CaMKII MEDIATES INPUT-SPECIFIC EARLY ODOR PREFERENCE LEARNING IN RATS

by
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Abstract

The synaptic tagging hypothesis explains how input specificity is preserved in long-term potentiation of synapses. This phenomenon requires new proteins that are synthesized in the nuclei and shipped cell wide, to be captured by a tag at synapses that signal a prior synaptic activity. One specific molecule called calcium/calmodulin Kinase II (CaMKII) plays a major role and has been postulated to function as a synaptic tag due to its unique properties. The behavioral relevance of synaptic tagging has not been extensively studied. Using an early odor-preference learning model, which occurs in a week-old rat pup when a novel odor is paired with a reward, we tested the hypothesis that CaMKII activation is critical for short and long-term memories as well as for input-specificity of the odor learning. Using behavioral pharmacology, we first tested whether blocking CaMKII with KN-62 infusion in the olfactory bulb blocks short-term (tested at 3 hr) and long-term memories (tested at 24 hr). Our results show that both memories were blocked. To test the role of CaMKII in input specificity of long-term memory, we used a PKA agonist, Sp-cAMP, to induce 24 hr memory and tested whether blocking CaMKII at the same time affects memories for the learned odor (peppermint) as well as a control odor (vanillin). Co-infusion of Sp-cAMP with KN-62 did not impair 24 hr memory for peppermint; however, the input specificity was lost since animals also showed preference to vanillin. Immunohistochemistry results show that phosphorylated CaMKII is primarily expressed in mitral cell dendrites of olfactory bulbs. These experiments help us understand the specific role of CaMKII in short and long-term odor memories, its role as a synaptic tag, and its role in memory specificity.

This work is dedicated to the loving memory of my father, Dr. Gholam Hossein Modarresi. He was a brilliant scientist, an amazing writer, and an incredibly gifted poet. He taught me how to be kind and how to love. My father continues to inspire me everyday.

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α-AR	Alpha-adrenoceptor
α1	Alpha1- adrenoceptors
α2	Alpha2- adrenoceptors
β-AR	Beta-adrenoceptor
β1	Beta1-adrenoceptors
2-DG	2-deoxyglucose
2pFLIM	Two-photon fluorescence lifetime imaging
5-HT	Serotonin / 5-hydroxytryptamine
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AOB	Accessory olfactory bulb
AON	Anterior olfactory nucleus
aPC	Anterior piriform cortex
BDNF	Brain-derived neurotrophic factor
CaMKII	Calcium-calmodulin Kinase II
cAMP	Cyclic adenosine monophosphate
cAMP-GEF	cAMP-guanine exchange factor
CNS	Central nervous system
CR	Conditioned response
CRE	cAMP response element
CREB	cAMP response element binding protein
CS	Conditioned stimulus
DAB	Diaminobenzidine
EP	External plexiform
Epac	Exchange protein activated by cAMP
EPSC	Excitatory postsynaptic current
ERK	Extracellular signal-regulated kinase
fEPSP	Field excitatory postsynaptic potential
FRET	Fluorescence resonance energy transfer
GABA	Gamma-aminobutyric acid
GC	Granule cell
GluR	Glutamate receptor
GPCR	G protein-coupled receptor
HFS	High-frequency stimulation
IA	Inhibitory avoidance
iGluR	Ionotropic glutamate receptor
IHC	Immunohistochemistry
LC	Locus coereuleus
LOT	Lateral olfactory tract
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase

MC	Mitral cell
mGluR	Metabotropic glutamate receptor
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NE	Norepinephrine / Noradrenaline
NMDA	N-methyl-d-aspartate
OB	Olfactory bulb
ON	Olfactory nerve
O/O	Odor only
OR	Olfactory receptor
ORN	Olfactory receptor neuron
O/S	Odor + stroking
PBS	Phosphate buffered saline
pCaMKII	Phosphorylated calcium-calmodulin Kinase II
pCREB	Phosphorylated CREB
PG	Periglomerular
PKA	Protein kinase A
PKC	Protein kinase C
PKM-ζ	Protein kinase C zeta
PND	Postnatal day
pPC	Posterior piriform cortex
PRP	Plasticity-related proteins
PSD	Postsynaptic density
STM	Short-term memory
TBS	Theta burst stimulation
TC	Tufted cell
Trk	Tyrosine kinase receptors
UCS	Unconditioned stimulus

Chapter 1 – INTRODUCTION

1.1 Overview

One of the biggest mysteries of the human brain is the process of learning and memory. The earliest theories of how the brain learns and memorizes came from the works of Aristotle in his article "On the Soul" around 2000 years ago. In this article, he compares the human mind to a blank slate, stating that humans are born without any knowledge and that they are the sum of their experiences. It wasn't until the 18th century that it was theorized by the English philosopher David Hartley that memories are encoded activities within the nervous system (Buckingham and Finger, 1997). Today, it is generally accepted amongst neuroscientists that learning and memory results from the plasticity of neurons that allows for encoding, storage, and retrieval of information.

To study memory at the neural system level, rat pups are ideal animals. At birth, these animals rely critically on their sense of olfaction to learn and survive in their environment (Leon and Moltz, 1971). They are blind and almost deaf up until around postnatal day (PND) 9. From PND 10 their eyelids (Eayrs, 1951) and auditory meatus start to open (Crowley and Hepp-Reymond, 1966). By PND 14, their ability to see and hear has fully developed (Eayrs, 1951; Crowley and Hepp-Reymond, 1966; Watanabe et al., 1996). Before PND 14, rat pups easily form preference towards an odor that is associated with the dam's care (Woo and Leon, 1987; Sullivan et al., 2000b). This gives researchers a time window to study associative memories that are formed by classical conditioning between rewards such as food and warmth, and different odors. This type of

learning is called *early odor preference learning* in neonate rats, and it is a unique model to study the cellular and molecular processes involved in learning and memory.

Memories are thought to be encoded by structural and functional changes that happen between synapses (Martin et al., 2000). Long-term potentiation (LTP) is the strengthening of synapses, and long-term depression (LTD) is the weakening of synapses. The synaptic tagging and capture hypothesis explains that activity at a particular synapse creates local molecular changes (synaptic tags) that mark synaptic plasticity as having occurred at that synapse (Frey and Morris, 1997). These local changes create a target for future plasticity-related proteins (PRP) and the incorporation of PRPs at the marked synapses is necessary for maintaining LTP and LTD at those synapses.

One specific molecule that has been called the 'memory molecule' in many scientific articles (Cammarota et al., 2003; Griffith, 2004) has a key role in regulating such changes at the cellular level. This remarkable molecule is Calcium-calmodulin Kinase II (CaMKII) and its role in olfactory associative memory is the main focus of this thesis project.

Extensive research has shown that CaMKII is essential for sustained LTP as shown by studies that have used CaMKII knockout mice and pharmacological inactivation of CaMKII. For instance, a study by Giese et al. (1998) provided evidence that CaMKII is required for hippocampal LTP and spatial learning in the Morris water maze using knockout mice. Another study by Tokumitsu et al. (1990) showed that using specific CaMKII inhibitors such as KN-93, LTP induction by tetanic stimulation could be prevented. Ever since the discovery of synaptic tagging in 1997, many studies have focused on the role of this molecule as a synaptic tag. CaMKII has been postulated to

function as a synaptic tag due to its unique properties including staying in an active (phosphorylated) state even in the absence of calcium (Ca^{2+}), a feature known as autophosphorylation of CaMKII. Numerous studies have been accumulated over the past two decades to support the hypothesis that CaMKII acts as a synaptic tag (Okamoto et al., 2009; Redondo and Morris, 2011). In addition, many studies assert the relevance of CaMKII as a synaptic tag on real memories and behavioral outcomes and not just in physiological models of memory. For example, in a study done by Moncada et al. (2011), they examined LTM of rats that explored an open field followed by inhibitory avoidance (IA) training. These authors investigated the involvement of glutamatergic, dopaminergic, and noradrenergic inputs on the setting of an IA learning tag and the synthesis of PRPs. Through pharmacological interventions during open-field and/or IA sessions, they found that hippocampal dopamine D1/D5- and β-adrenergic receptors were required to induce PRP synthesis and that activation of the glutamatergic NMDA receptors was required for setting the learning tags, and this machinery further required CaMKII activity (Moncada et al., 2011). Although over the past 20 years many researchers have investigated the role of CaMKII in learning and memory, much of the work done on the route to unraveling the roles of this molecule as a synaptic tag has been in hippocampus and it is still unclear whether it has the same functions in other brain regions. Furthermore, while the role of CaMKII as a synaptic tag has been looked at in behavioral paradigms (as in Moncada et al., 2011) and it has been established that CaMKII has an important role, these studies lack specificity in that they do not provide information about how memory and consolidation are affected without the tag. Is memory completely diminished, or will some aspects of memory still be there? What kinds of memory are affected? Thus, a study

that examines the behavioral relevance of CaMKII as a synaptic tag in brain regions other than the hippocampus is needed to fill in the gaps. The goal of this thesis project was to answer these questions by scrutinizing the role of CaMKII as a synaptic tag in olfactory associative memories in rat pups.

In this Introduction I will begin by explaining the concept of memory, the different theories of memory (from early classical theories to the more modern), the classification of memory and a brief description of the molecular mechanism of learning and memory. Then I will introduce the cellular mechanisms of memory in the olfactory system, and give a brief overview of the structure of olfactory bulbs (OBs). I will then describe the specific molecular mechanisms of CaMKII and the cellular cascade that it is involved in. I will review the concept of synaptic tagging and introduce candidate synaptic tags and what is known about the role of CaMKII as a synaptic tag. Finally I will review my experimental design and the specific questions that this study sets out to address.

1.2 What is Memory?

Learning and memory are fundamental higher brain functions that allow the individual to adapt to the environment (Benfenati, 2007). As we go about our daily lives we learn different things and memories are formed. Sometimes we forget things that shouldn't be forgotten and sometimes we remember the not-so-important events. Frederich Nietzche in his essay "On the Advantage and Disadvantage of History for Life" (1874) argues that we can only move on and live in happiness if we learn to forget, or in other words to live un-historically. The questions that arise are: how are memories formed? What determines whether we will forget or remember an event? How is it that

we might remember one thing but not another? The ability to memorize and remember is one of the most fascinating phenomena one can study. Without the ability to remember, life becomes meaningless and virtually impossible. Many diseases of the brain affect our memories (e.g., Alzheimer's disease). The quest to finding an ideal treatment for these diseases and solve one of the biggest puzzles we face about the brain has led scientists to delve more deeply into the workings of memory formation and retrieval within the brain.

But what exactly is memory? Generally it is defined as the process of encoding, storage, and retrieval of information (Smith, 1980). How might it be represented at the neural level? According to Ramon y Cajal (1893) (regarded by many as the father of modern neuroscience), modifications in synaptic plasticity lead to creation of memories in the mammalian brain. Today, it has been postulated that due to experience, neurons and neural networks get altered, and memory is the persistence of such changes (Okano et al., 2000).

1.3 Theories of Memory

The search for an answer to what memory is and how memories are formed started thousands of years ago and it continues to the present day. Some theories from early classical periods are: Memory is craft perfected through philosophical dialogue (Plato), memories are imprinted on the mind like a carving on a wax tablet (Aristotle), and the mind is a storehouse of memories that can be recalled for rhetorical practice (Cicero). It wasn't until the 18th century that David Hartley, an English philosopher, shed more light on the concept of memory and hypothesized that memories are encoded through hidden motions in the nervous system (Buckingham and Finger, 1997). During the mid-1880s

German philosopher Herman Ebbinghaus advanced the understanding of memory by investigating it scientifically. His experiments involved the usage of syllables with no meaning which were then associated with meaningful words. Some of his findings from this work (such as his classification of the three distinct types of memory: sensory, short-term and long-term) are still widely used and cited in today's publications. In 1904, Richard Semon was the first scientist to propose that experiences leave physical traces, which he referred to as 'engrams'. In 1949 the Canadian Donald Hebb in his book "The Organization of Behavior" put forward the idea that "neurons that fire together, wire together". Hebb states: "Let us assume that the persistence or repetition of a reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability… When an axon of cell *A* is near enough to excite a cell *B* and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that *A*'s efficiency, as one of the cells firing *B*, is increased." To simplify, in this statement Hebb implies that memories are created by the creation of connections between neurons and this happens through repeated use. This concept is referred to as Hebb's Rule, The Hebbian Theory, Hebb's Postulate, or Cell Assembly Theory. Hebb's significant discovery gained more support when the concepts of LTP, LTD, and neuroplasticity were discovered during the 1970s. Today Hebbian Theory remains as the most significant theory to explain the concept of learning and memory. Eric Richard Kandel's work (He was the recipient of the 2000 Nobel Prize in Physiology or Medicine for his research on the basis of memory storage in neurons) was central in demonstrating Hebb's Theory and identifying the physiological and molecular changes that occur during learning.

1.4 Neuroplasticity

In his groundbreaking book “Principles of Psychology” William James presented the concept of neuroplasticity around 120 years ago. However, this idea was ignored for over 50 years. In 1948 Jerzy Konorski, a Polish neuroscientist suggested that the brain and neurons undergo plastic changes. However, it was Paul Bach-y-Rita that applied the theory of neuroplasticity to experimental applications. His work was mostly concentrated on finding an ideal treatment for stroke patients. As the name suggests, neuroplasticity is obtained from the two words neuron and plastic, implying that nerve cells can undergo change and re-organization (plasticity). These changes are essential for many different purposes like regaining a lost brain function after a trauma, regaining a function after a stroke, and of course learning and memory. Nowadays the term neuroplasticity and the notion that the brain is plastic are generally accepted.

Numerous studies have been published to support the concept of neural plasticity in different paradigms. In 2006, Maguire et al. published a paper about neuroplasticity in the hippocampus of London taxi drivers using Magnetic Resonance Imaging (MRI) technique. They showed that London taxi drivers have a relatively larger hippocampus in comparison with London bus drivers and they suggest that the increase in the hippocampal gray matter volume in taxi drivers is a form of neural plasticity that occurs with learning and memory. Neural plasticity has also been observed in brains of bilinguals. Mechelli et al. (2004) provided evidence by comparing the brains of bilinguals and monolinguals. The authors suggest that learning a second language increases the density of grey matter in the left inferior parietal cortex. Structural and functional neural

plasticity also happen throughout the olfactory system by being exposed to and learning new odors. Plasticity in the olfactory system can happen from the level of receptors (Jones et al., 2008; Claudianos et al., 2014) or throughout the OB and cortex (Yuan et al., 2003b; Lethbridge et al., 2012; Morrison et al., 2013; Jung et al., 1990).

The concept of neuroplasticity tells us that the brain is not at a fixed state, it is rather plastic, malleable, and changeable. In other words, the brain never stops changing and adjusting. A quote by Dr. Marvin L. Misky from his book "Society of the Mind" puts it best: The principle activities of the brains are making changes in themselves (1986). However, the question that arises at this stage is how do these changes occur and what are the molecular mechanisms responsible for these changes?

1.4.1 The Synaptic Mechanism of Neuroplasticity

Broadly speaking and simply put, two types of modifications happen in the brain during learning; one is the change of the internal structure of neurons and two is an increase or decrease in the number of synapses between neurons (Drubach, 2000). However, the reigning theory to explain neural (synaptic) plasticity is that plasticity is achieved through strengthening or weakening of synapses in response to increases or decreases in their activity (Hughes, 1958) for which the terms LTP and LTD are used (Bliss and Lomo, 1973).

1.4.1.1 Long-term Potentiation and Long-term Depression

The phenomenon of LTP was first described by Terje Lømo and Tim Bliss in 1973. These two researchers were able to show that a burst of tetanic (100 Hz) stimulus on

perforant path fibers led to a dramatic and long-lasting augmentation in the post-synaptic response of cells onto which these fibers synapse in the dentate gyrus. In 1975, Douglas and Goddard proposed "long-term potentiation" as a new name for the phenomenon of long-lasting potentiation (Douglas and Goddard, 1975). Today, the meaning of LTP is generally accepted to be a persistent strengthening of synapses based on recent patterns of activity. These patterns of synaptic activity produce a long-lasting increase in signal transmission between two neurons (Cooke and Bliss, 2006). During the early 1980s, about a decade after the discovery of LTP, LTD was discovered. LTD is the opposite of LTP in that it produces a long-lasting decrease in synaptic strength (Lynch et al., 1977). It is important to note that LTD and the interplay between LTP and LTD are also significant elements in learning and memory. Both of these two opposing processes (LTP and LTD) appear to be triggered by the same triggering signal (i.e., Ca^{2+} influx). Early work by Mulkey and Malenka (1992) showed that intracellular Ca^{2+} buffers can block LTD and what determines whether LTP or LTD occurs is the *level* of Ca^{2+} influx (Lisman, 2001). It is normally thought that memory formation is based on the LTP of synapses but these two changes have to occur in both directions to protect the network from the saturating effects of potentiation alone (Martin and Morris, 2002). LTD may also have its own role in altering neural circuits to support memory (Nabavi et al., 2014; Connor and Wang, 2015).

1.4.1.1.1 The Mechanism of Long-term Potentiation

Neural activity leads to a series of molecular occurrences such as Ca^{2+} influx, activation of neurotransmitter and kinase systems, gene translation and expression, regulation of proteins, and others that are essential to establish the plastic changes

underlying memory formation (Ramirez-Amaya, 2007). It has been shown that one of the most important neurotransmitter systems in neuroplasticity and learning and memory is the glutamatergic system (Dudai, 2002). Glutamate is the most prevalent excitatory neurotransmitter in the central nervous system (CNS). It is also the metabolic precursor of Gamma-Aminobutyric Acid (GABA), the chief inhibitory neurotransmitter (Petroff, 2002). Activity at glutamatergic synapses contributes to both the loss and preservation of synapses (Penn and Shatz, 1999). Glutamate receptors (GluRs) fall into two main categories, ionotropic and metabotropic. The classification is based on how they give rise to a postsynaptic current (Palmada and Centelles, 1998). Metabotropic glutamate receptors (mGluRs) belong to the family of G-protein Coupled Receptors (GPCRs) and are divided into 3 groups (Groups I, II, and III) and a total of 8 subtypes (Pin and Acher, 2002). Group I mGluRs (mGluR1 and mGluR5), especially mGluR5, play an important role in the regulation of neuronal excitability and synaptic plasticity (Niswender and Conn, 2010). There are 3 main classes of ionotropic glutamate receptors (iGluRs): α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA), N-methyl d-aspartate (NMDA), and kainate receptors, each of which includes an assembly of subunits (Monaghan et al., 1989). Ionotropic receptors are fast acting. Once opened, these receptors can produce large changes in current flow. When the ligand (glutamate) binds to an ionotropic receptor, the channel undergoes a conformational change. This change in conformation happens to allow an immediate influx of sodium and efflux of potassium ions. This causes excitatory postsynaptic current (EPSC). This current, which is typically caused by the opening of AMPA receptors, depolarizes the membrane in the post-synaptic cell and may cause an action potential (Jonas and Spruston, 1994). In its resting

condition, the NMDA receptor, which is permeable to Ca^{2+} ions, has a magnesium (Mg^{2+}) blockade (Nowak et al., 1984). The depolarization of the cell by AMPA receptors will lead to removal of the Mg^{2+} blockade from the NMDA receptors and ultimately lead to activation of these receptors and Ca^{2+} influx (Johnson and Ascher, 1990). It is well accepted that this is the triggering mechanism for LTP induction (Malenka and Nicoll, 1999) although NMDA receptor and calcium-independent LTP also exists (Grover and Teyler, 1990; Grover, 1998; Stricker et al., 1999; Villers et al., 2014).

