 2000).

The CaMKII enzyme is reported to interact with two sites on the NR2B subunit of NMDA receptors, providing another mechanism for the glutamate-induced translocation of the kinase to the PSD. This interaction brings CaMKII close to the site of Ca^{2+} entrance into the cell and also suppresses inhibitory autophosphorylation of CaMKII. On top of this it believed that sequestering the protein to this microdomain physically prevents PP1 from accessing and dephosphorylating CaMKII. The NR2B interaction also leads to trapping of CaM that may reduce down-regulation of NMDA receptor activity (Ehlers et al., 1996). CaMKII binds to the NR2B subunit only if it is autophosphorylated at T286 even after the removal of CaM*; unphosphorylated wild-type kinase and mock autophosphorylated T286A mutant forms fail to bind.

Binding of CaM* to CaMKII is inhibited by autophosphorylation at T305 and T306, which is initiated when CaM dissociates from an autonomous enzyme (Colbran and Soderling, 1990; Hanson and Schulman, 1992). Binding of CaMKII to NR2B suppresses the inhibitory phosphorylation of T305/T306 (Shen et al., 2000), thereby supporting the kinases autonomous activity. Thus, NR2B binding generates an autonomous and CaM-trapped state that is far more efficient then if the enzyme had only autophosphorylation of T286 to rely on. In addition to this, it appears that autophosphorylation at T286 promotes CaMKII interaction with a second site on NR2B (Bayer and others, 2001).

In rat sensory neurons, Millikan et al. (2002) found that CaM activation peaked after the maximum Ca2+ influx, with maximum activation a half second after the calcium influx ended. CaM activation did not immediately fall to baseline when the Ca2+ level in the dendritic spine did, but fell to a slowly decaying plateau persisting for tens of seconds. In this experiment, it is also interesting to note that Ca2+ clearance was not as rapid in the cytosol as in spines, where it persisted for a slightly longer duration.

One protein sensitive to activated CaM (CaM*), is CaMKII. CaM* binding to CaMKII appears to expose an autophosphorylation site, allowing CaMKII to become persistently activated even when CaM* is no longer present. Giese (1998) substituted alanine for T286 (T286A) in mice such that CaMKII was unable to reach its CaM-independent state. It was confirmed that the mutation affected the autophosphorylated state of CaMKII, but not the CaM* dependent activity. LTP was tested by using extracellular field recordings in hippocampal slices in the CA1 region. The mutant mice showed decreased LTP and deficits in the Morris water maze. It was concluded that the

autophosphorylated state of CaMKII is necessary for spatial learning. These studies (Silva et al., 1992; Giese and others, 1998) support the notion that CaMKII is necessary for LTP and certain types of learning.

Earlier, Silva et al. (1992) had already reported LTP deficits in αCaMKII mutant mice. Using field potential and whole cell recordings, the study demonstrated increased synaptic strength in wild type mice and unchanged synaptic strength in mutant mice following LTP induction. Beyond that, the study also found similar results as above; that is, mutant rats' performance in the hidden platform version of the Morris water maze was poor as compared to wild-type rats.

Interestingly, knock-in mice expressing a constitutively active mutant form of CaMKIIα demonstrate that persistent phosphorylation of CaMKIIα decreases the association of CaMKIIα with the PSD and blocks both LTP and learning (Elgersma et al., 2002). It therefore appears that both the activation and inactivation of CaMKII is critical for appropriate plasticity and learning. Transgenic mice with a constitutively activated form of CaMKII exhibit spatial learning deficits (Rotenberg et al., 1996). NMDA associated LTP was not found to occur in the hippocampus of these mice, and they were unable to engage in spatial learning as measured by the Morris water maze.

The specific CAMKII inhibitor, KN-62, has been used frequently in the past to explore LTP in neurons of the hippocampus. Ito et al., 1991, found that bathing rat hippocampal neurons in KN-62 prevents LTP in the CA1 region between Schaefer and commissural neurons without affecting basal synaptic transmission. No significant effect on LTP between mossy fibers and CA3-pyramidal neurons was noted, however. Evidence suggests that KN-62 interacts with the regulatory domain of CaMKII (Tokomitsu et al.,

1990), and is competitive with respect to CaM but not ATP. Tokomitsu (1990) found that hippocampal tissue incubated with KN-62 exhibited normal CaMKII CaM*-independent activity; demonstrating that the compound affects interactions between the CaMKII and CaM*, rather than ATP or substrate associated enzyme activity.

Commonly, when one thinks of the biological mechanism behind memory, LTP is strongly considered to be, at the cellular level, the process underlying memory encoding. In blocking the formation of the active form of CaMKII, KN-62 has been shown to effectively reduce neural outgrowth and differentiation in fetal and adult rat hippocampal cells. This is attributed to lack of phosphorylation of the NeuroD transcription factor at Ser336 by CaMKII activity (Gaudilliere et al., 2004, Kim et al., 2005), as well as a lack of CaMKII dependent calbindin, yet to be well described (Kim et al., 2005). Results from Kim et al. (2005) indicated that KN-62 inhibited a calcium-dependent phosphorylation of CaMKII and ensuing CaMKII-mediated phosphorylation of NeuroD at Ser336, which ultimately reduced neurite outgrowth.

KN-62 has been previously used to block the action of CaMKII on molecular measures of synaptic plasticity *in vivo* (Vaynman et al., 2003). A dose of 7.2 μg/μl in DMSO (Vaynman et al., 2003; Wolfman et al., 1999) has been shown to block CAMKII mediated memory in the water maze task. In addition to the highly specific inhibitory action on CaMKII, KN-62 also shows some inhibition of CaMKI and –IV (Wayman et al., 2004),and the CaMKI pathway has been shown to play an important role in normal axon outgrowth (Wayman and others, 2004). Therefore, the possibility that CaMKs other than CaMKII may contribute to and/or regulate the pathway downstream of calbindin cannot be ruled out.

1.8 Hypothesis and Objectives

As mentioned above, early odor preference learning in rats is associated with increases of phosphorylated CREB (pCREB) in the mitral cells of the olfactory bulb. The cAMP-triggered phosphorylation of CREB as well as the indirect CaMKII associated phosphorylation of CREB has long been implicated as a mediator of learning and memory in various animals as reviewed above and in Frank and Greenberg (1994). In the present study we investigated whether there is a role for CaMKII in conditioned associative olfactory training as might be predicted given the proposed general role of the phosphorylation of this protein in learning as well as it role in PSD changes. This is merely a first step in characterizing the role of CaMKII in early odor preference learning. The kinase has many targets that could potentially act in parallel to one another.

To test the hypothesis that CaMKII signaling is essential for odor preference learning pups were fitted with a cannula on postnatal day (PND) 5 which allowed direct infusion of a CaMKII antagonist, KN-62, into each olfactory bulb. On PN6, pups were trained for 10 min using a standard conditioned olfactory learning paradigm in which a conditioned stimulus (CS), odor, was either used alone or paired with an unconditioned stimulus (US), isoproterenol (Iso). The pups were sacrificed at various time points after the training, or examined the following day for development of odor preference. Given that it is unclear as to whether or not CaMKII is involved pre- or post synaptically, and for what period of time its involvement in synaptic plasticity is required, we infused the CaMKII antagonist at various times before and after training.