Many researchers have specifically focused on the triggering mechanism of Ca^{2+} influx in LTP and memory. Early experiments in 1983 by Lynch et al provided evidence that preventing the rise in postsynaptic Ca^{2+} with Ca^{2+} chelators blocks LTP. Another study investigated the role of Ca^{2+} in LTP induction using a caged Ca^{2+} compound in CA1 pyramidal cells (Yang et al., 1999). These researchers showed that raising the amount of postsynaptic Ca^{2+} activates LTP. In addition, using two-photon microscopy to image fluorescence with high resolution in strongly scattering tissue, Yuste and Denk (1995) measured calcium dynamics in spines from CA1 pyramidal neurons in slices of rat hippocampus and demonstrated that increases of Ca^{2+} within dendritic spines are due to NMDA receptor activation (Yuste and Denk, 1995).

Generally the NMDA receptor is known for *induction* of LTP and the AMPA receptor for *expression* of LTP. Activation of NMDA receptors leads to Ca^{2+} influx, which in turn leads to activation of protein kinases and neurotrophins. These cellular activities all play roles in activation of gene transcription, neuroplasticity and LTP (Sheng and Kim, 2002; Johnston et al., 2003). The role of the NMDA-receptors in LTP in the hippocampus and in learning and memory has been confirmed in multiple studies. The

advent of transgenic mice has made possible very specific manipulations. For example, Tang et al (2001) using the NR2b transgenic mice, in which NMDA receptor function is enhanced via the NR2b subunit transgene in neurons of the forebrain, demonstrated both larger LTP in the hippocampus and superior learning and memory. These gain-of-function findings further support the important role of NMDA receptors in LTP and memory. Expression of LTP through AMPA receptors is made by insertion of more AMPA receptors (Scannevin and Huganir, 2000; Malinow and Malenka, 2002; Bredt and Nicoll, 2003). According to Bailey and Kandel (1993) structural neuroplasticity refers to either production of new synapses or re-organization of existing synapses. As an example of the changes seen with LTP expression, Maren et al. (1993) examined the role of AMPA receptors in hippocampal LTP. They used quantitative autoradiography to assess the binding properties of AMPA receptors in frozen brain sections obtained from rats. Induction of LTP resulted in a selective increase in AMPA binding and increases in AMPA binding were highly correlated with the magnitude of LTP recorded. Their results indicated that LTP induction resulted in an increase in the number of AMPA receptor binding sites and they suggested that a modification in postsynaptic AMPA receptors plays a role in the expression of synaptic enhancement following LTP induction in the hippocampus (Maren et al., 1993).

Using primary cultures of mouse striatal neurons and a phosphospecific mitogen-activated protein kinase (MAPK) antibody, another group of researchers addressed the involvement of AMPA receptors in intracellular control of synaptic plasticity. They asked whether AMPA receptors could activate the MAPK cascade (Perkinton et al., 1999). The reason they chose the MAPK cascade is that the MAPK cascade has been shown to be

involved in gene expression and synaptic plasticity (Sweatt, 2001; Thomas and Huganir, 2004). They found that in the presence of cyclothiazide (an AMPA receptor agonist), AMPA caused a robust Ca^{2+}-dependent activation of MAPK through MAPK kinase and the activation was blocked by GYKI 53655 (an AMPA receptor antagonist) (Perkinton et al., 1999).

Some researchers have looked at the role of small GTPases in AMPA receptor trafficking and LTP expression. Zhu and colleagues (2002) examined the function of the small GTPases Ras and Rap in the postsynaptic signaling underlying synaptic plasticity and showed that Ras relays the NMDA receptor and CaMKII signaling that drives synaptic delivery of AMPA receptors during LTP. In contrast, Rap mediates NMDA receptor-dependent removal of synaptic AMPA receptors that occurs during LTD (Zhu et al., 2002).

As just mentioned, the molecule CaMKII has a role in regulating LTP through regulating AMPA receptors. Barria et al. (1997) showed that induction of LTP increased the phosphorus-32 labeling of AMPA receptors and this AMPA receptor phosphorylation appeared to be catalyzed by CaMKII. These authors argue that this was correlated with the activation and autophosphorylation of CaMKII, and the CaMKII inhibitor (KN-62) blocked it (Barria et al., 1997). These results not only provide confirmation for the involvement of AMPA receptors in LTP and synaptic plasticity but also present us with a candidate key molecule for generating and maintaining LTP. There is now considerable evidence that CaMKII is a key element of the molecular machinery of LTP. Since the topic of this thesis project is CaMKII, I will explain this molecule in more detail in a separate section.

Over the past 30 years, the concept of LTP has been the object of intense scrutiny by neuroscientists due to the widely and rightfully believed notion that LTP is a key factor leading up to the formation and storage of memories (Teyler and DiScenna, 1987; Bliss and Collingridge, 1993). A study done by Whitlock et al. (2006) is an example of many such studies. In this study, the investigators found that one-trial IA learning in rats produced the same changes in hippocampal glutamate receptors as induction of LTP with high-frequency stimulation (HFS) and caused a spatially restricted increase in the amplitude of evoked synaptic transmission in CA1 in vivo. Because the learning-induced synaptic potentiation occluded HFS-induced LTP, they concluded that IA training induces LTP in CA1 (Whitlock et al., 2006).

1.4.1.1.2 Neuroplasticity and LTP in Olfaction

In vertebrates, olfactory receptor neurons (ORNs), mitral cells (MCs), and piriform cortical pyramidal neurons are all glutamatergic, and express both NMDA and AMPA receptors (Shipley and Ennis, 1996). Ennis et al. showed that ORNs to MCs synapses could express LTP following HFS (Ennis et al., 1998), which was further confirmed using TBS of the olfactory nerve (ON) (Yuan, 2009). The MC and granule cell (GC) synapses have also been shown to undergo synaptic plasticity and LTP following learning (Wilson et al., 1985; Nissant et al., 2009; Gao and Strowbridge, 2009).

Glutamatergic receptor mediated neural plasticity and LTP have been observed and extensively studied in the olfactory system (Lethbridge et al., 2012; Morrison et al., 2013). The study done by Morrison et al. (2013) investigated neural changes in the anterior piriform cortex (aPC) and observed plasticity following learning in an odor

associative learning paradigm. Using a locally infused NMDA receptor antagonist (which prevented learning) the authors demonstrated that the neuroplastic changes were NMDA receptor mediated.

Synaptic plasticity in the aPC has also been shown using *ex vivo* slices from rat pups given multi-trial, spaced lateralized odor preference training (Fontaine et al., 2013). A 10-minute training session yields 24-hour memory, and four daily sessions produce 48-hour memory (Fontaine et al., 2013). Lateralizing the odor preference memory through naris occlusion reduces response variability (permitting within – subject control). Following one 10 minute training session, the AMPA receptor-mediated field excitatory postsynaptic potential (fEPSP) in the aPC to lateral olfactory tract input was shown to be enhanced at 24 hours but not at 48 hours. Following four spaced lateralized training sessions, the AMPA receptor-mediated postsynaptic response was enhanced in the trained aPC at 48 hours. In addition, calcium imaging in the same preparation revealed an increase in the activation of pyramidal cells in aPC after training. These results provide evidence that 4 sessions of odor preference training increased odor input responsiveness and increased synaptic strength. The authors showed that the increase in aPC network activation corresponded with the behavioral memory, which is an excellent illustration of neural plasticity linked to memory within the olfactory system.

The notion of glutamatergic-mediated neuroplasticity within the olfactory system has also been illustrated *in vitro* using ON stimulation paired with beta-adrenoceptor (β-AR) activation, which potentiates MC firing (Lethbridge et al., 2012). The results of the Lethbridge study showed that the potentiation was blocked by an NMDA receptor antagonist (D-APV) and by increased inhibition and that glomerular disinhibition itself

induces NMDA receptor-sensitive potentiation. Behavioral learning was prevented by infusion of an NMDA receptor antagonist or a $GABA_A$ receptor agonist. Further, a glomerular $GABA_A$ receptor antagonist paired with odor can induce NMDA receptor-dependent learning. *Ex vivo* experiments using OBs from trained rat pups revealed an increase in the AMPA/NMDA EPSC ratio post-training, consistent with an increase in AMPA receptor insertion and/or the decrease in NMDAR subunits. These results provide yet another example to support the concept of neural plasticity within the olfactory system using the rat pup odor preference learning paradigm.

Besides AMPA receptor changes, the NMDA receptor can undergo plasticity itself. A recent study done by Mukherjee and colleagues (2014) investigated the change in NMDA receptor number in the aPC in rat pups following odor preference learning. The results of this study demonstrate an activity- and experience-dependent refinement of neural circuitry that occurs during repeated learning, and that may have implications for learning optimization.

Using an olfactory rule-learning paradigm (olfactory discrimination learning) in rats, Quinlan et al. (2004) showed that rule learning regulates the composition of NMDA receptors in the piriform cortex, resulting in receptors with a higher complement of the NR2a subunit protein relative to NR2b. They also showed that rule learning reduces the LTP induced by HFS of the intracortical axons in slices of piriform cortex. As NR2a-containing NMDA receptors mediate shorter excitatory postsynaptic currents than those containing NR2b, they suggest that learning-induced regulation of NMDA receptor composition constrains subsequent synaptic plasticity, thereby maintaining the memory encoded by experience (Quinlan et al., 2004).

One question that arises at this point is what biochemical pathways Ca^{2+} influx (the triggering mechanism of NMDA plasticity) activates and what pathways or molecular cascades are required for translating the Ca^{2+} signal into an increase in synaptic strength, (or in other words production of LTP)? A review of the literature generates a large list of candidate signal transduction molecules that could be responsible for this phenomenon. However, there are only a few molecules for which the evidence of a key, mandatory role in LTP is compelling. These essential components are protein kinase A (PKA), cyclic adenosine monophosphate (cAMP), cAMP response element binding protein (CREB), and exchange protein activated by cAMP (Epac), and will be discussed in more detail in later sections.

1.4.1.1.3 Basic Properties of LTP

LTP has been shown to have 3 basic properties: a) input-specificity, b) associativity, and c) co-operativity (Bliss and Collingridge, 1993).

The first property (input-specificity) refers to the fact that, when LTP is elicited at one set of synapses on a postsynaptic cell, other adjacent synapses that were not activated during the induction protocol do not show LTP. This property is explained by the requirement that, to elicit LTP, synaptic NMDA receptors must be activated, leading to a spatially restricted increase in intracellular Ca^{2+} in the relevant dendritic spine (Bliss and Collingridge, 1993; Malenka, 2003). This property is very important and advantageous because it increases the storage capacity of individual neurons (Malenka and Nicoll, 1999) and memory formation depends on this specific activation of synapses.

The second property (associativity) refers to the fact that LTP can be induced at synapses that are activated by low-frequency, sub- threshold stimuli or in other words, by a weak input if their activation is happening around the same time as an LTP-inducing stimulus (a strong input) at another set of synapses on the same cell (convergent) (Bliss and Collingridge, 1993). This property is explained by the fact that the LTP-inducing stimulus provides the required depolarization, which is rapidly transmitted to synapses in which the NMDA receptors were simultaneously activated but by a weak stimulus (Malenka, 2003). The important notion to grasp is that through this property we can understand the mechanism by which LTP associates two pieces of information being conveyed by different sets of afferents that synapse on the same postsynaptic cell (Malenka, 2003). This property of LTP has been proposed to be the cellular analog of classical conditioning (Malenka and Nicoll, 1999). The first two properties can be explained best by the synaptic tagging and capture hypothesis, which will be discussed thoroughly in a later section.

The third property (co-operativity) is somewhat similar to the second property, associativity, but it is indeed different and valuable. Co-operativity refers to the fact that when using HFS to induce LTP, a critical number of fibers must be simultaneously activated, in other words they must cooperate to induce LTP (Bliss and Collingridge, 1993). To put it in simpler terms, weak 'HFS', which activates relatively few fibers, does not produce LTP whereas strong stimulation at the same frequency and for the same duration does produce LTP, so a threshold stimulus intensity during HFS is required for synaptic enhancement (Ikezu and Gendelman, 2008) and below this threshold LTP does not occur (Levine, 2000). This property is explained by the fact that, to trigger LTP, the

postsynaptic cell must be sufficiently depolarized to allow current (particularly Ca^{2+}) to flow through the NMDA receptor channel (Malenka, 2003). The question that comes up at this point is that what mechanism is responsible for the input selective property of LTP?

The concept of synaptic tagging and capture hypothesis provides an excellent explanation and framework. In the following sections I will explain this concept, experiments that have evolved out of this hypothesis, and molecules that have been proposed to be involved in this process.

1.5 Synaptic Tagging and Capture Hypothesis

Any given neuron can have thousands of synapses with an enormous amount of information coming in, however single synapses can undergo highly selective modifications and each of them can be modified in an independent manner. Because this type of synaptic modification requires both transcription and translation, the problem of targeting gene products from the nucleus to the few activated synapses in a vast dendritic tree arises (Martin and Kosik, 2002). The synaptic tagging hypothesis has been put forward as a way to address this problem. This hypothesis proposes that the products of gene expression are delivered throughout the cell, but that they function to increase synaptic strength only at synapses that have been 'tagged' by previous synaptic activity (Frey and Morris, 1997).

Frey and Morris first proposed the synaptic tagging hypothesis in 1997. In their experiment they used 53 transversal hippocampal slices prepared from 53 male Wistar rats (7 weeks old). The authors stimulated two different sets of Schaffer collateral fibers

that synapsed on the same population of CA1 neurons. They then recorded fEPSPs associated with each stimulus on either S1 or S2 pathways to produce early LTP (E-LTP) and late LTP (L-LTP) on different synapses within the same neuron. Their results showed that E-LTP produced by weak stimulation could be turned into L-LTP if a strong S2 stimulus was delivered before or after. They also showed that the ability to convert E-LTP to L-LTP decreased as the interval between the two stimulations increased, implying temporal dependence. When they blocked protein synthesis prior to the delivery of strong S2 stimulation, the conversion to L-LTP was prevented, which showed the importance of translating the messenger ribonucleic acids (mRNAs) produced by the genomic cascade (Frey and Morris, 1997). Frey and Morris stated that LTP initiates the creation of a short-lasting protein-synthesis-independent 'synaptic tag' at the potentiated or stimulated synapse which leads to establishment of late LTP through protein synthesis. They also proposed the notion that synaptic tags decay in less than three hours and they are not permanent. These findings indicate that the persistence of LTP depends not only on local events during its induction, but also on the prior activity of the neuron (Frey and Morris, 1997). Later work showed that a L-LTP inducing stimulus induces two independent processes: one is creation of a tag that identifies the synapse as having been stimulated, and another is a genomic cascade that produces new mRNAs and proteins which are now called plasticity related products (Rudy, 2008). While weak stimulation also tags synapses, it does not produce the genomic cascade that leads to the production of new proteins and other plasticity related products. Therefore, if no protein support occurs, the state of the synapse will go back to its baseline levels even though the tags have been set

(Redondo and Morris, 2011). So as Frey and Morris discovered, the tag is temporary and will disappear if no protein is synthesized to be captured by the tag.

1.5.1 Evidence for Synaptic Tagging

Evidence for the concept of synaptic tagging emerged from pioneering studies on cultured Aplysia neurons (Martin et al., 1997). This study used a culture system, in which a single bifurcated Aplysia sensory neuron formed synaptic contacts with two spatially separated motor neurons. In this elegant study delivery of five puffs of serotonin (5-HT) to one contact selectively enhanced synaptic efficacy at that synapse without altering the efficacy of the other contact. The authors suggested that the increase in synaptic potency, which lasted for more than 24 hours, depends on transcription as it could be blocked by the transcriptional inhibitor actinomycin D. These results were interesting because they showed that a single cell could change the strength of its connections in a manner that depends on transcription, but was spatially restricted to a single subset of synapses. In the same study evidence for synaptic tagging were presented when a single puff of 5-HT was delivered to one contact and five puffs were applied to the other connection. The single puff of 5-HT produced short-term facilitation (E-LTP), but if five puffs were applied to the other contact, the facilitation produced by the single puff was converted to long lasting or L-LTP (Martin et al., 1997). These observations indicated that long-term synaptic changes at one synapse could trigger a process that is captured by another synapse that has experienced a lower level of activation. In other words, the E-LTP generates tags that can then be used to capture PRPs generated by L-LTP in other synapses of the same sensory neuron. Furthermore, the authors also show that this capture

phenomenon has a transient lifetime. These findings are in agreement with the synaptic tagging hypothesis and provide illustrious evidence for this concept. After the initial discovery and the early pioneering evidences many investigators explored the hypothesis and provided data to support it (Barco et al., 2002; Dudek and Fields, 2002; Fonseca et al., 2004; Young and Nguyen, 2005).

Some studies have also focused on taking advantage of the idea of synaptic tagging and capture to improve memory (in a paradigm that would otherwise produce only short-term memory) by associating weak training with something else, for example exploration of a novel spatial context. A study by Moncada and Viola (2007) is one such study. These authors used an IA paradigm in which rats were given foot shocks after they stepped down from a platform, in this way the rats were trained to suppress the step down behavior the next time they are placed on a platform and this behavior was used as a parameter to assess their memory. To figure out whether they can improve rats' memories, they combined this IA paradigm with the exploration of a novel field. Their results showed that weak training in the IA task produced short-term memory (STM), lasting no longer than 15 minutes but when a 5-minute session of spatial novelty was given just before the IA task, the STM was converted into a persistent memory lasting 24 hours.

These results resembled the electrophysiological experiments, where a strong tetanus applied with a weak tetanization in a different pathway resulted in L-LTP in the weakly stimulated pathway (Frey and Morris, 1997). Moncada and Viola (2007) hypothesized that in this study some synapses are tagged by the weak IA training and PRPs are produced by the exploration of the novel field. To test this assumption, the

authors blocked new protein synthesis after the open field exploration session. Infusions of the protein synthesis inhibitor anisomycin into CA1 immediately after the open field session disrupted long-term memory (LTM) for the weak IA training. However, anisomycin had no effect on strong IA training. These results provide conclusive evidence that new protein synthesis triggered by spatial novelty was necessary and sufficient to drive memory consolidation for IA training. Also consistent with the synaptic tagging hypothesis, the authors demonstrated a restricted temporal window within which coincidence of the two behavioral experiences leads to LTM for the weak IA training (Moncada and Viola, 2007).