Chapter II: Material and Methods

2.1 Animals

Sprague-Dawley (Charles River) pups of both sexes were used in this study. Rats were housed in polycarbonate cages containing hardwood chips in the animal care facility of the Health Sciences Centre at Memorial University of Newfoundland. The dams were maintained under a 12 hr light/dark cycle at 21 degrees Celsius with *ad libitum* access to food (Prolab RMH 3000 rat diet, Brentwood, MD) and water. Litters were culled to 12 pups on PND 1, where the day of birth is considered PND 0. All procedures were approved by the Memorial University Institutional Animal Care Committee and conformed to standards set by the Canadian Council on Animal Care. A total of 38 pups from 5 litters were used for behaviour analysis at 24 hours, 24 pups from 4 litters were used for Western blot analysis and 6 pups from 2 litters were used for immunocytochemistry.

2.2 Drugs

We used the specific CaMKII inhibitor KN-62 (Ito et al., 1991), acquired from Calbiochem Bioscience, La Jolla, CA, USA. KN-62 is a specific inhibitor that interacts with the regulatory domain of CaMKII (Tokomitsu et al., 1990) to block the active form of CaMKII (Kim et al., 2006). Each animal received a 1 µl infusion of 3.6 µg/µl KN-62, dissolved in 100% dimethyl suphoxide (DMSO), into each olfactory bulb for a total dose of 7.2 µg of drug (Vaynman et al., 2003; Wolfman et al 1999). This dose has been shown to block CAMKII mediated spatial memory in the water maze task (Wolfman et al., 1999).

Rat pups (PND6) were subjected to conditions that had been shown previously (Sullivan et al., 1989, 1991; McLean et al., 1993) to produce conditioned olfactory preference learning (Odor + 2mg/kg Iso) or not (Naive, Odor only, 2 mg/kg of Iso only, Odor + 6 mg/kg of Iso), and four prospective conditions (KN-62 only, Odor + KN-62, Odor+2mg/kg of Iso+KN-62, Odor+6 mg/kg Iso + KN-62).

2.3 Surgery

On PND 5, pups were anaesthetized by hypothermia (5-min on ice) and had bilateral cannula implanted into the skull over the olfactory bulbs as described previously (McLean et al., 1996; Rumsey et al., 2001). Guide cannulae were made from dental acrylic poured into a play-dough mould. To fashion these cannulae, two steel guide cannulae (6mm long/23 gauge tubing, Small Parts Inc., Florida) were first placed into moulded squares (4 mm x 4 mm), 2 mm apart from one another, extending approximately 1 mm from the top and bottom of the moulded square. Subsequently, dental acrylic (Lang Dental mfg. Co., Lang Fast Curing Jet Acrylic Resin, Wheeling, IL) was poured into the imprint. When the acrylic had hardened, excess acrylic was trimmed off to make the cannulae as smooth fitting as possible. Insect pins (size 00, Indigo Instruments, Waterloo, ON) were placed into each guide cannula to act as stylets and prevent the ascension of blood and cerebrospinal fluid into guide cannula. The insect pins were then trimmed and sanded so as not to protrude from the end of the guide cannula.

Anaesthetized pups were placed on ice into a stereotaxic holder that held their head in position for surgery. A number 15 scalpel blade was used to make a sagittally oriental rostro-caudal incision along the skin of the pup's skull. The skull was then cleaned using a cotton swab. Two holes were drilled through the Dura mater and the skull

without damaging the brain. Fragments of connective tissue were removed using fine forceps. Subsequently, the moulded cannula assembly, which was comprised of stereotaxic equipment with a modified clip to hold the moulded cannula, was carefully lowered such that the guide cannulae entered the drilled holes and rested just above the olfactory bulb. A small plastic screw (MN0440-02F-C, Small Parts Inc., Florida) was glued to the skull just behind the cannula to act as an anchor. Additional dental acrylic was placed around the plastic screw and cannula and allowed to harden. The skin was sutured using 4-0 suture, thus covering most of the implanted cannula and screw. The pup was then placed on a warm pad until it fully regained consciousness at which point bitter orange (Gourmet Pet, Georgia) was spread around the surgical area in order to discourage the dam from damaging the implant.

2.4 Training: The kinase, CaMKII, is reportedly involved with the consolidation of associative memory principally in the first 10-30 mins following UCS/CS exposure as its autonomous activity allows the kinase to continue acting on its substrates after calcium is reduced in the cell to pre-exposure levels. The window of time during which CaMKII activation is required for memory consolidation following odor exposure was previously unexplored in our model and was investigated in the present study using various post exposure infusion times. The rate of infusion for KN-62 directly into the olfactory bulb was 1 µl/8min. The 8 min infusion was commenced at one of five different time points: 10 min before training, 10 min post-training, 30 min post-training, 1 hr post training and 180 min post-training.

The procedure for odor conditioning using isoproterenol has been described previously (Langdon et al., 1997; Yuan et al., 2000). In brief, on PND6 saline or

isoproterenol (2, or 6 mg/kg; Sigma, St. Louis, MO) was injected subcutaneously into pups 40 min before exposure to the peppermint odor. Each pup was removed from the dam 30 min after injection and placed on fresh bedding. The pups were then placed on normal or peppermint-scented bedding (300 μl of peppermint extract/500 ml of bedding, aired out for 5-10 minutes for the evaporation of alcohols before use) 40 min after injection of saline or isoproterenol for a 10 min period. After odor exposure, pups were returned to their dams. Norepinephrine-like activation by isoproterenol (a β-adrenoceptor agonist) acted as the UCS. All pups were infused with vehicle (DMSO) or an inhibitor of CaMKII (KN-62) into the olfactory bulbs at one of the aforementioned time points.

For Western blot analysis after the various training conditions, the pups were sacrificed by decapitation at 5 min post exposure. For immunohistochemical analysis, pups were sacrificed 10 min after training by overdose with sodium pentobarbital (Somnotol) and perfused transcardially with fixative as described previously (McLean et al., 1993).

For immunohistochemistry, on PND6, pups underwent odor-preference training for ten minutes after having received a subcutaneous injection of Iso (2 mg/kg). During the 10 min prior to training, animals were infused with 1 μl of vehicle to one bulb and 1 μl of KN-62 (3.6 μg /μl) to the other bulb. The infusion was administered by inserting a 30 gauge/7 millimeter long stainless steel infusion cannula (Small Parts Inc.) into each of the guide cannula. The infusion cannula was attached to polyethylene tubing, which was connected to a 10 μl Hamilton syringe (Hamilton Company). One microliter of solution was infused into each olfactory bulb over an 8 minute period. The infusion cannulas were

left in the guide cannula for an additional two minutes to allow the solution to diffuse throughout the olfactory bulb. The pup was then placed on peppermint scented bedding for 10 minutes, where it was allowed to freely move. After the odor exposure, the pup was placed back with the dam. Ten minutes after that, the pup was sacrificed by decapitation and perfused with saline followed by paraformaldehyde.

2.5 Immunohistochemistry

After immunohistochemistry specific drug-induced learning procedures (KN-62 in one bulb and vehicle in the other) pups were sacrificed and then perfused 10 minutes after the peppermint odor exposure, as this was determined to be a critical time point for the phosphorylation of CREB (McLean et al., 1999). The olfactory bulbs and brains were removed from the skull and immersed in 4% paraformaldehyde in 0.1M phosphate buffer for 24 hours at 4°C. The brains were then placed in 20% sucrose for 12 hours. The brains were removed from the sucrose solution and quickly frozen on dry ice and cut in the coronal plane at 30 μm using a cryostat set at -18°C. Each section was kept and placed on a series of slides, such that each pair of olfactory bulbs was 60 μm apart from the adjacent pair.