1.5.2 Candidate Synaptic Tags

The first question that comes to mind after being introduced to the concept of synaptic tagging is what are these tags, in other words what could serve as a synaptic tag? Any candidate should fulfill the following criteria. First, it should be local. Second, it should be time-limited and reversible. Third, it should be able to interact with cell-wide molecular events that occur after strong stimulation to produce long-term, synapse-specific strengthening (Martin and Kosik, 2002). Broadly speaking anything that provides a spatially restricted trace of activity could serve as a synaptic tag. Examples of such activities and subsequent changes include the addition of AMPA receptors (Shi et al., 1999), the lateral mobility of NMDA receptors between synaptic and extra synaptic sites (Tovar and Westbrook, 2002), HOMER-mediated insertion of glutamate receptor mGluR5 into the membrane (Ango et al., 2001), and palmitate cycling on postsynaptic density protein 95 (PSD95) (El-Husseini et al., 2002). Such events could serve as

localized traces of previous synaptic activity that are able to produce synaptic strengthening on their own within a limited time period (Martin and Kosik, 2002). The slate of candidates is wide ranging. In the following section I will describe some of the other molecules and processes that have been suggested to function as synaptic tags.

1.5.2.1 Kinases

Protein kinases meet the criteria for a tag, as they allow a synapse to 'remember' previous activity in a spatially restricted and reversible manner (Martin and Kosik, 2002). There are several protein kinases, many of which have been suggested to work as a synaptic tag in maintaining LTP. The atypical protein kinase C zeta known as protein kinase Mζ (PKM-ζ) has been shown to be necessary for the maintenance of LTP in the hippocampus (Ling et al., 2002).

Drier et al. (2002) used a Pavlovian olfactory learning task in Drosophila, and found that induction of the mouse PKM-ζ transgene enhanced memory. The enhancement was temporally specific, with optimal induction at 30 minutes after training. Chelerythrine, an inhibitor of PKM-ζ, inhibited memory. The authors suggest that PKM-ζ is not only necessary for, but can also enhance LTM for olfactory learning in Drosophila (Drier et al, 2002).

Sajikumar et al. (2005) investigated whether PKM-ζ is involved in tagging by providing strong and weak tetanization to two independent pathways and then disrupting the function of the kinase by a selective myristoylated zeta-pseudosubstrate inhibitory peptide. The authors found that persistent PKM-ζ activity maintains potentiated responses, not only of the strongly tetanized pathway, but also of the weakly tetanized

pathway. In contrast, an independent, nontetanized pathway was unaffected by the inhibitor, indicating that the function of PKM-ζ was specific to the tagged synapses (Sajikumar et al., 2005). From the results of these experiments it can be inferred that local, persistent changes in the activity of these kinases could serve as synaptic tags.

PKA has also been postulated to function as a synaptic tag in creating LTP and LTM. A study done by Young et al. (2006) showed that hippocampal slices from transgenic mice that have genetically reduced hippocampal PKA activity display impaired synaptic capture of L-LTP. Also, an inhibitor of PKA, KT-5720, blocked synaptic capture of L-LTP. Moreover, pharmacological activation of the cAMP/PKA pathway can produce a synaptic tag to capture L-LTP expression, resulting in persistent synaptic facilitation. Collectively, these studies argue that PKA can function as a synaptic tag in maintaining LTP (Young et al., 2006). Although PKM-ζ and PKA have both been suggested as synaptic tags, their activity depends strongly on the activity of another kinase molecule, CaMKII, which may be the most important synaptic tag as it is upstream of other suggested synaptic tags. CaMKII is the topic of this thesis project; hence in the following section more detail on the workings of this molecule will be provided.

1.5.2.1.1 CaMKII

CaMKII is a ubiquitous serine/threonine protein kinase involved in a broad variety of cellular functions (Colbran and Soderling, 1990; Kennedy et al., 1990; Kelly, 1991; Waxham et al., 1993). CaMKII is found in most tissues, but it is present in especially high concentrations in neurons, in which it may be up to 2% of total protein in some brain regions (Erondu and Kennedy, 1985). The CaMKII family consists of 28 isoforms,

derived from four genes (α, β, γ, and δ) but the α and β subunits are predominant in the brain (Li et al., 2001). Each isoform has 3 domains, a catalytic domain (substrate binding site), an autoinhibitory domain, and a self-association domain. The self-association domain causes the formation of a dodecameric holoenzyme. In basal conditions, the catalytic domain is inhibited by the autoinhibitory domain (Lisman et al., 2012). When the calcium concentration rises, the calcium/calmodulin complex subsequently formed can bind to the autoinhibitory domain (inhibition of the autoinhibition), and this will cause the substrate to bind to the catalytic domain (Chao et al., 2011). One other consequence of inhibition of the autoinhibitory domain is the autophosphorylation of Thr 286 site on the α subunit and the Thr287 on the β subunit. Phosphorylation of these sites reduces the dissociation of calmodulin (Meyer et al., 1992). Autophosphorylation makes CaMKII activity persist even after the calcium levels fall to baseline levels (Miller and Kennedy, 1986), and even after dissociation of the calcium/calmodulin complex, making the enzyme autonomous, with a calcium independent activity (Hoffman et al., 2011). The active state caused by autophosphorylation is transient and is affected by phosphatase activity (Lisman, 1994).

Following calcium influx into the post synaptic neuron, CaMKII diffuses to the synapse and accumulates in the postsynaptic density (PSD), where it binds to NMDA receptors through the carboxy-terminal domain of the NR2b subunit (Leonard et al., 2002). The binding sequence of NR2b blocks the autoinhibitory gate of CaMKII, leaving it in an active state, even after dissociation of the calcium/calmodulin complex and without autophosphorylation. This active state is transient, ranging from seconds to

minutes, but is not affected by phosphatase activity (Bayer et al., 2001). The binding of CaMKII to NMDA receptors increases calmodulin affinity for the kinase (this phenomenon is called trapping). It may be a way of preventing dissociation of CaMKII from synaptic sites (Shen et al., 2000), but it also works to facilitate further autophosphorylation around the ring (Bayer et al., 2001). Besides its functional role, the binding of CaMKII to NMDA receptors has structural consequences as well, as it allows addition of AMPA receptors into the PSD (Sanhueza and Lisman, 2013). In its autonomous state the molecule specifically binds to proteins in the PSD (Strack et al., 1997).

CaMKII has been known as an integral component in mediating LTP. Testing whether CaMKII is required for LTP was made possible by the development of specific inhibitors of this kinase, such as KN-62 and KN-93 (Malinow et al., 1989). Many studies have provided evidence that injection of these inhibitors of CaMKII or genetic disruption of CaMKII blocks the ability to generate LTP (Malenka et al., 1989; Silva et al., 1992). Many studies have also provided evidence that activation of CaMKII leads to enhancement of synaptic transmission and that LTP will be reduced by increasing the concentrations of constitutively active CaMKII (Pettit et al., 1994; Liedo et al., 1995). Together, the pharmacological and genetic evidence strongly argue that a large fraction of LTP is dependent on CaMKII and that this enzyme is involved in a core process of LTP (Lisman et al., 2002). CaMKII mediates LTP by addition of AMPA receptors into the PSD (Sanhueza and Lisman, 2013), phosphorylating the already existing AMPA receptors (Malenka and Nicoll, 1999) and also by activating other proteins (Saneyoshi et al., 2008).

The role of CaMKII in learning and memory *in vivo* has also been extensively studied. The first of such studies was made in αCaMKII knockout mice. These mice were deficient in LTP and also in hippocampus- dependent spatial learning tasks (Silva et al., 1992). A few years later in 1998, Giese et al. were able to test the spatial memory of a mouse with the knock-IN CaMKII T286 mutation and provided evidence that these animals were strongly deficient in spatial memory tasks. Pharmacological manipulations of CaMKII have also yielded the same result of impaired memory in the Morris water maze (Wolfman et al., 1999; Vaynman et al., 2003).

Thus far I have summarized data indicating that CaMKII is a key molecule in mediating LTP and is necessary for hippocampal-dependent memory formation. But what has more recently received much attention is that CaMKII has been suggested to function as a synaptic tag in maintaining LTP and LTM. CaMKII has several properties that make it an ideal candidate for a synaptic tag. Firstly, as mentioned previously, is that CaMKII remains activated even when Ca^{2+} levels go back to baseline. This property of CaMKII (autophosphorylation) opens up a time-window for new proteins to be synthesized and to be shipped to the tagged synapses (synapses with activated or phosphorylated CaMKII).

As mentioned before, LTP is a synapse-specific event (Andersen et al., 1977) and recently optical methods have been able to provide further evidence for it (Matsuzaki et al., 2004). The second characteristic of CaMKII that makes it an ideal tag is its synapse-specific activation. Takao et al. (2005) were able to visualize the activity of CaMKII using a CaMKII reporter called Camui. Camui is a fluorescence resonance energy transfer (FRET)-based indicator that reports the transition from the basal state to the activated

conformation as an increase in CFP/YFP ratio. Through the use of this sensor and two-photon fluorescence lifetime imaging (2pFLIM), the conformational change in CaMKII that is associated with activation can be monitored at the level of a single spine in real time. Takao et al. (2005) provided evidence that CaMKII activation has the localization that is required for the synapse specificity of LTP. This distinctive pattern of activation provides evidence for why CaMKII is a good synaptic tag model.

One recent study perfectly demonstrated the role of CaMKII as a synaptic tag that mediates synapse-specific LTP. Villers et al. (2014) took on the task of studying LTP in αCaMKII-T286A mutant mice using slice electrophysiology (CA1 region of hippocampus). In their study the authors provided evidence that several forms of LTP can actually be induced in these mice but this happens at the expense of synaptic specificity. A nonspecific LTP was observed in mutant and wild-type (control) mice after a recovery period in a submersion chamber that was independent of NMDA receptors. Their findings suggest that αCaMKII autophosphorylation is not necessary to induce LTP but the increase in synaptic strength was not restricted to stimulated synapses. Villers et al. (2014) propose that because synapse specificity is a key element in the learning capacity of the neuronal networks, this cell-wide LTP cannot be related to specific learning processes and therefore αCaMKII autophosphorylation seems necessary to guarantee the synapse specificity of plasticity and learning and memory.

There are other molecules that have been proposed to function as a synaptic tag; some of them are discussed below. However, these studies all also provide evidence that CaMKII is an ideal candidate to function as a synaptic tag.

1.5.2.2 Actin Network

Actin is the major cytoskeletal protein in dendritic spines, where it serves both as a framework for the mechanical stability of spine structure and as a scaffold for recruiting various other postsynaptic proteins (Cingolani and Goda, 2008). One of the most discussed potential candidates for a synaptic tag is the actin microfilament network at the synapse. The actin network in neurons is extremely dynamic, and these dynamics have been shown to change with activity (Star et al., 2002). It has been suggested that changes in the actin microfilament network are likely to underlie the growth of new synaptic structures that has been observed after repetitive stimulation of hippocampal synapses (Engert and Bonhoeffer, 1999). New actin networks are formed at the dendritic spine during LTP induction and they fulfill all of criteria for synaptic tagging including the fact that actin stays polymerized for at least 30 minutes (Fukazawa et al., 2003). Actin has also been shown to serve as a major docking site for postsynaptic proteins that directly and indirectly bind to F-actin (Okamoto et al., 2004). Hence, the increased number of binding sites conferred by the formation of new actin filaments might be the mechanism that selectively captures the LTP-related proteins synthesized in the cell body and transported into dendrites (Okamoto et al., 2009).

Ramachandran and Frey (2009) investigated the role of actin in synaptic tagging in the CA1 region in hippocampal slices and showed that pharmacologically disrupting the actin filament (using latrunculin A or cytochalasin D) during LTP induction prevented setting of the synaptic tag. In addition, Colicos et al. (2001) have shown that repetitive tetanic stimulation can lead to remodelling of the actin network at the synapse. Such local

changes in the cytoskeleton could combine with the products of gene expression induced by strong synaptic stimulation to produce enduring, yet spatially restricted alterations in synaptic strength (Martin and Kosik, 2002). These results all point to the notion that actin networks can act as synaptic tags during LTP induction. However Sajikumar et al. (2007) showed a critical role for CaMKII in this actin filament behavior. These authors investigated the synaptic tagging hypothesis using hippocampal slices, providing evidence that activation of CaMKII controls the status, activation and content of actin filaments, which ultimately controls the structural plasticity of the spine and supports synaptic tagging.

1.5.2.3 Ca^{2+} Concentrations

As mentioned previously, the initiation of any input selective change is through the influx of Ca^{2+} ions into the postsynaptic neuron. Michmizos et al. (2011) proposed that the synapses under potentiation are tagged for dendritic transport by the increased Ca^{2+} microconcentration at the dendritic shaft directly under their spine. The authors argue that there are at least four types of voltage-gated Ca^{2+} channels (Westenbroek et al., 1990; Ben-Fredj et al., 2004) and that these channels create Ca^{2+} gradients and direct dendritic transport accordingly. However, one factor to consider in this argument is that long lasting cellular changes follow an elevated amount of Ca^{2+} in the PSD through the opening of NMDA receptors and these changes cannot be initiated with a difference in microconcentration caused by voltage gated Ca^{2+} channels. Furthermore, the Ca^{2+} rise in the PSD is very short lasting.

1.5.2.4 Local Translation

The activation of local translation meets the requirements for a tag; it can alter the composition of that synapse over an extended period of time, and thereby leave a relatively persistent trace of previous activity (Martin and Kosik, 2002). mRNAs that are delivered from the cell soma will be preferentially translated at sites at which translation is active (Martin and Kosik, 2002). When polyribosomes are present in dendritic spines, the involvement of local protein synthesis in the modification of synapses becomes more apparent (Ostroff et al., 2002). A study done by Ostroff et al. in 2002 looked at the amount of polyribosomes after the induction of LTP in hippocampal slices of rats. The percentage of spines containing polyribosomes increased from 12% ± 4% after control stimulation to 39% ± 4% after tetanic stimulation. The authors found that after the induction of LTP, the area of the PSD was significantly larger in the spines that contained polyribosomes than in spines that lacked them, indicating that the presence of polyribosomes produces structural changes, which, in turn, produce changes in synaptic strength (Ostroff et al., 2002). The question that comes up at this point is that how might synaptic stimulation produce increases in local translation? Krichevsky and Kosik (2001) provided evidence that RNA granules serve as storage sites for mRNAs, and that depolarization releases mRNAs from the granules to polysomes, where they are actively translated. Granules oscillate around a cluster of synapses, and the release of mRNAs from granules could deliver translational products to several synapses within a discrete locale (Rook et al., 2000). This would lead to an increase in the translation of a subset of mRNAs and can function as a tag to capture PRPs. A product of local translation,

activation of translation itself, or the reorganization of the RNA in the region of activity, could serve as a tag that can be recognized by transcription-dependent products (Martin and Kosik, 2002). Although necessary for synaptic tagging, this model is likely to operate in concert with other models (e.g., CaMKII activation) and its activation depends on the activation of upstream molecules (Redondo and Morris, 2011).

1.5.2.5 Protein Degradation

Protein degradation by the proteasome is another model that has been suggested for synaptic tagging. Proteasomes are protein complexes inside all eukaryotes and their main function is to degrade proteins by proteolysis (Peters et al., 1994). Proteasomes are required for synaptic plasticity (Hedge, 2004) and maintenance of LTP (Fonseca et al., 2006) and formation of LTMs (Lopez-Salon, 2001). Cai et al. (2010) provided substantial evidence that proteasomes play an essential role as a synaptic tag for maintenance of hippocampal LTP. However, this model also requires the activation of CaMKII. CaMKII autophospholyrlation promotes proteasome recruitment to spines and stimulates proteasome activity by phosphorylating proteasome subunit Rpt6 on Serine 120 (Bingol et al., 2010). So this model of synaptic tagging also depends on other processes and molecules. Now that we have briefly reviewed the topic of memory formation machinery, it is appropriate to introduce the concept of associative memory to have a better understanding of the issue at hand.

1.6 Associative Memory

Associative memory has been widely used in animal studies of memory mechanisms. It is defined as the ability to learn and remember the relationship between unrelated items such as someone we have just met and the aroma of a particular perfume (Suzuki, 2005). The most basic type of associative learning is called classical conditioning. Developed by the Russian scientist Ivan Pavlov, classical conditioning is the type of learning wherein a conditioned stimulus (CS) is paired with an unconditioned stimulus (UCS). The CS is usually a previously neutral stimulus (e.g., the sound of a tuning fork) and the UCS is biologically potent (e.g., the taste of food). The result of this pairing is a conditioned response (CR) to the conditioned stimulus when it is presented alone. Pavlov originally was studying the digestive processes of animals but he noticed that the dogs in the experiment began to salivate in the presence of the technician who normally fed them, rather than simply salivating in the presence of food. So he started analyzing this observation by ringing a bell and then giving the dog food. After a few times, the dogs started to salivate in response to the bell ringing. Pavlov called the ringing of the bell the CS (because its effect depended on the association with food), called the food the UCS (because its effect didn't depend on previous associations) and the response to the CS, he called the CR. Pavlov established the laws of classical conditioning in 1927 and since then many experiments on classical conditioning amounts have been conducted and they have led to some of the most significant discoveries with respect to the workings of brain and behavior.

1.6.1 Olfactory Associative Memory

As with other associative memories, olfactory associative memories are formed by associating two different and usually unrelated stimuli. However in this case, one of the stimuli is an odor. It might seem effortless to form and remember connections between odors and other stimuli, but a physiological understanding of how our brains instantiate these associations remains vague. Studying olfactory associative memories and their underlying mechanisms is particularly important, since, as most people have experienced scents evoke very vivid recollections. For example, the smell of sunscreen brings the ocean to mind or a whiff of perfume calls forth a long-ago friend. Studying olfactory memory provides an ideal research model because of the uniqueness of the olfactory system and pathways. Unlike other sensory systems the sense of smell does not pass through the thalamus, instead cortical and sub-cortical processing occurs before thalamic relay. Also the relatively simple structure of the OBs and piriform cortex and their convenient location on the ventral forebrain provides structures readily amenable experimental manipulations. The local circuitry and principle cell types (which I will describe in later sections) resemble those of the more complex neocortex.

In addition, olfaction is involved in behaviors essential for survival of species, including identification of predators, recognition of individuals for procreation or social hierarchy, location of food, as well as attachment between mating pairs and infant-caretaker dyads (Sullivan et al., 2015). This broad role for odors appears highly preserved across species. Because of these reasons and others, much research has been directed at understanding olfactory memory including studies of associative olfactory memory in

slices as well as, through pharmacological and other manipulations in behavioral studies. To study olfactory associative memory multiple behavioral models have been developed, which can be broadly categorized into IA training and preference training paradigms. Relevant to the current project is odor preference training, and to be more specific we used a unique paradigm called 'early odor preference learning'.

1.6.1.1 Early Odor Preference Learning

Neonate rats are born blind, they almost don't hear anything and they are unable to thermo-regulate. Therefore, they must stay near their mother in order to receive warmth and food. For these reasons pups rely heavily on odor cues to locate their mother (Leon and Moltz, 1971). Taking advantage of this biological fact, early odor preference learning is a type of classical conditioning during which neonate rats are conditioned to prefer novel odors (CS) using stimulation that mimics the way their mother cares for them (UCS) (Sullivan et al., 1986). Research has shown that different stimuli can be used for this kind of UCS, including the nesting environment (Galef and Kaner, 1980; Alberts and May, 1984), milk presentation (Johanson and Hall, 1979; Johanson and Teicher, 1980), stroking or tactile stimulation (Pedersen et al., 1982; Sullivan and Leon, 1986; Sullivan and Hall, 1988; Weldon et al., 1991; Moore and Power, 1992; McLean et al., 1993), tail pinch (Sullivan et al., 1986), the odor of maternal saliva (Sullivan et al., 1986), and mild foot shock (Camp and Rudy, 1988; Roth and Sullivan, 2001; Sullivan, 2003; Moriceau et al., 2006).

Sullivan and Leon (1986) showed that training rat pups for 10 minutes per day with tactile stimulation creates a clear preference for the trained odor (CS). One main factor in

this paradigm is that there is a sensitive period for the development of early odor preference in rats (Woo and Leon, 1987). Pups trained with odor and tactile stimulation after the first ten days of life do not develop a preference for the trained odor on PND 19 (Woo and Leon, 1987). Therefore this training has to be done within the first ten days or in other words within the sensitive period. Relevant to this project, a single 10-minute pairing of tactile stimulation and odor produces an odor preference lasting 24 hours (Wilson and Sullivan, 1994; Yuan et al., 2014). Also, peppermint is traditionally used as the CS in early odor learning experiments because pups have a natural aversion to the smell and because the glomeruli that respond to peppermint are located on the dorsolateral quadrant of the OB, which is a convenient location to access for chemical stimulation and electrical recording (McLean et al., 1999). Finally, the learning effect is easily quantified in this unique model as pups clearly move towards or away from odor sources during testing. The percentage of time pups spend on scented bedding versus normal beddings scent over a 5-minute period is calculated (McLean et al., 1996; Yuan et al., 2003).

1.6.1.1.1 Intracellular Cascades Involved – cAMP/PKA/CREB Hypothesis

As mentioned previously, one of the most rigorously investigated problems in neuroscience is to decipher the mechanisms by which experience-induced changes in the brain will lead to learning and memory. The most intensely studied aspect of this has been the cellular and molecular cascade responsible. In addition the field of olfaction has experienced explosive growth over the past decade toward understanding the molecular

events underlying signal transduction (Wilson et al., 2004). In this section I will describe the intracellular and biochemical pathways and the molecules that have been shown to be involved in learning and memory (olfactory classical conditioning – early odor preference learning) in the olfactory system (OBs - to be precise) of rats with a focus on the cAMP/PKA/CREB hypothesis.

1.6.1.1.1.1 Cyclic Adenosine Monophosphate

In 1971 Earl Wilbur Sutherland won a Nobel Prize in Physiology or Medicine for his discoveries concerning the mechanisms of the action of hormones via second messengers namely cyclic adenosine monophosphate which is also called cAMP, cyclic AMP, or 3'-5'-cyclic adenosine monophosphate. Ever since its discovery, research has been done on the role of cAMP as an intracellular signal in associative learning. The first insights came from the works of Brunelli et al. in 1976 when they injected cAMP directly into pre synaptic cells of Aplysia and found that it produced pre synaptic facilitation (Brunelli et al., 1976). Today a growing body of evidence supports the hypothesis that the cAMP/PKA/cAMP response element binding protein (cAMP/PKA/CREB) cascade might be a universal mechanism underlying learning and memory (McLean et al., 1999).

cAMP is activated by norepinephrine (NE) via β-adrenoceptors (β-ARs), which critically mediate the US in early odor preference learning (Langdon et al., 1997; Sullivan et al., 2000). The specific role of cAMP in early odor preference learning was scrutinized in a study that proposed the interaction of noradrenergic and serotonergic input with odor occurs in the MCs of the OB through activation of cAMP (Yuan et al., 2003b).

Yuan and colleagues used selective antibodies and immunofluorescence imaging, and demonstrated that β-ARs and 5-HT (2A) receptors colocalize primarily on MCs. Using a cAMP assay and cAMP immunocytochemistry, they found that β-AR activation by isoproterenol and stroking, significantly increase bulbar cAMP. 5-HT depletion of the OB did not affect basal levels of cAMP but prevented isoproterenol-induced cAMP elevation (Yuan et al., 2003b). These results provide evidence that MC cAMP mediates the UCS. The authors suggested that the MC cAMP cascade converges with a Ca^{2+} pathway activated by odor (CS) to recruit CREB phosphorylation and memory-associated changes in the OB (Yuan et al., 2003b). So according to these data, β-AR activation and increases in MC cAMP mediate the UCS and converge with a Ca^{2+} pathway activated by odor (CS). Two years later, another study revealed a causal role for the cAMP increases in odor preference learning by manipulating cAMP levels with the phosphodiesterase IV inhibitor, cilomilast (McLean et al., 2005). Phosphodiesterase IV inhibitors prevent cAMP breakdown. The inhibitor converted a low learning-ineffective dose of isoproterenol into an effective UCS and cilomilast paired with peppermint odor prolonged memory at least 4 times longer than without the drug (McLean et al., 2005).

One interesting feature of cAMP is that it has been suggested to work in an oscillatory or pulsating pattern in early odor preference learning. The first suggestion that more than increased cAMP levels alone were implicated came from observations that higher than 2mg/kg doses of isoproterenol did not induce learning but did increase cAMP levels in the OBs (Yuan et al., 2003b). A few years later a study was done to measure

changes in cAMP in the OB prior to, during and following odor preference training in rat pups (Cui et al., 2007). The authors found that learning was associated with an increase in cAMP at the end of training (after 10 minutes), followed by a decrease 5 minutes later. In addition, they did not observe elevated cAMP when an ineffective isoproterenol dose that did not produce learning was used unless this ineffective dose was combined with a phosphodiesterase inhibitor, in which case they observed the pulsatile pattern in cAMP levels (Cui et al., 2007).

So thus far, we know that NE input, adrenoceptor activation, 5-HT input, and Ca^{2+} influx through activation of glutamatergic receptors, and cAMP activation are all implicated in the intracellular signaling pathway in early odor preference learning. As mentioned above, the cAMP/PKA/CREB cascade has been shown to be critical in learning and memory in nearly all organisms as it is highly conserved among species. So the next molecule that I will be reviewing is PKA.

1.6.1.1.1.2 Protein Kinase A

Edwin Krebs and Edmond Fischer discovered PKA one decade after the discovery of cAMP by Sutherland. PKA is also known as cAMP-dependent kinase because its activity is dependent on the cellular levels of cAMP (Johnson et al., 2001), and its activation is the primary role of cAMP (Taylor et al., 1990; Dell'Acqua and Scott, 1997). Most of the actions of cAMP are mediated through PKA (Kandel, 2012). When the level of cAMP rises in cells, cAMP binds to the regulatory subunits of PKA, causing them to undergo a conformational change that frees the active catalytic subunits and allows them

to phosphorylate PKA substrates. In 1992 Krebs and Fischer won the Nobel Prize in Physiology or Medicine for this discovery.

To explore the role of PKA in memory, Abel et al. (1997) generated transgenic mice that express R (AB), which is an inhibitory form of the regulatory subunit of PKA. In these transgenic mice, hippocampal PKA activity was reduced, and LTP was significantly decreased. These deficits were paralleled by behavioral deficits in spatial memory and in long-term, but not short-term, memory for contextual fear conditioning (Abel et al., 1997). These results provided evidence for a role of PKA in memory especially in the consolidation of LTM.

This specific feature of PKA has also been investigated in our model of early odor preference learning in rat pups. Intrabulbar infusions of the PKA antagonist (Rp-cAMPs) prevent odor preference learning and CREB phosphorylation, which is consistent with a causal role for the cAMP/PKA/CREB cascade (Grimes et al., 2012). Pairing of the PKA agonist, Sp-cAMP infusion in the OB with odor (CS) creates 24-hour odor preference memory (LTM) and it can be said that here PKA activation acts as the UCS. The results of this study also showed that activating PKA using the agonist Sp-cAMP would result only in LTM and not STM at 3 hours (Grimes et al., 2012). Thus consistent with the above-mentioned study by Abel et al. (1997) the cAMP/PKA/CREB cascade is selectively involved in late-phase synaptic plasticity and LTM.

The first sign of a preference memory for the conditioned stimulus (novel odor) was seen 5 hours following training (Intermediate-term memory). This suggests that PKA plays a causal role in intermediate-term and long- term preference memory in the rat pup

odor preference model, but not in STM. These data argue strongly for separate and parallel cellular memory mechanisms underlying normal learning (Grimes et al., 2012).

So in understanding the cAMP/PKA/CREB cascade in early odor preference learning, we have looked at and provided evidence for the involvement of cAMP and PKA. The next molecule that will be discussed in the following section is CREB.

1.6.1.1.1.3 cAMP Response Element-Binding Protein

In 1986, Marc Montminy and R.H. Goodman first defined a conserved DNA sequence in the promoter elements that are activated by cAMP, called cAMP Response Element (CRE). CRE is one of the DNA response elements contained within the control region of a gene. The binding of different transcription factors to these response elements regulates the activity of RNA polymerase, thereby determining when and to what level a gene is expressed (Kandel, 2012). In 1987 Marc Montminy and L.M. Bilezikjian described cAMP Response Element Binding protein (CREB) as a cellular transcription factor that binds CRE (Montminy, 1997). CREB functions as a transcriptional factor after it is phosphorylated.

More than a decade later after its initial discovery, CREB's role in LTM became evident by molecular manipulations of it (Tully et al., 2003). Many studies have provided evidence that agents that disrupt the activity of CREB block the formation of LTM, whereas agents that increase the amount or activity of CREB accelerate the process (Yin and Tully, 1996). For example, one study studied fear conditioning and water maze learning in mice with a targeted disruption of the alpha and delta isoforms of CREB and found that LTM was profoundly impaired and STM was normal (Bourtchuladze et al.,

1994). The authors also showed that LTP in hippocampal slices from CREB mutants decayed to baseline 90 minutes after tetanic stimulation but paired-pulse facilitation and post-tetanic potentiation were normal. These results provide evidence for the critical role of CREB in LTM.

The role of CREB in the early odor preference-learning paradigm has also received much attention. A significant increase in phosphorylated CREB (pCREB) has been observed in the OBs 10 minutes following olfactory conditioning training using stroking as a UCS (McLean et al., 1999) and isoproterenol as a UCS (Yuan et al., 2000). CREB activation also seem to be temporally focused; as mentioned, a significant increase is observed 10 minutes following training, after 30 minutes elevated levels can still be observed, and at 60 minutes it is not seen anymore (McLean et al., 1999; Yuan et al., 2014).

Results from a Yuan et al. (2000) experiment revealed that pCREB levels are correlated with learning, when a higher than optimal dose of isoproterenol was used, learning did not occur and an increase in pCREB levels was not seen. In addition, in another scenario when learning was prevented by selective 5-HT depletion in the OBs, it could be restored by a higher dose of isoproterenol and in this case an increase in pCREB levels was observed. These results provide additional supporting evidence for a role of pCREB in olfactory learning and are consistent with the phrase that CREB is a memory gene.

One interesting feature of CREB workings in the olfactory learning studies is that it exhibits an inverted U-curve effect, meaning that it is effective when an optimal amount is activated (not less and not more) (Yuan et al., 2003). This finding came from

experiments that used a Herpes simplex virus expressing either CREB (HSV-CREB) or a dominant-negative mutant CREB (HSV-mCREB). As expected, injection of HSV-mCREB prior to training prevented learning but unexpectedly, injection of HSV-CREB increased pCREB levels and interfered with learning as well. The authors argue that it is unlikely that such exaggerated CREB levels would occur in normal rat pups (Yuan et al., 2003).

Although the focus of this thesis project is on olfactory memory, it is important to note that CREB-dependent gene expression has also been implicated in other types of memories, such as learned fear in the amygdala (Josselyn et al., 2001, 2004), and other forms of learning- related plasticity in the cerebellum and diverse regions of the cortex (Ahn et al., 1999; Barth et al., 2000; Pham et al., 2004).

So data from the associative olfactory learning model is consistent with the hypothesis that CREB and its associated cAMP/PKA/CREB cascade play central roles in learning and memory.

1.6.1.1.1.4 Exchange Protein Activated by cAMP

The complexity of the intracellular pathway to learning and memory does not stop at the cAMP/PKA/CREB cascade. There are other molecules that get activated in this process and that have been proven to be essential, some only in short-term and some only in LTMs. In 1998, exchange protein activated by cAMP (Epac), also known as cAMP-guanine exchange factor (cAMP-GEF), was identified in an attempt to understand the mechanism involved in the cAMP-dependent activation of the small GTPase Rap1 that was independent of PKA (de Rooji et al., 1998).

Ever since its fairly recent discovery, many studies have focused on the role of Epac in neural plasticity and learning and memory. Using pharmacologic and genetic approaches to manipulate cAMP and downstream signaling, Ouyang et al. (2008) demonstrated that Epac signaling is required for hippocampus-dependent memory retrieval. Another study using mice and pharmacological manipulations provided evidence for the role of Epac in memory. In this study using the Epac agonist 8-pCPT-2'-O-Me-cAMP it was shown that activation of Epac within the hippocampus enhanced LTM in a PKA independent fashion (Ma, 2011). The author also provided evidence for a correlation between impaired memory and decreased Epac activity when the animals were injected with Epac2shRNA adeno-associated virus (Ma, 2011).

Epac signaling has recently been studied in the early odor preference-learning paradigm. Grimes et al. (2015) examined the process using the same agonist (8-pCPT-2-O-Me-cAMP) infused into the OBs of rat pups and paired this activation with novel odor input. The authors showed results similar to Ma's (2011) findings. The pairing of Epac activation and novel odor resulted in long and short-term learning of a preference for the novel odor.

The authors also found that normal odor preference training led to an increase in extracellular signal-regulated kinases (ERK) phosphorylation, which has been shown to be consistent with a role for Epac activation in normal learning (Grimes et al., 2015). Further, it was shown that using an ERK antagonist spared short term and prevented intermediate and LTMs (Grimes et al., 2015). The authors suggest that Epac and PKA play parallel and independent, as well as likely synergistic, roles in creating cAMP-

dependent associative memory in rat pups and that there might be a novel ERK-independent pathway in the mediation of STM by Epac.

1.6.1.1.2 Memories of Various Durations

With duration as a criterion at least 2 types of memories have long been recognized: STM and LTM. As the names imply, STM is generally defined as the temporary storage of information (demonstrating temporal decay) and LTM is thought of as a more permanent mode of information storage. Neuroscientists investigating this subject more deeply have discovered a physiological difference between these two storage mechanisms. It is now accepted that a major difference between these two forms of memory is the synthesis of new proteins. As mentioned in previous sections, Ca^{2+} influx initiates the cascade that leads to long lasting cellular changes. One of the steps to the formation of these long lasting changes is the production of new proteins in the cell soma and integration of these proteins at stimulated synapses.

Many lines of research have shown that LTM for hippocampus-dependent contextual fear conditioning is critically dependent on protein synthesis (Flexner et al., 1965; Abel et al., 1997; Bouchouladze et al., 1998; Schafe et al., 1999). Similarly, studies have also shown that in hippocampal area CA1, maintenance of the late phase of hippocampal LTP (L-LTP) (which as I explained is the underlying mechanism for LTM) is also dependent on protein and RNA synthesis (Frey et al., 1988; Nguyen et al., 1994).

In the same way, decades of research have shown that induction of STM does not depend on protein synthesis (Davis and Squire, 1984; Goelet et al., 1986; Houpt and Berlin, 1999; Nader et al., 2000). A recent study examined this notion in an associative

olfactory memory context. Guerrieri et al. (2011) investigated whether the memories formed upon olfactory learning that are retrievable several days after training are based on protein synthesis. The authors addressed this question in the ant Camponotus fellah using a conditioning protocol in which individually harnessed ants learned an association between odor and reward. They showed that LTM consolidation was impaired by the ingestion of cycloheximide, a protein synthesis blocker, prior to conditioning. However cycloheximide did not impair STM.

Another study also investigated this concept in associative olfactory memory but in mammalian system. Grimes et al. (2012) used anisomycin, a translation inhibitor, and actinomycin, a transcription inhibitor to disrupt the cAMP/PKA-dependent odor preference memory in neonate rats. When these drugs were infused into the olfactory bulb after the pairing of peppermint odor and the β-AR agonist isoproterenol (2 mg/kg), STM (1 or 3 hours) was intact, but LTM (24 hours) was disrupted. In the current project, LTM is considered to be 24 hours and STM 3 hours as well. These results all point to the fact that STM and LTMs are dissociable and that a major difference lies in the synthesis of new proteins during LTM formation.

1.7 Olfactory Circuitry

The olfactory system contains several components including the ORNs, ON, OBs, and piriform cortex among others. Signal transduction starts when odor molecules (odorants) from the environment interact with ORNs in the nasal cavity in an area called the olfactory epithelium. The olfactory epithelium contains several million ORNs (Morrison and Costanzo, 1990). Dendrites of ORNs end in cilia projections that contain

ORs. ORs are a form of GPCRs that bind with odor signals (Jones and Reed, 1989). The axons of ORNs form bundles which together are called the ON (Price and Sprich, 1975). ORN axons project from the olfactory epithelium through the cribriform plate and terminate in the OB (Pinching and Powell, 1971a; Morrison and Costanzo, 1990).

1.7.1 Olfactory Bulb

The mammalian brain contains two OBs. Each OB has several layers. From the exterior of the OB moving inward, the layers of the OB are the ON layer, the glomerular layer, the external plexiform layer (EPL), the MC layer, the internal plexiform layer (IPL), the GC layer and the subependymal zone (Price and Powell, 1970a; Price and Powell, 1970b; Pinching and Powell, 1971a; Pinching and Powell, 1971b; Pinching and Powell, 1971c). Multiple studies have shown that direct infusion of drugs into the OB affects early odor preference learning (Sullivan et al., 1989a; Wilson and Sullivan, 1994; Sullivan et al., 2000b; Christie-Fougere et al., 2009; Grimes et al., 2012; Lethbridge et al., 2012). These results led Sullivan to suggest that the OB was both necessary and sufficient for early odor preference learning (Sullivan et al., 2000b).