Immunohistochemical procedures were followed as standard for this laboratory as published previously (McLean et al. 1999, 2001). Briefly, a rabbit phosphorylated CaMKII antibody (Promega) was diluted to 1:750 or 1:1000 in phosphate buffered saline with 0.2% Triton X-100, 0.02% sodium azide, and 2% normal goat serum and placed on the tissue for 48 hours in a humidified chamber at 4°C. The tissue was removed from the cold room and went through further incubation using the avidin-biotin complex technique

(Vectastain, Vector Labs). The sections were incubated in diaminobenzidine chromogen with 0.01% H_2O_2 for 3-4 minutes to visualize antigen sites. The sections were then dehydrated by being immersed for 3 minutes for each alcohol percentage (70%, 95%, and 100%) and 5 minutes in xylene. The sections were then covered using Permount and a cover slip. All sections from the same experiment had identical incubation times in the antibodies and identical exposure to chromogen solutions.

2.6 Western Procedure

After sacrifice, both olfactory bulbs were immediately dissected, frozen on dry ice and subsequently stored in 1.5 ml micro centrifuge tubes at − 80°C until use. On the day of the Western blot procedure, an ice-cold lysis buffer containing: 50 mM Tris-HCL (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1X protease inhibitor cocktail, 1X phosSTOP cocktail, 1mM EDTA and 1mM PMSF was added to frozen bulbs in the microcentrifuge tubes they were stored in. Olfactory bulbs were then homogenized using a plastic homogenizer that fit a 1.5 mL centrifuge tube (Fisher Scientific, Canada). The homogenate was left on ice for 30 min and then centrifuged at 13,000 rpm for 15 min at 4°C to remove tissue debris. The protein concentration of the lysate from each pair of olfactory bulbs was determined using a Pierce BCA Protein Assay kit.

Samples were prepared in 4 µl of 5× sample buffer (0.25 M Tris-HCl, 10% SDS, 50% glycerol, 0.025% bromophenol blue, and 0.5 M dithiothreitol) added just prior to use plus sufficient sample and water to constitute 20 µl of sample per well. For CREB and pCREB detection, positive controls were forskolin-treated 3T3 cells which showed the appropriate band for pCREB. Forskolin activates the AC/cAMP/PKA pathway in these cells, which leads to CREB phosphorylation.

Samples and positive controls were boiled for 5 min and placed on ice until use. Equal weights of protein (100 μg) from each sample were then loaded into a lane. Proteins were separated on the basis of gel mobility using SDS-PAGE. Color-coded molecular weight standards (Bio-Rad) were loaded into a separate lane for each blot.

The gel-running buffer was a solution containing 25 mM Tris, 192 mM glycine, and 0.1% SDS at pH 8.8. Once proteins were loaded into one of 15 lanes, each gel apparatus was attached to a Bio-Rad power supply set to 100 mV. The protein was then transferred at 4°C from the gel to nitrocellulose paper (Hybond ECL, Amersham) using a Bio-Rad power supply set to 0.35 A for 1hour. The transfer solution contained 25 mM Tris, 192 mM glycine, and 20% methanol at pH8.3.

After the transfer, the nitrocellulose blots were rinsed for 5 min in 5 ml of TBS and then placed in Ponceau S to visualize the total amount of protein transferred. Ponceau S was used on every blot as a qualitative assessment of protein loading equality between lanes. Blots were then immersed in blocking buffer (5% non-fat milk in TBS/T for 1 hr). Following 3 × 5 min rinses in TBS with 0.05% Tween 20 (TBS/T), blots were immersed in the appropriate primary antibody.

For pCREB, the blot was immersed in a polyclonal antibody to phospho^{133}-CREB (1/1000 dilution, upstate Biotechnology) in primary antibody buffer (TBS/T and 5%BSA) overnight on a shaker at 4°C. Following three further five minute washes in TBST and incubation (1 hr) in anti-rabbit IgG-horseradish peroxidase (1/20,000) in secondary antibody buffer (TBS/T and 5% non-fat milk (NFM)), the blot was rinsed in TBS/T and visualized using enhanced chemiluminescence (Thermo Scientific, Rockford, IL). This procedure was used for all antibodies used for Western blot procedures with the exception

of the total CaMKII antibody which required an anti-mouse secondary antibody (1/20,000). For total CREB protein, the blot was immersed in a polyclonal antibody to CREB (1/1000, Millipore, Billerica, Mass.). For active CaMKII, the blot was immersed in a polyclonal antibody to CaMKII phosphorylated at threonine 286 (1/1000, Promega, Madison, WI). Following detection of active CaMKII the blot was stripped and used for total CaMKII, for which the blot was immersed in a polyclonal antibody to total CaMKII (1/1000 dilution, Invitrogen, Burlington, ON).

2.7 Western Blot Analysis

Chemiluminescence was detected using Hyperfilm ECL (Amhersham Hyperfilm ECL, GE Healthcare, UK). Film was exposed to chemiluminescent western blots for various periods of time to collect enough data so as an appropriate exposures could be chosen (all exposed films must be of similar density for precise Image J analysis.) Once collected, films were scanned for analysis into image J on the lab computer. Each blot was loaded into image J, a software package available online to the public. The intensity of various blots was measured (Appendix 1). Measurements resulted in a histogram reflecting optical density from which the base (background) was removed in order to control for non-specific background signal. This measurement was repeated three times for each blot and averaged using statistical software.

2.8 Statistical Analysis

For analysis of behavior, one-way analysis of variance (ANOVA) was used followed by post-hoc Dunnett's tests. For Western blot analysis repeated measure one-way analysis of variance (ANOVA) was used followed by post-hoc Dunnett's tests.

Chapter III: Results

3.1. Behavioural Analysis

3.1.1 Pre-odor infusion of KN-62 specifically disrupted the development of 24 hr odor preference memory in the neonate rat model of long-term olfactory preference memory.

In order to assess whether the CaMKII inhibitor KN-62 can disrupt 24 hr memory following the training paradigm described above, KN-62, or vehicle in the control condition, was infused 10 min prior to pairing of odor with a subcutaneous injection of Iso (2 mg/kg) and conditioned odor preference was assessed 24 hrs later. In addition, two other groups of animals were included which had been injected with a non-learning dose of Iso (6 mg/kg; Langdon, et al. 1997) plus an infusion of either vehicle or KN-62. On analysis, a significant treatment effect was observed ($F_{(5,53)} = 22.526$; $p < 0.0001$). As shown in Figure 2, both saline-injected pups receiving vehicle and saline-injected pups receiving KN-62 demonstrated no significant learned odor preference. *Post hoc* Dunnett's comparisons showed that the pups receiving Iso (2 mg/kg) paired with odor spent significantly more time over peppermint odor at 24 hr testing compared with the saline and vehicle control group ($p < 0.01$). There was a significant disruption of this odor preference in the group receiving Iso 2mg/kg and KN-62. Surprisingly, there was significant odor preference learning in the group receiving an Iso (6mg/kg) injection and KN-62 ($p < 0.01$) compared to the saline/odor only control. Animals receiving Iso (6 mg/kg) with vehicle demonstrated a modestly significant learned odor preference as compared to saline plus vehicle controls ($p < 0.05$).