MCs are the largest cell type in the OB (Price and Powell, 1970a). MCs are also the primary output neuron of the OB. MC axons from the OB come together with tufted cell (TC) axons from the OB and MC axons from the accessory OB (AOB) to form the lateral olfactory tract (LOT) on the olfactory peduncle (Price and Sprich, 1975). The LOT projects to sub-cortical and cortical structures (for review see: Haberly, 2001). These include, but not limited to, the anterior olfactory nucleus (AON), the piriform cortex, the olfactory tubercle, and the entorhinal cortex, which together form the olfactory cortex.

1.7.2 Piriform Cortex

As already stated, odor information is transmitted from the OB via the LOT to the piriform cortex. The piriform cortex is a three-layered structure; layer I is subdivided into two layers with physically isolated inputs. Layer Ia receives afferent inputs from the LOT and layer Ib receives associational cortical inputs. Layers II and III contain pyramidal cell bodies. The piriform cortex is divided into an anterior portion, aPC, and a posterior portion, posterior piriform cortex (pPC). Kucharski and Hall were the first to provide evidence for cortical involvement in early odor preference learning (Kucharski and Hall, 1987). About almost two decades later Sullivan and colleagues presented more evidence for this notion by showing increased c-fos activation in both the OB and the aPC following odor preference training in rat pups (Roth et al., 2006). More recently, Yuan and colleagues also provided conclusive evidence for a role of aPC by demonstrating that transient silencing of the aPC using either lidocaine or muscimol during training prevents the expression of an odor preference memory acquired with a fully functional OB (Morrison et al., 2013). Pairing adrenoceptor activation in aPC and odor can induce early odor preference memory. These results provide evidence that an odor preference memory itself can be created in the piriform cortex.

1.8 Hypothesis

It is natural to think of ‘memory formation’ in one’s chosen model as a discrete set of interacting events triggered at a particular moment in time. However reality is otherwise, with memorable events happening before and after others. The memorability of an apparently isolated episode is likely to be affected by what has happened before that

episode (Redondo and Morris, 2011). With this notion in mind, and having gone over the basics of intracellular cascades involved in learning and memory in the early odor preference paradigm, and the synaptic tagging and capture hypothesis, an intriguing question arises: Can we find evidence for the hypothesized existence of synaptic tagging in this associative learning paradigm?

What has been lacking in the literature is a behavioral model to show the workings of CaMKII as a synaptic tag in a learning and memory paradigm other than hippocampal-dependent paradigms. The hippocampus and electrophysiological work on hippocampal slices has been an excellent model by which to elucidate the role of CaMKII as a synaptic tag in LTP, the question remains as to whether this form of plasticity and synaptic tagging occurs in brain regions other than the hippocampus. In addition, in studies of olfactory associative memory, the puzzle of how one odor is specifically associated with an event needs more investigation. In other words, the mechanism that leads to input-specificity in olfactory associative learning is vague and begs for more scrutiny. Therefore, this project aimed to solve these shortcomings by examining the specific role that CaMKII plays as a synaptic tag in an olfactory associative memory model (early odor preference learning) in rats.

We hypothesize that CaMKII mediates input-specific early odor preference learning in rat pups. So the main goal of this project is to decipher the role of CaMKII as a synaptic tag in early odor preference learning in rats. To do so, firstly we asked whether blocking CaMKII impairs short term as well as LTM of rats that have been trained in our early odor preference-training model. Secondly, we delve into the problem more deeply and hypothesize that if we activate PKA at the same time that we are blocking CaMKII,

LTM can be rescued. But two questions arise at this point and we ask would LTM be lost without CaMKII activity because newly synthesized PRPs have nowhere to be shipped? Or would input-specificity be lost because PRPs are shipped everywhere? We hypothesize that the latter is the case in the light of the study by Villers et al. (2014).

1.9 Review of Experimental Design

To answer the above questions our experimental design consisted of two parts. In the first part we test the role of CaMKII by pharmacological manipulations in our well-established behavioral paradigm early odor preference learning. First we infuse CaMKII inhibitor KN-62 directly into the OB of rat pups and observe the effects on their short- and LTMs for the trained odor. Then to see whether CaMKII does actually function as a synaptic tag to preserve input-specificity we activate LTM by infusing a PKA activator (as an UCS) and expose the pups to the odor, while at the same time we also block CaMKII (i.e., co-infusion of CaMKII blocker and PKA activator). Then we test the pups with a control odor, vanillin, to see whether input-specificity has been preserved or not.

In the second part of our study, we perform immunohistochemistry (IHC) on brain slices from rat pups that have been trained in our behavioral model. In this part we investigate the staining patterns of CaMKII and pCaMKII.

Chapter 2 - MATERIALS AND METHODS

2.1 Ethics Statement

All experimental procedures involving animals were approved by the Animal Care Committee at Memorial University of Newfoundland (protocol number: 15-01-QY) and adhered to the guidelines of the Canadian Council on Animal Care.

2.2 Animals

Male and female Sprague Dawley (Charles River) rat pups from PND 5 to 8 were used in this study. Total number of animals utilized was 91. Animals were bred and pups were born on-site at the Animal Care Facility of the Health Sciences Center at the Memorial University of Newfoundland. Breeding of the animals was done consistently on Monday morning of each week. The day of birth was assigned to be PND 0. On Mondays, after breeding was done, litters were culled to 12 pups with equal numbers of males and females (6F+6M). Dams were maintained under a 12:12 h reverse light/dark cycle with the light phase beginning at 8:00 AM and dark phase beginning at 8:00 PM. Animals had unrestricted access to food (standard rodent diet) and water.

2.3 Behavioral Studies Overview

In the behavioral portion of this study, the standard protocol of early odor preference learning (previously established by Sullivan and Leon, 1987; McLean et al., 1999) was used. According to this protocol, the groups were either exposed to odor only (O/O) or they were exposed to odor while receiving stroking at the same time (O/S).

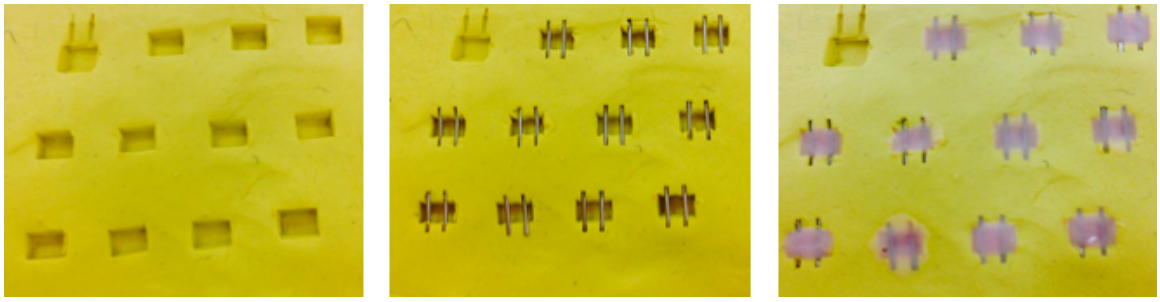
Three different experiments were conducted to examine the specific role that CaMKII has on STM, LTM and input-specificity. In the first experiment, STM was examined 3 hours following training and the intracranial infusion of either vehicle or the drug (CaMKII antagonist). In the second experiment, LTM was tested 24 hours after training and infusion of either vehicle or the drug. The third set of experiments, in which input-specificity was tested; pups were infused with vehicle, the CaMKII antagonist, or a combination of the CaMKII antagonist and a PKA agonist. These pups were trained with a specific odor (peppermint) and were later tested with peppermint as well as a control odor (vanillin). One-way ANOVAs were used to determine the statistical significance of the results.

In the following sections I will outline the specifics of how this behavioral model and testing was done. The first step was to make cannulae, perform surgery on pups and implant the cannulae on PND 5. The second step (the next day) was to prepare the drugs, infuse them into the OBs of pups, and train them in the behavioral model. The third step was to test their memory (their preference) for the trained odor.

2.4 Cannula Production

For this project, cannulae were made in the laboratory (Figure 1). In order to create a negative mold, a lump of commercial modeling clay was flattened and using a guide cannula (a ready-made cannula) imprints were made into the clay. For each cannulae set 2 pieces of stainless steel tubing (Vita Needle Company: Needham, MA, USA; 23 gauge – outer diameter 0.025”, inner diameter 0.013”; 6 mm length) were placed in the imprints, parallel to each other. Dental acrylic (Lang Dental: Wheeling, IL, USA) was used to fill

the mold (a small hollowed rectangle around the imprints) and was left to dry for 30 minutes. After 30 minutes cannulae in the molded acrylic block were removed from the clay mold using forceps and washed with warm water. Cannulae were then placed in a beaker with distilled water and sonicated for about 10 minutes. Using a drill, excess dental acrylic was trimmed off and insect pins were inserted into the cannulae to prevent blocking.



A. **B.** **C.**

Figure 1. Custom Made Cannulae. A. Imprints made into clay using a guide cannula. B. Stainless steel tubing placed in parallel 2 millimeters apart. C. Dental acrylic was used to fill up the hollow area and also covered the central portion of the stainless steel tubing.

2.5 Intracranial Cannulae Implantation

This surgical procedure was always done during approximately the same time of the day (starting in the mornings at 9 AM) and at the same location, on the laboratory's surgery table (Figure 2). On PND 5, the dam was brought upstairs and pups underwent the surgery one by one. Pups were numbered with a permanent marker and anesthetized

by insertion in ice for approximately 10 minutes (hypothermia). Pups were placed in a stereotaxic apparatus with the skull horizontal and fixed firmly (Figure 3). During the first 15 minutes of the procedure, ice was placed on the pup's body to avoid the body heating and the pup waking up. After fixing the skull in place, a horizontal incision was made using a #10 blade, from posterior of bregma to just posterior to the nose. Using two sutures, the skin of the skull was retracted, so the skull was made visible and enough working space was available. Using glue, a plastic screw was placed firmly just anterior to bregma and two points were marked on the two OBs using a permanent marker. The distance between the two points was approximately 2 millimeters. Using a drill, two holes were made at the marked points on the OBs. The cannulae were then lowered to the surface of the skull and inserted in the holes. Dental acrylic was used to fix the cannulae to the skull. After approximately 3 minutes when the dental acrylic hardened, pups were removed from the stereotaxic apparatus. One suture was made anterior to the cannulae, and three posterior to the cannulae (Figure 4). Pups were then placed on warm bedding for approximately 30 minutes to recover before being placed back with the dam. The whole process from start of hypothermia to placing the pups on the warm bedding did not take more than 40 minutes. Thirty minutes after finishing the last surgery, all the pups would be back with the dam. The dam was then taken downstairs to the animal care facility and pups were allowed to recover from this surgery for 24 hours.

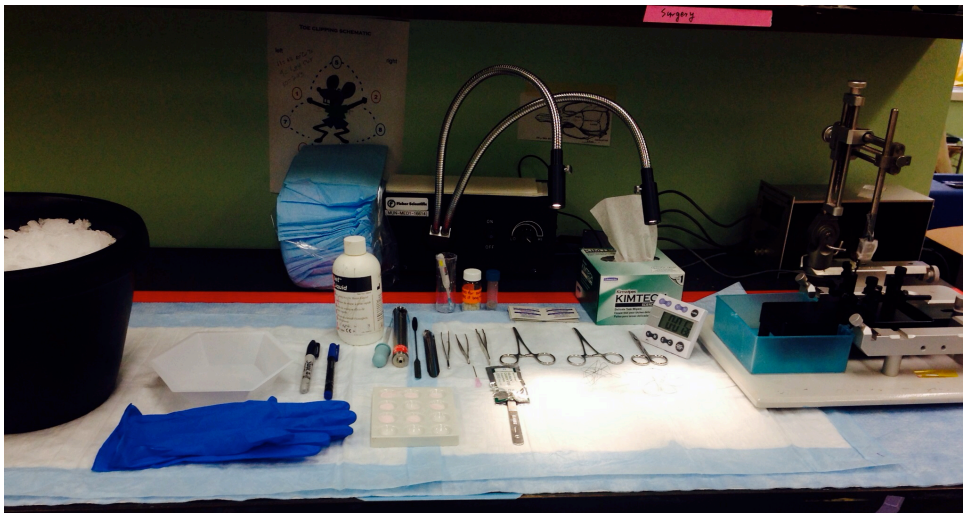


Figure 2. Laboratory's Surgery Table and Set up

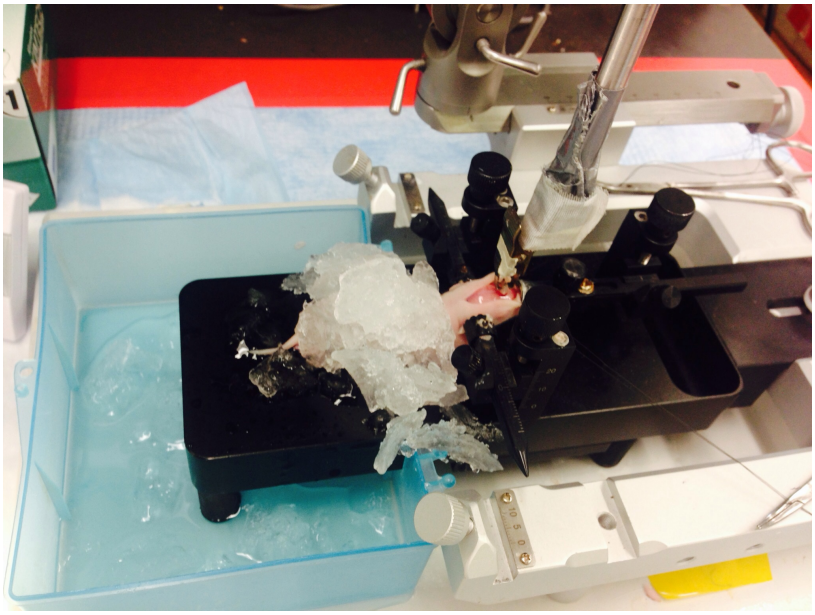


Figure 3. Cannulae Implantation Surgery

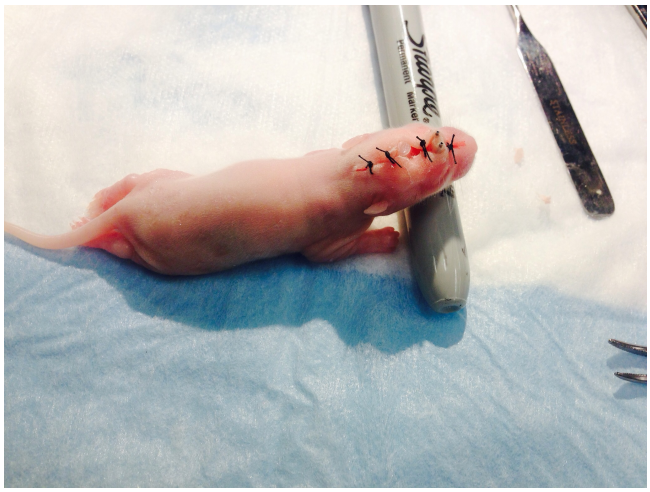


Figure 4. Pup with Implanted Cannulae After Surgery

2.6 Drugs

For this project we used 3 different drugs or mixtures (KN-62, Sp-cAMP, and KN-62 + Sp-cAMP). These 2 drugs were both purchased from Tocris Bioscience (Bristol, United Kingdom). To block CaMKII we used the specific CaMKII inhibitor KN-62 (Ito et al., 1991). KN-62 interacts with the regulatory domain of CaMKII (Tokumitsu et al., 1990) to block the active form of CaMKII (Kim et al., 2006). Each pup received bilateral1µl infusions of 3.6 µg/µl KN-62, dissolved in 75% dimethyl sulfoxide (DMSO) and 25% saline into each OB for a total dose of 7.2 µg of drug (Vaynman et al., 2006). To activate PKA we used the specific PKA activator Sp-cAMP. Each pup received a 1µl infusion of 18 µg/µl Sp-camp dissolved in saline into each OB for a total dose of 36 µg of drug (Grimes et al., 2012). The third type of drug was a mixture of the two previous drugs

(KN-62 + Sp-cAMP) in the same concentrations dissolved in 65% DMSO and 35% saline. The corresponding vehicles were infused into control pups.

2.7 Intracranial Pharmacological Infusion

Twenty-four hours following surgery (on PND 6) and 10 minutes prior to odor training, pups received pharmacological infusions. The infusions were either CaMKII blocker (KN-62), Sp-cAMP (PKA activator), a combination of the two drugs (Sp-cAMP + KN-62) or the vehicle. The above-mentioned drugs were prepared in the laboratory and maintained on crushed ice. The dam was taken to the room and pups were taken out of the cage and infused one by one. Pups were placed on a plastic weighing dish, insect pins were removed, and they were then infused with drug or vehicle at the rate of 1 μl/min for a total of 1 μl (Figure 5). In order to confirm patency of the cannulae, after each infusion the amount of liquid that had come down the tubes was marked. Pups that had cannula blockage were eliminated from the experiment. After the infusion, pups were allowed to rest in a cage with neutral bedding for 10 minutes before training. This allowed the drug to infuse completely into the brain and also allowed the pups to recover from the stress associated with the infusion procedure. The exact time of each infusion was noted so the training and testing time points were precise.

Figure 5. Drug Infusion

2.8 Odor Training

Peppermint scented bedding was prepared just prior to training with 0.3 ml of peppermint (using a syringe labeled for peppermint) and 500 ml of unscented bedding mixed together in a plastic bag and placed in a peppermint-designated box. Ten minutes following infusion, pups underwent odor training. Animals were assigned to two groups of odor only (O/O) or odor plus stroking (O/S). The O/S group is the classical conditioning group and the O/O is the control group. Pups in the O/S group were placed on the peppermint-scented bedding and stroked with a paintbrush for 30 seconds, followed by 30 seconds rest, alternating for a total of 10 minutes (Figure 6). Pups in the O/O group were placed on the peppermint scented bedding for 10 minutes but they did not receive stroking. After training was complete, pups were cleaned of any peppermint

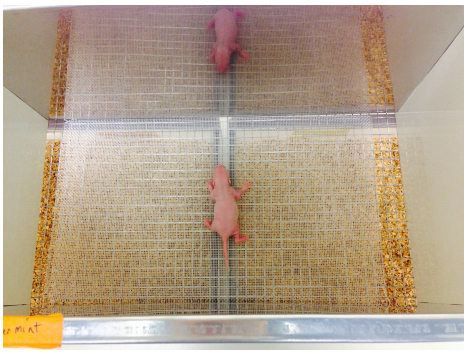
scented bedding on their skin and were returned to their home cage. In order to prevent odor contamination of the home cage, the dam and the home cage were placed in a separate room from the training room.. If a contamination happened, the results could be falsified by formation of associations of the peppermint scent with the mother's care, food, or the warmth of the cage.