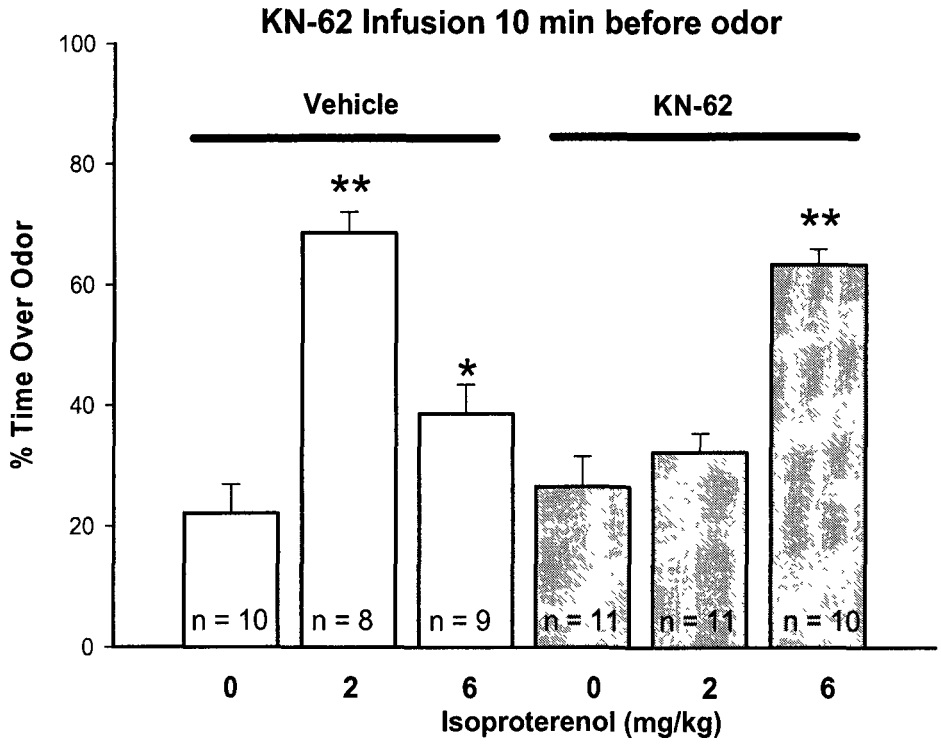
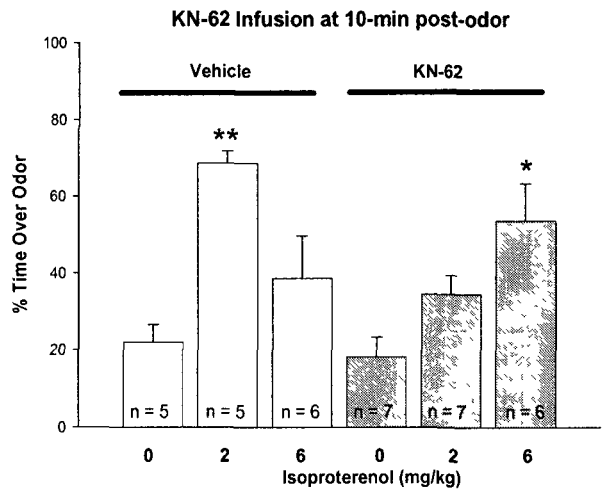


Figure 2: KN-62 infusion 10 min prior to odor exposure interferes with 24 hour odor preference learning. Animals receiving a learning associated dose of Iso (2 mg/kg) develop a 24 hour odor preference when peppermint is presented for 10 min, 30 min after Iso injection ($p < 0.01$) compared to odor only control. Infusion of the CaMKII inhibitor KN-62 10 min prior to odor exposure prevents the development of odor preference in animals receiving an injection of Iso (2 mg/kg), whereas KN-62 results in the development of odor preference in animals receiving an Iso (6 mg/kg) injection. A significant difference between animals receiving an injection of saline and infusion of vehicle and animals receiving an injection of Iso (6 mg/kg) and infusion of vehicle was also noted ($p < 0.05$). * $p < 0.05$; ** $p < 0.01$.

3.1.2 Infusion of KN-62 at various time points following odor training demonstrates a temporal window of sensitivity to CaMKII antagonism.

In order to assess precisely when the development of long-term odor preference learning is sensitive to interference by KN-62, the CaMKII inhibitor was infused at various time points following training. When KN-62 was infused 10 min post-odor or 30 min post-odor, similar behavioral results occurred as seen with infusion at 10 min before training (Figure 3). Animals receiving Iso (2 mg/kg) + KN-62 showed no significant difference from non-learning controls.,while animals receiving Iso (6 mg/kg) +KN-62 were different from non-learning controls. However, when KN-62 was infused at 1 hr or 3 hr post-training the drug effects disappeared and the pattern of results observed for the 3 hr time point reverted to a non-drug like condition (Figure 4). KN-62 no longer disrupted 24 hr preference memory in animals receiving an injection of Iso of 2 mg/kg and KN-62 infusion no longer promoted memory in animals injected with Iso 6 mg/kg.

A



B

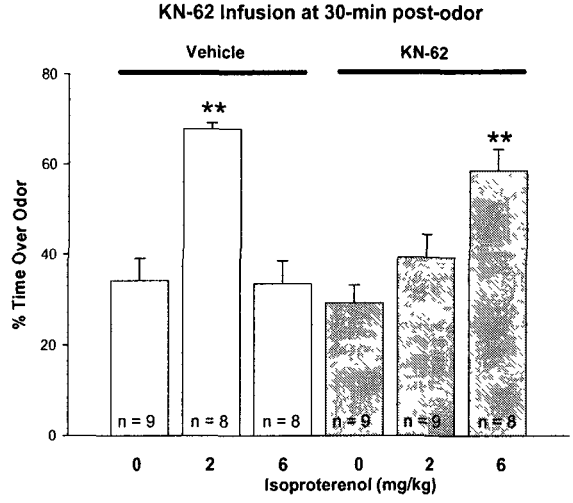


Figure 3: KN-62 infusion 10 min (A) or 30 min (B) post-odor exposure interferes with 24 hour odor preference learning. (A)Animals receiving a 2 mg/kg dose of Iso paired with odor develop a 24 hour odor preference ($p < 0.01$, B; $p < 0.01$). However, animals receiving Iso (6 mg/kg) do not develop this preference. Infusion of KN-62 at 10 min and 30 min after training eliminates the development of odor preference in animals receiving an injection of Iso 2 mg/kg, whereas post hoc Dunnett's test reveals that the combination of KN-62 infusion and high Iso (6mg/kg) injection results in the development of odor preference (A; $p < 0.05$, B; $p < 0.01$) compared to Saline/vehicle control.

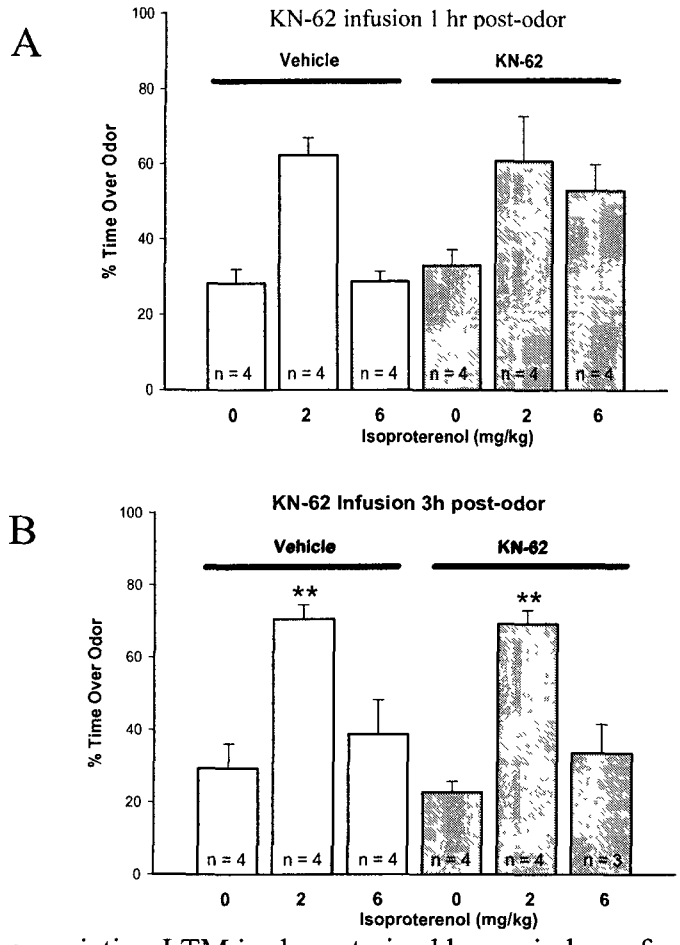


Figure 4: Odor association LTM is characterized by a window of sensitivity to infusion of KN-62. KN-62 infusion 1 hr (A) or 3 hr (B) post-odor exposure ceases to interfere with 24 hr odor preference learning. Animals receiving a dose of Iso (2 mg/kg) develop a 24 hr odor preference when peppermint is presented for ten minutes 30 min after Iso injection. Animals receiving 6 mg/kg of Iso do not develop this preference. Infusion of the CaMKII inhibitor KN-62 failed to eliminate the development of odor preference in animals receiving an injection of 2 mg/kg Iso. Post hoc Dunnett's test also showed that KN-62 failed to enhance the development of odor preference in animals receiving a 6mg/kg injection of Iso compared to Sal/vehicle controls.

3.2 Protein Expression Analysis

3.2.1 Infusion of KN-62 ten minutes prior to odor pairing affects the phosphorylation of CaMKII and CREB in Western blots.

Following behavioral testing, it was necessary to confirm that levels of the phosphorylation of CaMKII changed during the learning and non-learning scenarios. Therefore, Western blot analysis was performed to assess levels of CaMKII phosphorylation (Figure 5) with and without KN-62 infusion prior to training. There were significant differences between the 6 groups tested ($F_{(5, 3, 15)} = 4.933$; $P = 0.0072$). *Post hoc* Dunnett's comparisons showed the pups receiving Iso (2 mg/kg) paired with odor had a significantly higher activation of CaMKII compared to control ($p < 0.01$), and this activation profile was lost with a high Iso dosage (6 mg/kg) or with KN-62 infusion 10 min pretraining (Figure 5). Interestingly, animals that received Iso (6mg/kg) and KN-62 infusion, and were found to have subsequently learned, did not display significant CaMKII activation compared to non-learning controls at 5 min post training.

We assessed levels of activation of CREB as well in our experimental conditions. It was expected that levels of the phosphorylation of CREB would be increased during learning and decreased with KN-62 injection and non-learning scenarios. Western blot analysis of animals sacrificed 5 min after training demonstrated no significant difference ($F_{(5, 3, 15)} = 1.763$; $P = 0.1813$) (Figure 6). *Post hoc* Dunnett's comparisons showed the pups receiving Iso (2 mg/kg) paired with odor demonstrated a non- significant trend toward a higher activation of CREB compared to controls ($q = 1.445$, $p > 0.05$), and this activation profile was lost with high Iso dosage (6mg/kg) and with KN-62 infusion

(Figure 6). Interestingly, animals which received Iso (6 mg/kg) and KN-62 infusion that learned did not demonstrate this trend toward pCREB activation.

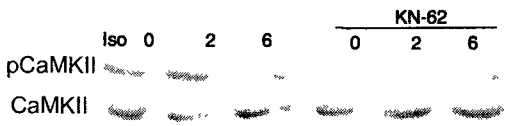
3.2.2 Unilateral bulbar infusion of KN-62 with infusion of vehicle into the opposite bulb 10 min prior to odor pairing also reveals qualitative effects on the phosphorylation of CaMKII in the olfactory bulb as revealed by immunohistochemistry.

Preliminary data exploring pCaMKII immunohistochemically in the olfactory bulb 10 min following training reveals qualitative insights into the distribution and effects of KN-62 on pT286 CaMKII in the olfactory bulb. Figure 7 shows qualitatively the widespread decrease of phosphorylated CaMKII (pCaMKII) expression in the olfactory bulb ipsilateral to KN-62 infusion relative to the vehicle infused bulb. The pCaMKII staining in the control side shows that this signal is abundantly present in the bulb. The pCaMKII expression appears to be especially present in mitral cells since expression was present in the mitral cell layer and in areas where the primary and secondary dendrites of mitral cells extend; the glomerular layer and deep portion of the external plexiform layer, respectively. The staining was cytoplasmic and not in mitral cell nuclei. The decrease in pCaMKII expression ipsilateral to KN-62 infusion was observed throughout the olfactory bulb in three out of four animals examined. In the fourth animal, there were equivocal changes. Quantitative analysis using image analysis optical density measurements will be performed in future studies to confirm the impression observed by visual microscopic inspection. I also hoped to examine expression of CREB phosphorylation (pCREB) in the olfactory bulbs following KN-62 infusion in order to correlate the pCREB results observed using Western blots of lysed olfactory bulbs (Section 3.2.1). Thus, olfactory

bulb sections were processed for pCREB immunohistochemistry in alternative sections stained for CaMKII immunohistochemistry. Unfortunately, for unknown reasons, the pCREB antibody did not work properly so the analysis could not be completed for this thesis.

Western Blot results for PCaMKII at 5-min post training

(A)



(B)

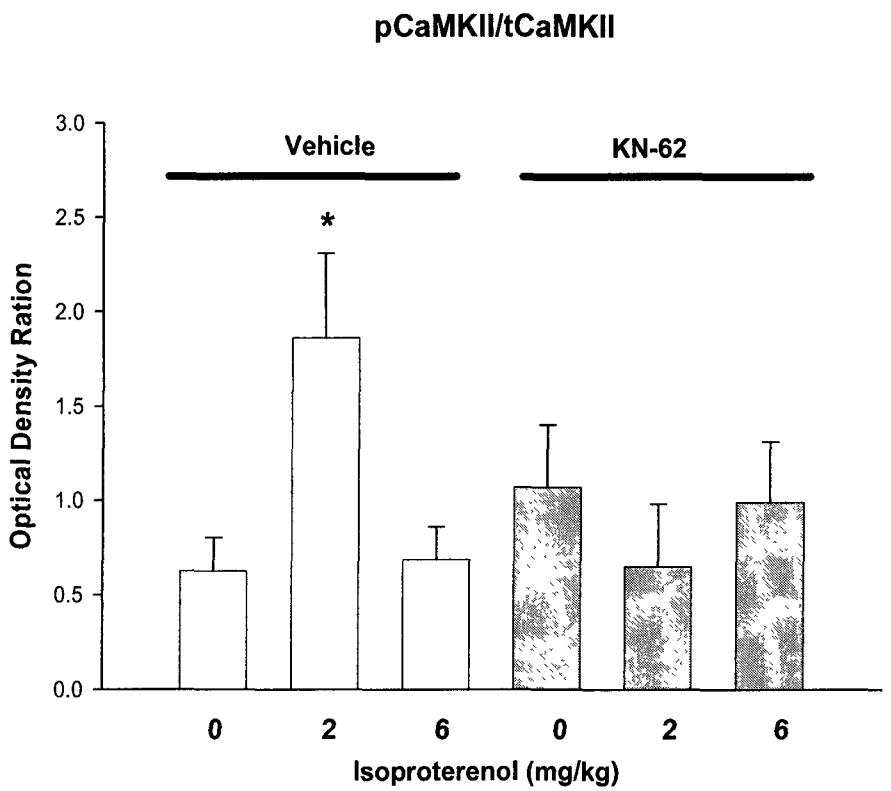
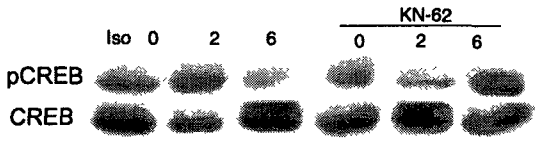