Figure 6. Early Odor Preference Training. Photograph of stroking a pup on scented bedding.

2.8.1 Odor Testing (3 hours/short-term memory)

In the STM testing experiment, pups were tested for their odor preference 3 hours following training on PND 6. For this experiment, the pups were infused with either KN-62 (CaMKII blocker) or vehicle. The home cage was kept in the room for 3 hours to avoid additional stress to the dam (the stress of changing the environment from their usual

animal care facility to testing rooms, then back to the facility and again to the testing rooms). Prior to testing, two new bedding boxes (peppermint and normal) were prepared (with the same concentration of peppermint as used for training) and placed in a separate room from the dam. The precise time of training was noted and pups were tested at exactly 3 hours following training (Figure 7A). Prior to testing, the testing chamber (30 x 20 x 18 cm) was cleaned with 70% ethanol to remove residual odors from previous experiments. In the testing room, the two bedding boxes were placed on a desk, separated by 2 cm, which was designated as the neutral zone. The testing chamber was a stainless steel box placed over the scented- and unscented bedding boxes. Two mesh sheets were used on the bottom of the stainless steel testing chamber. Between each testing session, the mesh was rinsed with 70% ethanol to avoid any scent contamination associated with the previous pup such as urine. Pups were placed on the neutral zone in the stainless steel chamber (Figure 7B) and timed for 1 minute using 2 different timers to determine the duration they spent on the peppermint side versus the normal bedding side (measured in seconds). After 1 minute, pups were removed from the testing chamber and allowed to rest for 30 seconds. The sequence was repeated for a total of 5 trials. The orientation of the pup was changed for each trial so that the initial facing directions were counterbalanced. The activity level of the pups was recorded for each trial on a 1-5 scale, 1 being the least active and 5 being the most active. Pups that had very low activity levels were eliminated from the experiment.



A. **B.**

Figure 7. Bedding boxes and Testing Chamber. A. Peppermint-scented box on the left and the normal bedding box on the right, separated by 2 cm (neutral zone). A separate timer is used for each side. B. The testing chamber placed on top of the two boxes and a rat pup placed in the neutral zone.

2.8.2 Odor testing (24 hours/long-term memory)

In this experiment, following training the home cage was returned to the animal care room until exactly 24 hours later. Pups were tested for their odor preference memory on PND 7. All other procedures and the infused drugs were the same as the 3-hour testing.

2.8.3 Odor testing (Input-specificity)

After completion of the short and LTM testing experiments, pup's odor memory was tested for its specificity (to test the role of CaMKII in input-specificity). In this experiment, the pharmacological manipulation had 2 additional elements. The groups

received either the vehicle, or the CaMKII inhibitor (KN-62), or the PKA agonist (Sp-cAMP) or a co-infusion of KN-62 and Sp-cAMP. Activating PKA in this case provides the role of the UCS. So in this series of experiments the pups were only exposed to the peppermint odor (CS) without receiving tactile stimulation.

Twenty-four hours following training, a group of pups was tested with peppermint (using the same procedural steps as the two previous experiments). Another group of pups was tested with a control odor, vanillin. Fresh beddings (scented and non-scented) were prepared just prior to testing. Vanillin-scented bedding was made using 0.6 ml vanillin and 500 ml bedding, mixed together in a plastic bag and placed in a box designated only for vanillin (to prevent any contamination with peppermint and falsification of the data). Testing procedure was identical with the previously explained testing method.

2.9 Cannulae Placement Verification

Following testing, all pups were brought upstairs to the lab to verify the location of the cannula and be sacrificed. One by one, pups received bilateral infusions of 4% methylene blue dye at a rate of 1 μl/min for a total of 1 μl, and were then anesthetized in the fume hood using isoflurane. Pups were sacrificed by decapitation 1-2 minutes following the dye infusion. Their brains were collected and the location of the cannula was verified by examining OBs for blue dye (Figure 8). Pups that had incorrect cannulae placements or did not have a blue spot (indicating cannulae obstruction) were eliminated from the experiment.

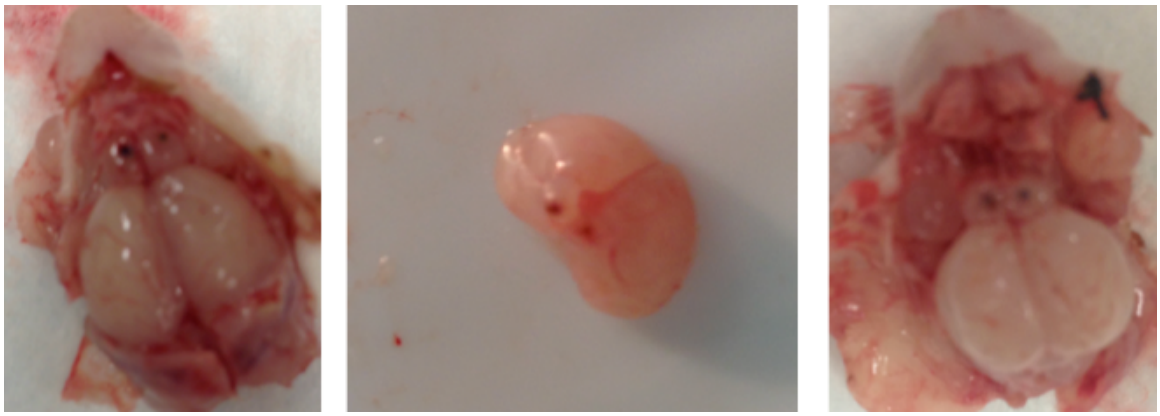


Figure 8. Cannula Placement Verification. Photographs of 3 different brains with correct cannulae placements.

2.10 IHC of CaMKII and pCaMKII

The second part of the methodology was performing IHC on the brains of pups that received training (O/S) and pups with no training, only odor exposure (O/O). Perfusions were done 10 minutes after the odor exposure or training was performed. CaMKII and pCaMKII primary antibodies purchased from Abcam (Cambridge, MA, USA) were used. Total numbers of animals used for IHC were 16. The groupings included were O/O with peppermint (n=9), O/S with peppermint (n=4).

2.10.1 Perfusions

Animals were deeply anesthetized and then perfused transcardially with ice-cold saline solution for 1 minute (until all blood was cleared) followed by perfusion of ice-cold fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) for about 30 minutes. Brains were then removed, post-fixed for 1 hour in the same fixative solution

and then immersed in buffered 20% sucrose solution overnight at -4C, and stored under these conditions until used for IHC.

2.10.2 Cryostat Sectioning

Sucrose-rich brains were mounted for slicing using a Cryomatrix embedding medium (Thermo Scientific: Marietta, OH, USA) and immediately placed in the cryostat at -20C until fully frozen. The OBs were cut into 30-micrometer coronal sections, collecting a slice every 200-micrometer. Slices were mounted on chrome-gelatin coated slides and kept inside the cryostat for the entire slicing period to keep them frozen. For each brain 2 chrome-gelatin coated slides were used, one for CaMKII and another for pCaMKII, about 10 slices on each slide. Slides were then removed from the cryostat and placed in a humid incubation box in the cold room (-4C) for 10 minutes before bringing them to room temperature.

2.10.3 Antibody application

Primary antibodies were made fresh and included CaMKII and pCaMKII (Abcam: Cambridge, MA, USA) with the concentrations of 1:1000 dissolved in phosphate buffered saline with 0.2% Triton-X-100, 0.02% sodium azide and 2% normal goat serum. Following the 10-minute period, the slides and box were removed from the cold room and were left to dry for about 8 minutes. When dried, the borders of the slides were marked with a PAP pen (Dako Pen – Dako: Glostrup, Denmark) to create a barrier between the label and the antibody solution. The box was then taken back to the cold room and the appropriate antibodies were applied to the slides. Water was poured in the bottom of the

box to prevent the antibodies from drying out. The box and slides were left covered and in a cold room overnight.

The next morning, after transferring the slides from the cold room to the laboratory (normal room temperature), the slides were washed 3 times for 10 minutes with phosphate buffered saline (PBS). Then the secondary antibody was made by mixing 10 ml of PBS plus 0.2% Triton X-100 with 150 µl of normal goat serum and 50 µl of biotinylated secondary antibody, goat anti-rabbit (Vectastain Elite, Vector Laboratories: Burlingame, CA, USA). The slides were incubated in this secondary antibody for one hour. Meanwhile, the avidin-peroxidase (A+B) (Vectastain Elite, Vector Laboratories: Burlingame, CA, USA) solution was made by adding 100 µl of A and 100 µl of B in 10 ml of PBS. Following incubation, the slides were washed 3 times for 10 minutes each with PBS, and then the A+B solution was applied for another 1-hour incubation period. The slides underwent another wash with PBS (3 times for 10 minutes). The next step was to make up the 3-3'-diaminobenzidine (DAB) solution by adding 50 mg of DAB to 50 ml of DI water in an Erlenmeyer flask and stirring until dissolved and while stirring, adding 50 ml 0.2M phosphate buffer and just prior to use, adding 30 µl of 30% H_2O_2.The slides were incubated with the DAB solution for 2-3 minutes (monitored under the light microscope for staining) before stopping the reaction with 0.1M phosphate buffer. After the staining step, the slides were washed with PBS 3 times for 10 minutes and dehydrated with progressive ethanol concentrations (50%, 75%, 95%, and 100%) and xylene and then cover-slipped with Permount (Fischer Scientific: Pittsburgh, PA, USA). The slides were left in the fume hood overnight to dry.

2.10.4 Imaging

Staining patterns were determined via a visual inspection of the treated slices. Photographs of the slices were taken through a Leica DMR SE upright microscope equipped with a DVC 1310 camera.

2.11 Statistical Analysis

OriginPro 9.0 Software (Originlab: Northampton, MA, USA) was used to analyse all the behavioral data. One-way ANOVAs and post-hoc Fisher tests were used to determine differences between the groups. The data are reported as mean±SEM. Significance was set at $p<0.05$.

Chapter 3 - RESULTS

3.1 Blocking CaMKII impairs short-term memory

To test whether blocking CaMKII would impair STM, the CaMKII antagonist, KN-62, or the vehicle (for control) was directly infused into the OBs of pups. Pups were divided into 2 groups of O/S and O/O and tested 3 hours later. A one-way ANOVA demonstrated significant group effects ($F_{(3, 27)} = 6.72$, $p=0.002$). As shown in Figure 9, pups in the O/O group that were infused with either the vehicle (41.86 ± 2.9%, n=7) or KN-62 (42.12 ± 5.71%, n=7) did not show a preference for the odor (peppermint). Pups that received training (i.e., O/S) and were infused with the vehicle showed a preference for peppermint (75.53 ± 5.42%, n=7), in other words their memory for the trained odor was intact. Pups that received training and were infused with KN-62 had impaired 3-hour memory (52.47 ± 6.73%, n=10). Post-hoc Fisher tests were done to determine the differences between the conditions. A significant difference was observed between the two groups of O/S + vehicle and O/S + KN-62 ($t = 2.9$, $p < 0.01$). These results show that blocking CaMKII results in an impairment of STM.

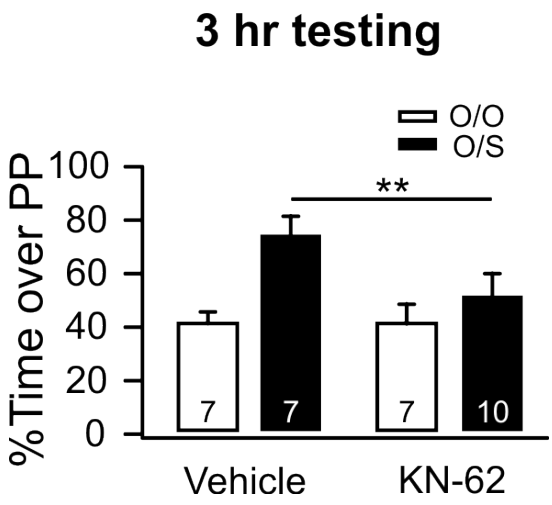


Figure 9. Blocking CaMKII Impairs Short-term Memory. Bars indicate the percentages of time spent in the peppermint side in a two-choice test box in different experimental groups. White bars indicate O/O group and black bars indicate O/S group. Error bars, mean ± SEM. Numerals on bars indicate number of animals in the group. PP on the vertical axis indicates peppermint. Animals that received a 2μl infusion of KN-62 + O/S at training spent significantly less time over the peppermint-scented bedding than the vehicle group when tested 3 hours following training.

3.2 Blocking CaMKII Impairs Long-term Memory

To test whether blocking CaMKII impairs LTM, the CaMKII antagonist, KN-62, or the vehicle was infused directly into the OBs of pups. Pups were divided into 2 groups of O/S and O/O and tested 24 hours later. A one-way ANOVA demonstrated significant group effects ($F_{(3, 16)} = 4.35$, $p=0.02$). As shown in Figure 10, pups in the O/O group that were infused with either the vehicle (38.41 ± 6.19%, n=4) or KN-62 (36.62 ± 5.77%, n=4) did not show a preference for the odor (peppermint). Pups that received training

(i.e., O/S) and were infused with the vehicle showed a preference for peppermint (62.12 ± 7.59%, n=6), in other words their memory for the trained odor was intact. Pups that received training and were infused with KN-62 had impaired memory (37.54 ± 4.21%, n=6). Post-hoc Fisher tests were done to determine the differences between the conditions. A significant difference was observed between the two groups of O/S + vehicle and O/S + KN-62 ($t = 3.05$, $p < 0.01$). These results show that blocking CaMKII results in an impairment of LTM.

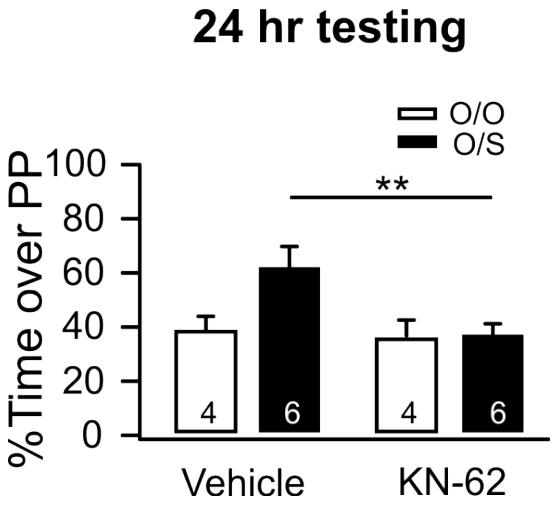


Figure 10. Blocking CaMKII Impairs Long-term Memory. Bars indicate the percentages of time spent in the peppermint side in a two-choice test box in different experimental groups. White bars indicate O/O group and black bars indicate O/S group. Error bars, mean ± SEM. PP on the vertical axis indicates peppermint. Numerals on bars indicate number of animals in the group. Animals that received a 2μl infusion of KN-62 + O/S at training spent significantly less time over the peppermint-scented bedding than the vehicle group when tested 24 hours following training.

3.3 Activating PKA acts as UCS and results in creation of memory

Consistent with Grimes et al. (2012) activating PKA acts as a UCS when rat pups are exposed to peppermint odor. In order to assess the input-specific role of CaMKII in LTM, we attempted to activate memory through a separate pathway that would not require synergistic activation of glutamate input and beta-adrenoceptors and we took advantage of the Grimes et al. (2012) finding. So in this experiment, we replicated the Grimes et al. (2012) work by infusing a PKA agonist (Sp-cAMP) and exposing rat pups to peppermint odor (O/O). We then tested the pups with peppermint scent to test their preference for peppermint. A one-way ANOVA demonstrated significant group effects ($F_{(2, 20)} = 13.76$, $p = 1.74E\text{-}4$). As Figure 11 shows (Sp-cAMP - white bar on the left), pups had a significant preference for peppermint when infused with Sp-cAMP (72.83 ± 5.24%, n=6) compared to control pups infused with the vehicle (39.35 ± 4.07%, n=6). Post-hoc Fisher tests were done to determine the difference between the conditions. A significant difference was observed between the two groups ($t = 4.85$, $p<0.01$).

3.4 Activating PKA Results in Creation of Specific Memory

Activating PKA (with Sp-cAMP) and exposing pups to peppermint scent results in a preference for peppermint and not other odors (e.g., vanillin). As already discussed and shown in Figure 11 (Sp-cAMP - white bar on the left), when pups were infused with Sp-cAMP and exposed to peppermint, and tested with peppermint they showed a significant preference (72.83 ± 5.24%, n=6) compared to the control group infused with vehicle (39.35 ± 4.07%, n=6). A post-hoc Fisher test demonstrated the difference between these two conditions ($t = 4.85$, $p<0.01$). But when pups were infused with Sp-cAMP and

exposed to peppermint and then tested with vanillin, which was the control odor, they did not show any preference for vanillin (34.46 ± 5.74%, n=6) (Sp-cAMP - black bar on the right) compared to pups that were infused with the vehicle (46.49 ± 8.11%, n=6). Post-hoc Fisher tests were done to determine the difference between these two conditions (t= 1.14, p>0.05). This suggests that activating PKA acts as an UCS for the specific odor (CS) that the pups were trained with.

3.5 Co-infusion of a PKA agonist with a CaMKII antagonist rescues LTM

So far the results show that infusion of CaMKII antagonist (KN-62) impairs LTM for the trained odor when trained with stroking, while infusion of a PKA agonist (Sp-cAMP) acts as UCS and creates a specific preference for the odor that pups were exposed to. At this point, we asked whether co-infusing PKA agonist (Sp-cAMP) with the CaMKII antagonist (KN-62) would prevent the loss of LTM caused by blocking CaMKII. As Figure 11 shows (Sp-cAMP + KN-62 - white bar on the right), when pups were infused with Sp-cAMP and KN-62 (co-infused) and exposed to peppermint odor, memory was preserved and pups showed a significant preference for peppermint (67.1 ± 4.54%, n=8) compared to pups that were infused with the vehicle (39.35 ± 4.07%, n=6). Post-hoc Fisher tests were done to determine the difference between the conditions (t = 4.27, p<0.01). Post-hoc Fisher tests showed no significant difference between the KN-62 + Sp-cAMP group (72.83 ± 5.24%, n=8) and the Sp-cAMP group (67.1 ± 4.52%, n=6) (t = 0.88, p>0.05), suggesting that activating PKA prevents the memory loss caused by CaMKII blocker given with stroking as the UCS.