Figure 5: (A) A representative blot of scanned images obtained from Western Blotting. (B) Ratio of p286-CaMKII over total CaMKII protein extracted from PND 6 neonatal rat pup olfactory bulbs 5 minutes after being exposed to a 10 minute odor exposure (n = 4 pups in all groups). The learning animal demonstrates significantly elevated CaMKII activation as compared to the control animal ($p < 0.05$).

Western Blot results for PCREB at 5-min post training

(A)



(B)

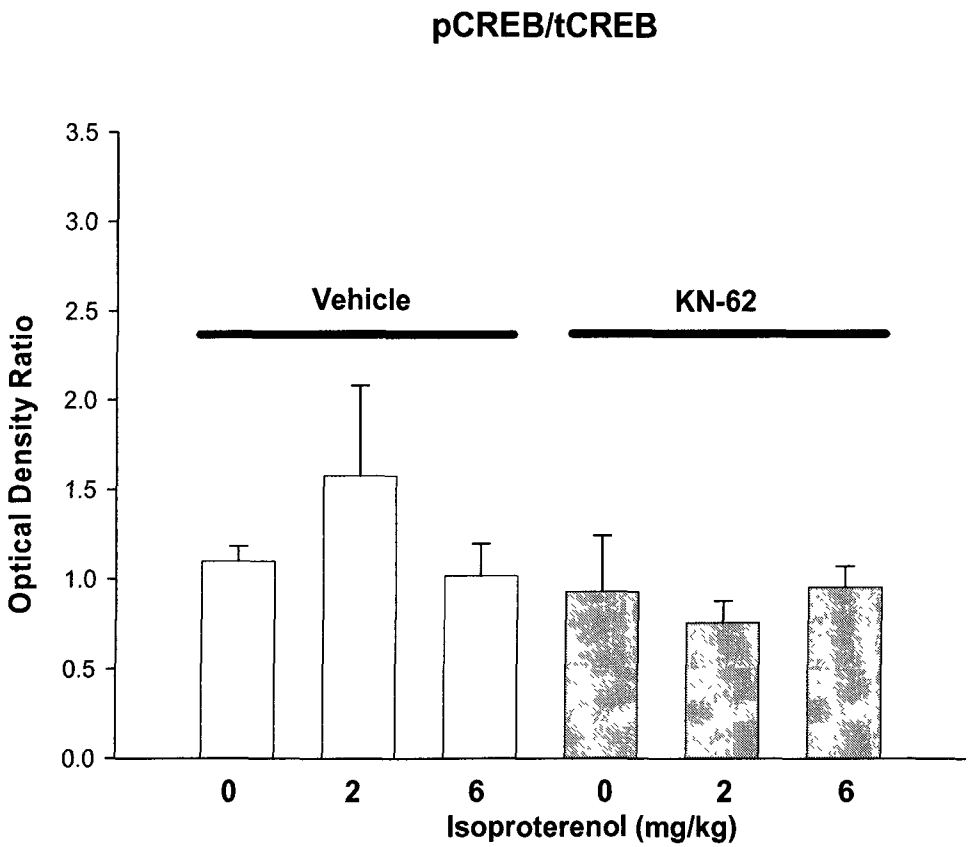


Figure 6: (A) An example of scanned images obtained from Western Blotting. (B) Ratio of p133-CREB over total CREB protein extracted from PND 6 neonatal rat pup olfactory bulbs 5 minutes after being exposed to a 10 minute odor exposure (n = 4 in all groups).

The learning animal demonstrates a non-significant trend towards elevated CREB activation as compared to the control animals.

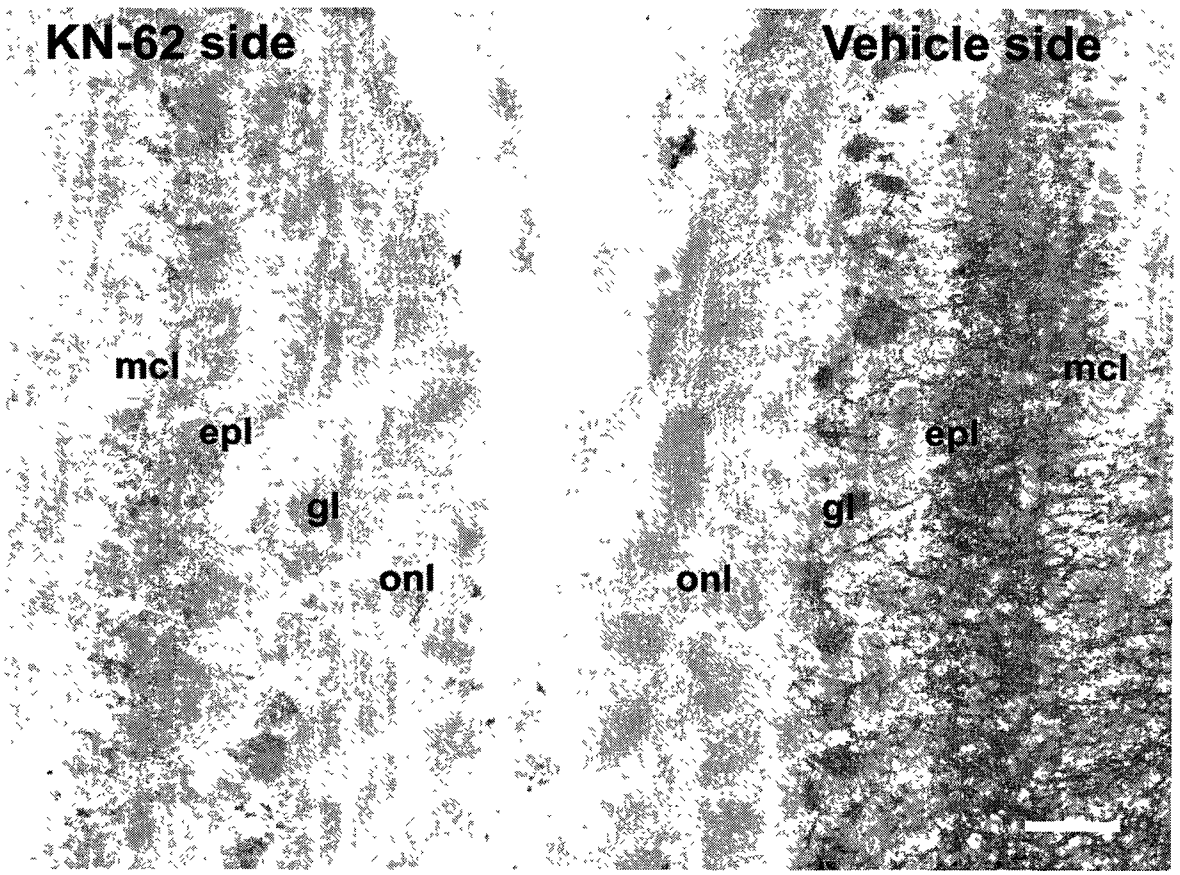


Figure 7: Immunohistochemistry of a slice taken from a neonate rat olfactory bulb showing the layers of the bulb; mitral cell layer (mcl), external plexiform layer (epl), glomerular layer (gl), olfactory nerve layer (onl). Staining for phosphorylated (T286) CaMKII was performed on the slide. KN-62 was infused into the left bulb of the animal prior to training and sacrifice while vehicle was infused into the other (control) bulb.

Chapter IV: Discussion

4.1 General Discussion

The present study investigated the previously unknown role played by CaMKII in the development of 24 hr odor preference memory in a neonatal rat model. It was discussed in the introduction that previous studies in older animals and different memory paradigms have demonstrated a reliance on CaMKII in the development of different types of LTM and have also elucidated a period of activation early on in memory consolidation. Activation of CaMKII occurs sometime shortly after the initiation of training until reasonably well after, although this time line varies between studies. Therefore our first goal was to establish that disruption of CaMKII signaling in the OB could disrupt learning, and secondly to determine the window of sensitivity during which inhibition of CaMKII had an effect on the development of odor preference learning. In addition to this, the effect on the memory molecule, CREB, was also examined as it is often considered to be a necessary marker of memory in cells.

In summary, it was demonstrated in this study that inhibiting CaMKII activation using the drug KN-62 both effectively reduced the level of T286 phosphorylated CaMKII as measured by Western blot analysis (samples from animals sacrificed at 5 min post-training, Figure 5) and disrupted learning normally induced by pairing of Iso (2mg/kg) with odor (Figure 2). Such disruption could be obtained by infusing KN-62 10 min before training, and 10 min and 30 min after training, but not 1 hr after training or 3 hr after training (Figure 2-4). Furthermore, a fluctuation in CREB at 5 min seen with normal learning animals was eliminated by CaMKII inhibition (Figure 6), although this only appeared as a trend in our Western results and was not significant. In previous work done

in the lab, the peak of CREB phosphorylation occurs at 10 min so this weaker effect is not so surprising.

One important discovery in this study was that in the rat neonate, CaMKII disruption up to 30 min after training prevented learned odor preference at 24 hr. Infusion 10 min previous to preference training presumably elevates the inhibitor level in the olfactory bulb for an extended period of time, so this study does not reveal whether or not disruption of CaMKII before training has an effect related to induction. Although, it does demonstrate that disruption affects consolidation, and thus it appears CaMKII in this case is important to consolidation even up to 30 min as evidenced in animals receiving KN-62 and normal (2 mg/kg) or high Iso (6smg/kg). As mentioned in the introduction, in rat sensory neurons, Millikan et al. (2002) found that CaM activation peaked after the maximum Ca2+ influx that would occur with CS/UCS pairing, and fell to a slowly decaying plateau persisting for tens of seconds. This seems temporally modest compared to extensive period of time that its target, CaMKII, appears to remain active (Lengyel et al., 2004). The present data support a functional role for extended CaMKII activation.

Hanson et al. 1994 demonstrated that bound Ca^{2+}/CaM was required for autophosphorylation and described Ca^{2+}/CaM-independent activity and CaM trapping, but the duration and nature of biochemical events that can modulate the duration of autophosphorylated CaMKII remain to be clarified. More recently it was found that CaMKII possesses an intriguing bistable autophosphorylation state in relation to Ca^{2+} concentration that is known to be regulated at least in part by PP1 (Zhabotinsky, 2000). PP1 and CaMKII share binding interactions with PSD95 and other PSD proteins, and PP1 only dephosphorylates CaMKII that is bound to the PSD (Makhinson et al., 1999).

Conversely, PP2A, another serine/threonine phosphatase, is able to dephosphorylate soluble CaMKII at Thr286, but not when the kinase is bound to the PSD. How long this process takes after training is still unknown.

In cell culture, CaMKII has been shown to remain in an autophosphorylated state up to 1 hr following the induction of LTP (Lengyel et al., 2004). LTP, the physiological correlate of memory also appears dependent upon CaMKII autophosphorylation (Hardingham et al., 2003). In terms of behaviour, it was found in adult mice that altering the activation state of CaMKII during the first 15 min of consolidation following training could disrupt spatial and fear memory at 24 hr (Wang et al., 2008). The present study finds that disruption up to 30 min after training can disrupt memory. This could be accounted for by the fact that we used neonate rat pups, whereas Wang et al. used adult mice. It is well known that many molecules involved with Ca^{2+} signaling are developmentally regulated (e.g. NMDA and AMPA receptors, ACs; Sala et al., 2000). There is also independent evidence from conditioned taste aversion studies that the process of consolidation is longer in neonate rat pups than in adult rats (Languille S et al. 2008).

There may even be significant differences between brain regions and the type of information being learned in molecular substrate time courses. Emotion, time, sound, taste and visual learning would all rely on disparate neuroanatomical substrates and really have not yet been well compared. Although, contrary to this notion, it is commonly recognized that the molecular cascades between these learning modes have many similarities (Izquierdo et al., 2006). The common events of consolidation involve acquisition via activation of AMPA, metabotropic and NMDA glutamate receptors

(Robinson et al., 1989; Bianchin et al., 1994; Pin and Bockaert, 1995; Riedel et al., 2003) resulting in Ca2+ influx, CaM activation and enhanced CaMKII activity in the first 60 min (Lledo et al., 1995; Barria et al., 1997; Cammarota et al., 1998). These studies and the present study are all supportive of a common mechanism for potentiation and functional circuit modification in neuronal networks that underlie memory.

The subject of this study, CaMKII, is known to be involved with neurotransmitter release (Liu et al., 2007), Ca^{2+} signaling, and maintenance of synaptic strength (Okamoto et al., 2007). It is also found in most cells along the pathway of interest so it is important to consider both postsynaptic and presynaptic effects. The present study verified the necessity of CaMKII signaling in the olfactory bulb in odor learning, but did not specifically look at markers of presynaptic neurotransmitter release profiles, changes at the synapses of mitral cells, or the Ca^{2+} signaling profile within mitral cells. In post-synaptic signaling, CaMKII takes over after NMDA receptor activation, whose blockage can disrupt learning up to a point immediately after training, but not thereafter (Weldon et al., 1997). Disruption of induction by CaMKII inhibition in the present model was probably not a factor given that adding the CaMKII inhibitor up to 30 min after training similarly disrupted learning. This suggests CaMKII has more to do with consolidation than induction in the olfactory bulb. Also, whereas a number of aspects of the molecular cascade leading to memory formation may be sensitive to disruptions for many hours following training, CaMKII inhibition may only disrupt memory if carried out within the first 15-30 min following training. In the present study, CaMKII inhibition at 1 and 3 hr had no effect on 24 hour memory. Taken together these data suggest that the kinase is involved with post-synaptic maintenance or initiation of early memory consolidation.