3.6 Blocking CaMKII Leads to Losing Specificity – CaMKII as a Synaptic Tag

Next we asked the fundamental question of whether CaMKII acts as a synaptic tag to preserve input-specificity in our early odor preference-learning paradigm. To determine that, we exposed the rat pups to odor only (peppermint), infused with either the vehicle, Sp-cAMP, or Sp-cAMP + KN-62 mixture, and tested with the control odor, vanillin. A one-way ANOVA demonstrated significant group effects ($F_{(2, 17)} = 6.16$, $p<0.01$). According to the results shown in Figure 11, rat pups in the O/O + Sp-cAMP group showed no preference for vanillin (34.46 ± 5.74%, n=6). Pups in the O/O + vehicle group also showed no preference for vanillin (46.49 ± 8.11%, n=6). Post-hoc Fisher test results show that there was no difference between these two groups ($t = 1.14$, $p>0.05$). But, rat pups in the O/O + [Sp-cAMP + KN-62] group- black bar on the right, showed a significant preference for vanillin (68.26 ± 7%, n=8) compared to pups in the control group O/O + vehicle (46.49 ± 8.11%, n=6). Post-hoc Fisher tests revealed that these two groups had significantly different results ($t = 2.2$, $p<0.05$). Post-hoc Fisher tests also showed that there was a significant difference between the two groups of O/O + [Sp-cAMP + KN-62] and O/O + Sp-cAMP ($t = 3.42$, $p<0.01$). These results show that pups infused with vehicle have the same preference for vanillin as the pups infused with the PKA agonist, Sp-cAMP and trained with peppermint. This further suggests that activating PKA acts as a UCS for the specific odor that the pups have been trained on. The results also show that rat pups that had been infused with the Sp-cAMP + KN-62 mixture prefer vanillin as much as they prefer peppermint. This result provides evidence that co-infusing

PKA agonist with CaMKII antagonist permits LTM, but at the same time blocking CaMKII has the adverse effect of disrupting input-specificity.

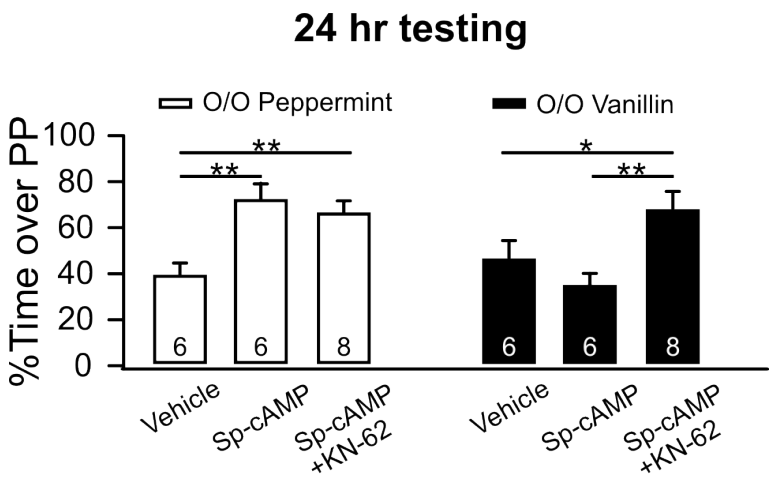


Figure 11. The Role of CaMKII in Long-term Memory Input Specificity. Bars indicate the percentages of time spent in the peppermint or vanillin side in a two-choice test box in different experimental groups. White bars indicate O/O group trained and tested with peppermint and black bars indicate O/O group trained with peppermint and tested with vanillin. Error bars, mean ± SEM. PP on the vertical axis indicates peppermint. Numerals on bars indicate number of animals in the group. Animals that received a 2μl infusion of Sp-cAMP + peppermint (O/O) at training spent significantly more time over the peppermint-scented bedding than the vehicle group when tested 24 hours later with peppermint following training. Animals that received a 2μl infusion of Sp-cAMP + KN-62 + peppermint (O/O) at training spent significantly more time over the peppermint-scented bedding than the vehicle group when tested 24 hours later with peppermint following training. Animals that received a 2μl infusion of Sp-cAMP + peppermint (O/O) at training spent significantly less time over the vanillin-scented bedding than the vehicle group when tested 24 hours later with vanillin following training. Animals that received a 2μl infusion of Sp-cAMP + KN-62 + peppermint (O/O)

at training spent significantly more time over the vanillin-scented bedding than the vehicle group when tested 24 hours later with vanillin following training.

3.7 IHC results show CaMKII and pCaMKII in the OBs

To visualize the expression of CaMKII in the OB, we performed IHC on OB slices harvested from rat pups at 10 minutes following training. Figure 12A shows a slice from a pup that had received O/S and with the CaMKII primary antibody. The staining pattern in the magnified area (Figure 12B) shows that CaMKII is especially present in the MC body in the MC layer, in the apical dendrites and EPL, as well as dense staining in the glomerular layer (arrows indicate MC body, apical dendrite and the glomerulus where the apical dendrite terminal tufts are located). Uneven staining was seen in the GC layer.

Slices for pCaMKII staining were taken from the same animals as for CaMKII. Figure 12C shows a slice from a pup that had received O/S training and a pCaMKII primary antibody. Figure 12D shows the staining pattern in the zoomed area. Similar to CaMKII, the pCaMKII expression appears to be especially present in MCs then in the glomerular layer and deep portion of the EP layer, respectively. Staining of pCaMKII was variable among the animals in both the O/O and O/S groups, so quantification of changes in CaMKII activation due to the training conditions (O/O vs. O/S) will be carried out by western blot (WB) in a separate cohort (see Discussion).

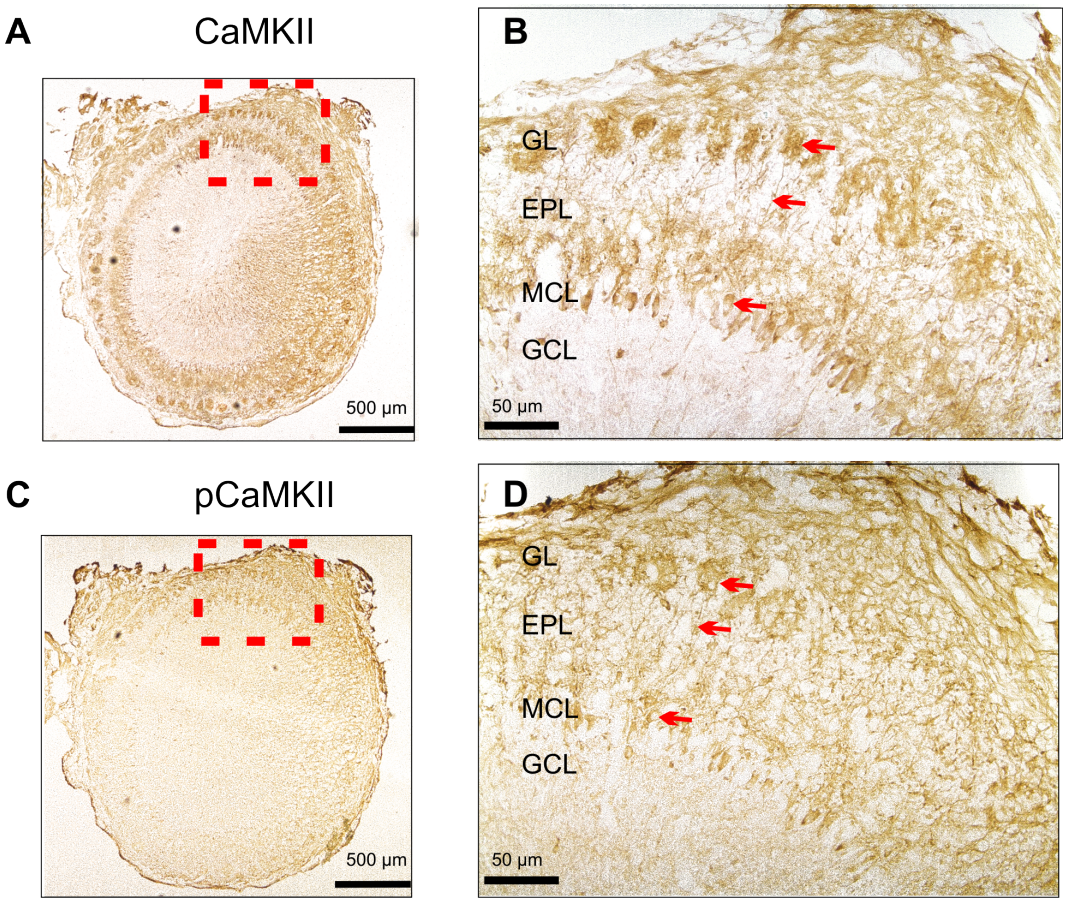


Figure 12. Immunohistochemistry Results Show CaMKII and pCaMKII in the Olfactory Bulbs. A. Slice from a pup OB that had received O/S and that was reacted with CaMKII primary antibody. B. Enlarged area indicated in A. The staining pattern shows that CaMKII is especially present in the MC layer and dendrites, and also in the glomerular layer. Minimal and uneven presence of staining can be seen at the GC layer. C. Slice from the same pup that was reacted with pCaMKII primary antibody. D. Zoomed image shows that pCaMKII expression appears to be especially present in MCs then in the glomerular layer and deep portion of the EP layer, respectively.

Chapter 4 – DISCUSSION

4.1 Gaps in the puzzle

One theme that has emerged from studies of synaptic tagging is that, just as multiple molecular mechanisms are involved in the production of LTP, there have also been multiple molecules and processes suggested to function as synaptic tags (structural changes, local translation, activation of different kinases, and several others). Each of these proposed synaptic tags might function under particular circumstances, and they might be differentially recruited by various stimuli and mediate plasticity over different time domains (Martin and Kosik, 2002). Decades of research has shown that CaMKII is one of the most important molecules in learning and memory. CaMKII fulfills the criteria for synaptic tagging as it is at upstream of some other proposed synaptic tags, its inhibition has been shown to cause memory deficits, and activating it has been shown to lead to memory formation. Overall, CaMKII can be called an ideal synaptic tag candidate. However an understanding of its specific working mechanism in a behavioral paradigm is lacking. Can this process be demonstrated in behavioral paradigms?

We attempted to address this question. Our results shed new light on the specific processes involved in neural plasticity and learning & memory.

4.2 Summary of Results

The present study sought to examine the role of CaMKII as a synaptic tag in early odor preference learning in rats. As reviewed, among the many molecules implicated in synaptic plasticity and synaptic tagging, CaMKII has been established as one of the most

important (Okamoto et al., 2009). CaMKII is highly abundant in the brain, especially in the postsynaptic density (Peng et al., 2004). CaMKII is a ubiquitous serine/threonine protein kinase involved in a wide variety of cellular functions (Colbran and Soderling 1990; Kelly, 1991; Lisman et al., 2002). NMDA receptor activation and the resultant Ca^{2+} influx trigger the activity of CaMKII and its autophosphorylation. The mobilized CaMKII can freely diffuse and phosphorylate other signaling targets and initiate multiple signaling pathways through phosphorylation of targets such as GluR1 or PSD-95 and through activation of the Rho family of small GTPases that regulate the actin filaments (Okamoto et al., 2009). Thus, CaMKII activation will also lead to remodeling of spine morphology. The presence of pCaMKII and its associated remodeled organization provides new binding sites for newly synthesized PRPs that can be captured at learning sites, sustaining the potentiated state for the long-term.

Our first goal in examining the role of CaMKII signaling in our behavioral model was to test whether disruption of CaMKII signaling in the OB would disrupt short-term and long-term odor preference memories. Our results support a normal role for CaMKII in both short and LTMs. Rat pups that had been infused with the drug KN-62 (CaMKII blocker) and trained with peppermint (O/S) did not show a preference for peppermint during testing both at 3 hours following training (STM) and at 24 hours following training (LTM). These results provide evidence that CaMKII is necessary for learning and memory in early odor preference learning and are in agreement with previous research using other paradigms (Silva et al., 1992; Giese et al., 1998; Wolfman et al., 1999; Vaynman et al., 2003).

Our second goal was to figure whether CaMKII confers input specificity in LTM. Grimes et al. (2012) showed that exposing rat pups to peppermint and activating PKA would lead to production of preference for peppermint (memory for peppermint) when tested 24 hours later. CaMKII is likely to have two roles in LTM. First, it would act synergistically with the cAMP pathway to promote UCS intracellular signaling and, second, it would confer input specificity. Direct PKA activation may bypass the requirement for synergistic cascades to promote memory, thus, this would give us an opportunity to activate LTM mechanisms directly via PKA, and look at the role of CaMKII only in the input specificity of the LTM.

We asked first what happens if we expose rat pups to peppermint, block CaMKII (with KN-62) and activate PKA (with Sp-cAMP), would LTM be lost because CaMKII is blocked? Or would LTM be intact because of direct PKA activation? So in the next set of experiments we infused a drug that was a combination of KN-62 and Sp-cAMP into the OBs of rat pups and exposed them to peppermint. Control groups consisted of a group of pups infused with vehicle and a group of pups infused with only the PKA activator. The pups were tested with peppermint. The results of this experiment were very interesting. As expected, we found that pups infused with vehicle did not show a preference for peppermint, and pups that were infused with only PKA activator showed a robust preference for peppermint (consistent with Grimes et al. 2012 study), however, pups that were infused with the KN-62 + Sp-cAMP drug also showed a preference for peppermint. These results provide evidence that activating PKA can directly induce LTM without the assistance of CaMKII. These results show that the normal role of CaMKII in creating memory can be by-passed by direct activation of PKA.

Next we asked if blocking CaMKII does not impair LTM when direct PKA activation is the UCS, can we show any role for CaMKII in this memory? The synaptic tagging hypothesis predicts input specificity would still be a function of CaMKII. If so, will input specificity be lost without CaMKII even in this model in which CaMKII is not necessary for engaging LTM processes.

The answer to these questions came from our next experiment. In this experiment we had the same control (vehicle and Sp-cAMP) and experimental (KN-62 + Sp-cAMP) groups. Training conditions were also the same as in the previous experiment in which rat pups were infused with either of the drugs and exposed to peppermint odor for 10 minutes. However this time, to check for input-specificity we tested the pups with a control odor (vanillin). So rat pups were trained with peppermint but tested with vanillin. The results of this experiment were fascinating. As expected, pups infused with vehicle did not show a preference for vanillin. Pups that were infused with PKA agonist (Sp-cAMP) also did not show a preference for vanillin because according to Grimes et al. (2012) activating PKA acts as UCS when the CS was peppermint and supports the associative memory for peppermint so it is logical that these pups did not show memory for vanillin. On the other hand, pups that were infused with the mixture drug showed a strong preference for vanillin. It is important to reiterate that these pups were trained only with peppermint, but still showed preference for vanillin. It can be inferred from these data that without CaMKII, and with PKA being activated, pups have memory for odors but not only the odor that they are supposed to remember (the odor they have been trained for). These results are very interesting in that they demonstrate precisely the role of CaMKII plays in input specificity.

Is input-specificity lost because newly synthesized PRPs have nowhere to be shipped, in other words no long-lasting changes or potentiation occured at any synapses? Or is input-specificity lost because newly synthesized PRPs are shipped everywhere since the tag (CaMKII) is lost? We argue in favor of the latter case because if the first case was true, pups would not show any preference for vanillin or any other odor (no memory), but our results showed that pups do show a preference for vanillin and peppermint.

Furthermore, we took the first steps towards understanding the cellular localization of CaMKII and pCaMKII in the OBs of rats following behavioral training and performed IHC. Both CaMKII and pCaMKII were observed in the MCs and their primary dendrites into the glomerular layer, while little staining was observed in the GC layer.

4.3 Results in Light of Previous Research

In this section I will review the literature and discuss our findings in light of previous research.

4.3.1 CaMKII in STM

It is generally accepted that STM is dissociable from protein-synthesis-dependent LTM (Goelet et al., 1986; Grimes et al., 2012). Previous research has shown that functional changes at synapses are required for production of STM. These changes comprise modification of pre-existing proteins and changes in the strength of pre-existing synaptic connections (Bailey et al., 2008). AMPA receptor modifications have been shown to be essential in mediating STM (Izquierdo et al., 1998; Sanderson et al., 2007; Cui et al., 2011; Sanderson et al., 2011; Taylor et al., 2011). The study done by Cui and

colleagues (2011) looked at AMPA receptor changes in the neonate rat using the early odor preference-learning paradigm. The results of this study revealed a significant increase in GluA1 subunits of AMPA receptors in OB glomeruli 3 hours following training and increased phosphorylation of GluA1 subunits from10-90 minutes after training. Sanderson et al. (2011) provided evidence that deletion of the GluA AMPA receptor subunit alters STM. Additionally, infusions of AMPA receptor antagonists have been shown to impair STM (Izquierdo et al., 1998). As discussed previously, one important molecule that has been shown to be playing an integral part of AMPA receptor modification and trafficking is CaMKII (Barria et al., 1997; Hayashi et al., 2000; Derkach, 2011; Opazo and Choquet, 2011; Sanhueza and Lisman, 2013).

Given the relationship between AMPA receptors, CaMKII, and STM, as explained above, one would expect that that inhibiting CaMKII would lead to disruption of STM and our results confirm this. In our model of early odor preference learning, blocking CaMKII with KN-62 leads to STM impairment.

Using transgenic mice, Wang et al. (2008) provides additional support for our argument. These authors tested STM in 3 different behavioral paradigms (novel-object recognition, contextual fear memories, and cued fear memories) and collectively with their electrophysiological data suggest that CaMKII activity plays an important role in STM. Another study that also provides evidence in favor of the involvement of CaMKII in STM is one by Rodrigues et al. (2004). The authors show that infusion of the CaMKII antagonist, KN-62, into the lateral nucleus of amygdala of rats prior to conditioning resulted in impaired short-term fear conditioning memories.

4.3.2 CaMKII in LTM

Considerable research exists to suggest that LTM formation is based on synaptic LTP (Bliss and Lomo, 1973; Bailey and Kandel, 1993; Bliss and Collingridge, 1993). CaMKII has been shown to be an essential component in maintaining LTP and the key experiments have been replicated in several laboratories using independent methods (Mallinow et al., 1989; Tokumitsu et al., 1990; Strack et al., 1997; Gardoni et al., 2000). Considering the foregoing, it is logical to draw the conclusion that inhibiting CaMKII would lead to impairment of LTM. Many lines of research provide evidence for this notion in many different learning paradigms (Elgersma et al., 2002; Irvine et al., 2006; Wayman et al., 2008; Lucchesi et al., 2011; Coultrap and Bayer, 2012). These results are in agreement with our finding that CaMKII blocks LTM in our early odor preference-learning model.

When examining this issue in more detail, we can suggest that a possible explanation for this finding (i.e., blocking CaMKII leads to blocking LTM) relates to the involvement of CaMKII in transcription, translation, and new protein synthesis. Evidence for the involvement of CaMKII in gene transcription comes from studies that blocked CaMKII with KN-93 and found that this blocking prevented CaMKII recruitment and the pCREB response (Wheeler et al., 2012; Ma et al., 2014). Although α and βCaMKII, which are the most dominant isoforms of CaMKII in the brain (Li et al., 2001), are not directly involved in gene transcription because they are not located in the nucleus (Giese and Mizuno, 2013), they do contribute to initiating the process of CREB phosphorylation and gene expression and activate cascades of molecules and events that are directly

involved with transcription (Ma et al., 2014). Other lines of evidence (using genetic and pharmacological approaches) suggest that one of the intermediate processes that shuttles CaM to the nucleus and activates transcription is the γCaMKII (Ma et al., 2014). Another molecule that has been postulated to be downstream of CaMKII activation and an up-regulator of gene transcription and translation is brain-derived neurotrophic factor (BDNF) (Blanquet et al., 2003; Redondo and Morris, 2011).

Our experiment however had an extra interesting dimension. We were able to obtain LTM in spite of blocking CaMKII, by activating another pathway at the same time. We co-infused CaMKII blocker and PKA activator and exposed rat pups to peppermint. Interestingly rat pups showed a preference for peppermint when tested 24 hours later. How can this be? We argue that CaMKII has a facilitative role in mediating LTM formation but directly activating later pathways that also lead to LTM formation can compensate for this function. In our model we activated the PKA pathway and we achieved LTM even though CaMKII was blocked.

One explanation for this outcome may relate to the common role of CaMKII and PKA in regulating proteasome activity. As mentioned before, proteasomes are required for synaptic plasticity (Hedge, 2004), maintenance of LTP (Fonseca et al., 2006) and formation of LTMs (Lopez-Salon, 2001). Evidence suggests that both CaMKII and PKA are capable of regulating proteasome activity through the phosphorylation of proteasome regulatory subunit Rpt6 at Serine-120 (Zhang et al., 2007; Djakovic et al., 2009). According to Upadhya et al. (2006) proteasome activity is higher at synapses than in the cytoplasm or nucleus and according to Bingol et al. (2010) and many other researchers

CaMKII is the most abundant postsynaptic protein. Thus it is likely that CaMKII is the primary regulator of proteasome-dependent synaptic plasticity but proteasome activity can be driven by PKA in the absence of CaMKII (Jarome et al., 2013). These results are in line with our finding that in the absence of CaMKII and when PKA is selectively activated, pups had intact LTM for the trained odor. Our results suggest that CaMKII is essential for LTM (i.e., impaired LTM when CaMKII was blocked) however, when CaMKII is blocked, and PKA is activated, PKA becomes the more forceful activator of proteasome activity, in other words PKA activation can overcome the loss of LTM normally caused by CaMKII inhibition.

Another explanation for this finding is that CaMKII and PKA work through pathways and strong activation of one (i.e., PKA) is sufficient to produce LTM even when the other one (i.e., CaMKII) is inactivated. Many studies have examined this mechanism of blocking one pathway can be overcome by over activation of another pathway. For example, Langdon et al. (1997) blocked 5-HT input to the OB, which blocked learning, but this was overcome by extra isoproterenol activation. Similarly, Grimes et al. (2015) blocked PKA but over activation with isoproterenol likely activated alternate pathways for learning.

As previously mentioned many studies have provided evidence that blocking either of these two leads to blocking LTM (Giese et al., 1998; Vaynman et al., 2003; Abel et al., 1997; Young et al., 2006; Grimes et al., 2012). There are also studies that have shown they work through independent mechanisms (Yao and Wu, 2001; Grimes et al., 2015). Yao and Wu (2001) using genetic and pharmacological analyses examined the roles of CaMKII and PKA in regulation of neuronal membrane excitability and K+ currents in

Drosophila neurons. According to these authors genetic perturbation of the catalytic subunit of PKA did not alter the action potential duration but disrupted the frequency coding of spike-train responses. In contrast, CaMKII inhibition lead to prolonged spike duration but did not affect the spike frequency coding. In addition, enhanced membrane excitability, was observed in CaMKII-inhibited but not in PKA-diminished neurons. These authors provided evidence that PKA and CaMKII have distinct effects in modulation of the kinetics and amplitude of different K+ currents, which leads to differential regulation of K+ channels. One recent study by Ghosh et al. (2015) used rats in an olfactory discriminatory task and provided evidence that using PKA inhibitors did not affect the IPSCs of pyramidal neurons of piriform cortex whereas manipulating CaMKII levels using the KN-93 inhibitor significantly affected them. Another study presented evidence that inhibiting CaMKII and PKA inhibits memory but they phosphorylate at different serine residues of GluA1, S831 and S845 respectively (Olivito et al., 2014). Therefore, putting all these pieces of information together we can say that CaMKII and PKA work on independent pathways, but strong activation of one could be sufficient for producing LTM as per the results of our experiment.

In vivo experiments provide additional support for our results. A study done by Yamagata et al. (2009) used αCaMKII mutant mice to examine long-term synaptic plasticity and behavioral learning. The authors used an IA learning model in which learning can be produced in a single trial. The results of this study show that no learning was established after one single training trial (as compared to control groups), however after repeated training trials these mice showed learning. These behavioral results were

correlated with electrophysiological results in which the mutant mice showed no LTP to a single tetanus but repeated tetani resulted in potentiation. This behavioral experiment can provide further support for our findings that blocking CaMKII can impair normal learning but selectively and strongly activating another pathway (either pharmacologically or through mass training) can rescue the lost LTM.

4.3.3 CaMKII as a Synaptic Tag

The influential study done by Villers et al. (2014) provides additional support for our argument that CaMKII acts a synaptic tag in preserving LTP's input-specific property. In this study they used αCaMKII-T286A mice (this mutation causes impairment in autophosphorylation) and investigated whether LTP can occur in these mice. The authors showed that in CA1 pyramidal cells of these mice, LTP induced after a recovery period in an interface chamber, is strictly dependent on postsynaptic αCaMKII autophosphorylation. However, the interesting part of their finding was that they demonstrated that αCaMKII-autophosphorylation-independent plasticity could also occur in the hippocampus but at the expense of synaptic specificity. This nonspecific LTP was observed in mutant and wild-type mice after a recovery period in a submersion chamber and was independent of NMDA receptors (an NMDA receptor antagonist was used). When slices prepared from mutant mice were pre-incubated with rapamycin, which has been reported to inhibit LTP through its action on mTOR-dependent translation (Gong et al., 2006), high frequency trains induced a synapse-specific LTP that was added to the nonspecific LTP. These results provide evidence that LTP (which as we discussed is the purported underlying mechanism for learning and memory) can be induced in mice with a

CaMKII mutation but this LTP is not specific to synapses that have already been stimulated. These *ex vivo* results are in complete agreement with the present *in vivo* results.

In an attempt to study the properties of bicuculline-induced aversive responses in rats, Okutani et al. (2002) found that infusion of the $GABA_A$ receptor antagonist, bicuculline, at a high dose into the OBs in the presence of an odor leads to developing aversive responses without sensory stimulation. Normally the acquisition of aversive learning requires pairing of an unfamiliar odor with foot shock, and this involves the disinhibition of the mitral/tufted cells induced by noradrenergic activation during the foot shock or the sensory stimulation (Okutani et al., 1999). The learning during this classical conditioning would be specific to the odor that the rats were exposed to during conditioning. The interesting part of Okutani et al.'s (2002) results was that after pairing bicuculline and odor, the learned response was not specific to the odor the rats were exposed to during conditioning. Their results showed that bicuculline infusion in the presence of an odor results in aversive responses to odors that have never been presented. These authors suggested that disinhibition of mitral/tufted cells from granule cells by bicuculline infusion makes rats aversive to strange odors non-specifically. This study was groundbreaking in the sense that it provided an insight into memory specificity and generalized learning. However, at the same time, it raised more questions regarding how it happens and what mechanism ensures memory specificity rather than generalized learning. Besides our IHC results, a study done by Zou et al. (2002) showed that CaMKII is selectively present in the GABAergic GC layer of the OBs. Infusing GABA receptor

antagonist drugs into the OBs can possibly disrupt the CaMKII functioning and lead to generalized learning.

Elgersma et al. (2002) showed, in a hippocampal-dependent behavioral paradigm, that αCamKII mutant mice could be conditioned but that they are unable to discriminate between conditioning and testing conditions. The authors used contextual fear conditioning. In this task mice learn to associate the context in which they are trained with a foot shock. Twenty-four hours later, they are placed back in the training chamber, and measuring freezing time assesses memory. The authors tested mice for their ability to discriminate between two similar chambers, one in which they receive a foot shock (chamber A) and another in which they do not (chamber B). Twenty-four hours after being shocked in chamber A, the mice were tested in both chambers A and B. Unlike wild type mice, mutant mice showed similar freezing responses in both chambers. The authors suggested that inactivation of αCaMKII autophosphorylation at T305 and T306 in mice does not impair contextual fear memory formation, but does block contextual discrimination. Based on these and other findings, Giese and Mizuno (2013) suggested that αCaMKII autophosphorylation is important for memory specificity. However, the study done by Elgersma et al. (2002) did not provide an underlying reason or explanation as to how CaMKII is involved in memory specificity. Our results can fill in these gaps by providing more clarification on how CaMKII preserves memory specificity.

Overall, our study provides *in vivo* evidence in a well organized behavioral paradigm for the conclusions of Villers et al. (2014) study and also presents further support for the Elgersma et al. (2002) and the Okutani et al. (2002) studies in a more

unambiguous, logical and clear way. In our study LTM is activated through activation of PKA, CaMKII is blocked (co infusion of KN-62 and Sp-cAMP), and rat pups that had been trained with peppermint show preference for the smell of vanillin. We propose the reason for this interesting finding to be that without CaMKII, input-specificity is lost and explain this further by arguing that CaMKII mediates the input-specificity. In this scenario CaMKII is impaired, or the tag is lost and therefore the input-specificity is lost.

At this point it is important to emphasize that synaptic tagging is much more complex than one single molecule being responsible for everything. Each of the suggested synaptic tags might function under particular circumstances, and they might be differentially recruited by various stimuli and mediate plasticity over different time domains. Looking at the concept from a broader perspective, we think that the molecular players and processes identified in this thesis document are all behind the neuronal algorithms that determine the persistence of synaptic and network changes and that synaptic tagging is actually a combination of structural changes and signaling molecules. However, in this project, and as others have suggested, CaMKII seems to have a particularly important role in plasticity and mediating memories because, as mentioned previously, CaMKII activation seems to be upstream of other synaptic tags.

4.3.4 CaMKII in IHC

Studies have been performed in this thesis in attempt to visualize the localization of CaMKII within the OBs. Prior odor learning experiments used [14C] 2-deoxyglucose (2-DG) within the OBs. Early experiments showed that pairing of a tactile stimulus and peppermint results in an increase in update of 2-DG in certain glomeruli (Sullivan and

Leon, 1986). Later, another study using the same technique showed that peppermint tends to produce dorsolateral labeling (Guthrie et al., 1990). A few years later, a study done by Johnson and Leon (1996) illustrated this concept more clearly. The authors mapped uptake throughout the GL in coronal sections of the OB. They showed that early preference training with peppermint increased 2-DG uptake in the posterior half of the mid-lateral bulb, whereas the ventro-lateral patches of uptake did not increase to the same extent.

Around the same time Mombaerts et al. (1996) used a genetic approach to visualize axons from olfactory sensory neurons expressing a given odorant receptor, as they project to the OBs. These authors presented evidence that showed odors activate distinct glomerular maps depending on odorant composition (Mombaerts et al., 1996). These studies all point to the likelihood that CaMKII activation within the OBs would be confined to a specific area or cell types. However, these results need to be complemented with experiments that measure protein levels using techniques such as western blots.

It has been previously reported that CaMKII mRNA levels are high in the MC layer and the GC layer in the OB and very high in layer II of piriform cortex (Benson et al., 1992). A study done by Zou et al. (2002) examined the normal expression patterns of the αCaMKII in the mouse OB. These authors showed that immunoreactivity to αCaMKII was heavy in the EP layer and the GC layer but minimal in the ON layer and the glomerular layer. At the cellular level, αCaMKII was selectively expressed in the granule cells but not in the periglomerular cells and no detection occurred in the mitral/tufted cells.

Our results seem not to be aligned with those of Zou et al. (2002) results, however many factors could account for this apparent discrepancy. Firstly, Zou et al. used a mouse model whereas ours utilized rats. Secondly, the age of their animals is PND 30 and 50, whereas our animals were 7 days old. Thirdly, our animals were trained in our behavioral model and exposed to peppermint odor prior to IHC and Zou et al. (2002) only examined the normal expression patterns. It will be beneficial to test the CaMKII expression with a different antibody and/or in mice as comparison.

Expression and the cellular localization of CaMKII and pCaMKII in the OBs of rats following behavioral training have not been previously investigated. In the present study we took the first step towards this understanding. The staining pattern of our IHC results show that CaMKII is present at the glomerular layer, MC layer, and at the dendrites of MCs. Similar patterns can be observed for pCaMKII.

4.4 Alternative Explanations

To broaden our perspective, we can look at other possible explanations for the outcomes of our study. One possible alternative could be that the drugs were infused in the OBs had spread to the level of ORNs. It has been shown that disrupting CaMKII using a specific peptide inhibitor, autocamtide-2-related inhibitory peptide, affects the ORNs (Leinders-Zufall et al., 1999). These authors propose that CaMKII controls the temporal response properties of ORNs during odor adaptation. Based on this, one might argue that the reason behind the impairment in memory specificity could be that the animals had trouble noticing the odors in the first place at the level of receptors, rather than encoding or retrieval during training and/or testing.

In 2008, Yamasaki et al. showed that αCaMKII is implicated in adult hippocampal neurogenesis and that this may regulate reorganization of LTM after contextual fear conditioning. Some might argue that this reorganization of memory is the reason that memory specificity is lost in pups that were infused with CaMKII blocker. This way of thinking, although not completely unreasonable, does not fit into our model of 24 hour testing because neurogenesis takes time. Therefore we doubt that the loss in memory specificity is due to an impaired neurogenesis and faulty reorganization of memory.

Some researchers provide another interesting explanation for memory specificity. When there is a talk of the September 11 attack, interestingly most people remember the exact place they were, or what exactly they were doing. Some memory researchers argue that this phenomenon is one specific type of autobiographical memory known as a flashbulb memory (Davidson et al., 2006; Hirst, 2009; Kvavilashvili et al., 2009), which is defined as a highly detailed, exceptionally vivid snapshot of a moment or circumstances in which surprising and consequential (or emotionally arousing) news was heard (Brown and Kulik, 1977). However, the concept of synaptic tagging provides a better explanation as to why we remember a lot of details about a specific day in the distant past (Clopath, 2012). According to this point of view, researchers argue that people tend to remember a lot of details *if* a strong event happens at the same time. This is similar to tagging experiments, where a weak induction alone (a regular day in the distant past) does not lead to consolidation unless paired with a strong induction (attack on the twin towers) in another pathway.

The findings of the present study and our proposed reasoning attempted to fill one of the biggest gaps in understanding the role of CaMKII in memory specificity. The

results provide us for the first time with direct evidence that CaMKII has an essential role in odor memory specificity and that without it, the ability to discriminate odors that are associated with different events would be lost. It is also the first time that such an investigation of synaptic tagging has been done in an appetitive behavioral paradigm that is free of other biases such as other cues to the subject and additional stress. Studies done on the hippocampus are not free of these extraneous biases (Kim et al., 2006).

4.5 Caveats of the Current Study

The main caveat of this study is that the IHC images and experiments are not complete. The IHC images of the O/O group are not included because staining in the O/O group was sufficiently variable that we could not argue differences in level of CaMKII and pCaMKII expression levels between the two groups. Also, our IHC images simply show the staining pattern of CaMKII and pCaMKII. As mentioned before, they only set the stage for more investigation regarding scrutinizing the localization of these enzymes following behavioral training.

4.6 Future Directions

In future, it will be interesting to test a few things in regards to CaMKII and memory formation. Performing IHC on brain slices harvested from pups that have been trained with vanillin can provide additional confirmation that activation of CaMKII is odor input-specific (through comparing the results with slices harvested from pups that have been trained with peppermint). This would strengthen our argument that CaMKII

activation is synapse-specific and it is in fact a synaptic tag mediating LTP and LTM formation.

To provide support for the activation of CaMKII following training and also to demonstrate the exact time window of this activation, WB experiments at different time points following training will be carried out. Providing the time course of activation in this paradigm can be a valuable proof for CaMKII as a synaptic tag as one of the key features of synaptic tagging is the lifetime of the tag. According to the synaptic tagging and capture hypothesis the tagged state of a synapse should have a limited lifespan (Frey and Morris, 1998).

AMPA receptor insertion has been shown to be one of the main products of CaMKII activation and memory formation (Hayashi et al., 2000; Zhu et al., 2002; Derkach, 2011). WB can also be done to test AMPA receptor insertion for the O/O + vehicle, O/S + vehicle, O/S + KN-62, and O/O + KN-62+ Sp-cAMP groups to determine the more downstream effects of CaMKII activation.

Another step that can be taken to provide added support for our results would be to add an experiment in which CaMKII is activated but PKA is blocked and pups are trained in our behavioral model. Could activating CaMKII compensate for the inactivation of PKA? Adding this extra experiment would help us better understand the workings of PKA and CaMKII.

Next, it would also be interesting to see how incorrect reorganization (following inactivation of CaMKII) of the brain could lead to impairments in memory specificity, possibly through looking at F-actin. The results could potentially further support our argument in favor of CaMKII as a synaptic tag.

As discussed before, NMDA receptor and CaMKII interactions are required for LTP maintenance (Sanhueza et al., 2011). It will be useful to see how impairment in this interaction affects impairments in memory storage and/or memory specificity.

Lastly, it is important to note that there appears to be a link between CaMKII dysfunction and memory impairment in certain diseases, for example, αCaMKII dysfunction has been shown to cause learning deficits in Alzheimer's disease models (Zeng et al., 2010; Reese et al., 2011). Another study done by Van Woerden et al. (2007) showed that in a mouse model of Angelman syndrome, restoration of dysfunctional αCaMKII by increasing phosphorylation at T305/T306 rescued hippocampal learning impairments.

Along these lines, it would be interesting to study the memory specificity of rats with these diseases in our behavioral model. Understanding what precise role CaMKII plays in memory specificity, the exact mechanism of its functioning, and observing these functions in a well-organized less biased behavioral paradigm can offer helpful clues into understanding many neurological diseases in addition to understanding the puzzle of the brain and its workings.

4.7 Conclusion

Given the complex biochemical cascade that is involved in LTP and the small distances that separate different synapses, it is remarkable that synapse-specific LTP can be achieved. For years numerous studies have tried to elucidate the molecular mechanisms that regulate this mechanism. Here, we have added to this growing literature by illustrating the role of the enzyme, CaMKII, as a synaptic tag in long-term odor

memory. This was accomplished through the results of behavioral experiments carried out in the present study. We were able to show that inhibiting CaMKII impairs short- and long-term odor memories. In addition we provided evidence that inhibiting CaMKII and activating PKA at the same time rescues LTM, and the specificity of the memory was gone, since the tag (CaMKII) had been lost. Thus we can suggest, with some confidence that CaMKII acts as a synaptic tag mediating odor-specific LTM in early odor preference learning.

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