Additional evidence for this conclusion comes from electrophysiological findings suggesting that LTP may be reversed in post-synaptic cells via CaMKII inhibition during the first 30 min following induction (Sanhueza et al., 2007).

Our findings are further supported by those of Cammarota (Cammarota et al., 1998) who also found that learning specific CaMKII activity significantly increased from 0 to 30 min after training. Their study noted that post-training infusion in the hippocampus of the CaMKII inhibitor KN-62 produced full retrograde amnesia of inhibitory avoidance training. Furthermore, these authors found an accompanying increase in phosphorylation of the GluR1 AMPA receptor subunit at 30 and 120 min post-training. As previously reviewed, the CaMKII molecule is involved with the trafficking of proteins to active synapses, the remodeling of the actin cytoskeleton and the increase of the phosphorylation of CREB in learning paradigms. Therefore, it is reasonable to hypothesize that the kinase may be involved with trafficking of learning specific *de novo* mRNA and proteins to active synapses. For example, it appears that AMPA receptor insertion is required for LTP maintenance (Kauer and Malenka, 2006), and AMPA insertion appears in part to rely on CaMKII activity (Thomas and Huganir, 2004; Krapivinsky and others, 2004). Exactly what new proteins are produced to maintain long term memory after training and where they need to be directed remains a key question in neuroscience, but recent data from our laboratory suggests increases in AMPA receptor insertion are correlated with learning performance (McLean, 2006).

In our behavior results, there was an unexpected result in the group receiving a KN-62 infusion 10-min prior to training and an injection of Iso (6 mg/kg) wherein the group performed significantly better than non-learning controls (figure 2). Generally,

findings in this lab and others have demonstrated that Iso (6 mg/kg) injected animals do not learn; the reasons why they fail to learn are unknown. We have speculated that higher Iso may disrupt a fine kinase/phosphatase balance, which is related to an anomalous profile of continually rising cAMP levels rather than the pulsatile increases associated with training (Cui et al. 2007). Alternatively the addition of extra 'stress' transmitter reflected in higher Iso doses may disrupt learning by other mechanisms as stress itself is known to do. One procedural difference between this study and those previous is that an infusion occurred 10-min prior to training and this additional invasive procedure may have affected the results. In all other groups investigated, Iso (6mg/kg) paired with vehicle did not differ significantly from non-learning controls.

As we had noticed in previous unpublished data in this lab that learning deficits at 24 hr, i.e. long term memory, in our model caused by inhibition of PKA with Rp-cAMPs could be overcome with a high Iso (6 mg/kg) dosage we had decided to explore the same thing with CaMKII inhibition. The results again suggested that a higher Iso dosage could overcome a learning disruption. There are a number of theories that might account for this, grounded in the notion that inhibiting CaMKII results in reduced AMPA receptor (Krapivinsky and others, 2004) and CREB activity (Choe and Wang, 2001) that is needed for learning. In the first theory for how this may come about, higher β-adrenoceptor activity could enhance EPAC (Gelinas et al., 2008) and, in turn, the Erk pathway which would compensate for deficiencies in the cAMP mediated CREB and AMPA phosphorylation pathways. In the second, high β-adrenoceptor activity could compensate for deficiencies in CREB phosphorylation via it increasing PKA activity. And in the third,

higher β-adrenoceptor activity could correct an imbalance in cAMP signaling. It is important here, again, to mention work indicating that PP1, whose activity would be theoretically increased by higher β-adrenoceptor activity may not be able to dephosphorylate CaMKII in the PSD. This is an important concept as one might think higher levels of PP1 activation would lead to further CaMKII inactivation, which appears not to be the case (Makhinson et al., 1999).

Our Western blot data indicated that KN-62 did inhibit the phosphorylation of CaMKII at T286, the site of enzymatic autophosphorylation. These data also indicated that in groups that received vehicle, CaMKII activity level rose significantly in the learning animals as compared to the non-learning controls. It is also important to note that there was no recovery of CaMKII phosphorylation in the high Iso (6mg/kg) group that received an infusion of KN-62 10 min prior to learning. This result could be consistent with the previously noted theories as to how high Iso (6 mg/kg) restores learning, but does not modify CaMKII activity. Against the hypothesis that Iso (6 mg/kg) offers alternate routes for enhancing the conventional CREB pathway was the result from the Western blot data for CREB activity. While the learning group that received vehicle showed a trend towards an increase in CREB phosphorylation at 5 min following learning, this activity was not restored in the high Iso (6mg/kg) animals receiving KN-62 infusion that learned. This pattern of results suggests alternate theories. Firstly, the cytosolic proteins needed for learning may be more efficiently scavenged and brought to active synapses without the need for CREB related de novo mRNA synthesis. Or, secondly, the activation of CREB may be delayed, and looking at later time points may reveal an increase. Previous data in this lab had found an increase in CREB at 10 min post

training which was still significant at 30 min, but that study did not look at the 5 min time point (Yuan et al. 2003). Thus, the present data demand further investigation of a CREB activity profile in the time following training. The most interesting outcome might be the identification of a novel nonCREB-dependent pathway to learning.

4.2 Conclusions

The primary object of this study was to firmly establish that phosphorylation of the enzyme, CaMKII, is a necessary step in our model for the development of odor preference learning. This was accomplished as per the results of behavior trials carried out in the present study. In the context of other studies investigating the role of this enzyme, this was expected. However, what follows the activation of CaMKII may only be theorized at the present time. The relative influence CaMKII has on various molecular pathways has yet to be described, and the relative importance of each pathway has yet to be characterized. A number of anomalies found in the present study support the notion of parallel pathways, and grant good insight into what directions this research should take.

The next step in this research should be to establish a link between AMPA receptor trafficking and/or insertion and CaMKII activity. As well, the relationship with CREB and Erk remains poorly described. The study stands as a good foundation for a great number of investigations to follow.

Chapter V: Reference List

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Appendix 1: Image J Instructions

1. File > Open Find scanned image to be analyzed.
2. Analyze > Gels > Gel Analyzer Options.
3. Check "Label" with percentages.
4. Select rectangular tool from toolbar.
5. Draw box around first blot.
6. Press "1" on keyboard (or Analyze > Gels > Select 1st lane).
7. Use arrow keys to move rectangle to the next lane. Press "2".
    a. Repeat for each lane, pressing "2" for each one.
8. When completed all lanes, Press "3" (or Analyze > Gels > Plot lanes).
    a. A new window appears "Plot of Filename" showing peak for each blot.
9. Select "Straight line tool" from toolbar. Draw line across each base of peak. Scroll by holding down mouse over plot and slide plots up to see all. Draw line on all.
10. Select Magic Wand Tool. Click inside each peak. Do all.
11. Go to Analyze > Gels > label Peaks. View results in new Results window to find **area** of peak.
12. Record data. It will not be saved by Image J unless you save data (Results > File > Save As).

NOTES:

- If rectangle is too small (if width is longer than 2x height),

  it will move back to the 1st position when attempting to go to next lane.
- Use same magnification factor/zoom size for all blots being compared.

  Area will vary as zoom changes.
- Save scanned image as TIFF files – 1200 dpi.