INVESTIGATION OF THE RELATIONSHIPS BETWEEN CA$^{2+}$-MEDIATED PROTEINS AND LEARNING ON TASKS DEPENDENT ON THE HIPPOCAMPUS AND STRIATUM

AN ABSTRACT

SUBMITTED ON THE SECOND DAY OF OCTOBER 2015 TO THE NEUROSCIENCE PROGRAM IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE SCHOOL OF SCIENCE AND ENGINEERING OF TULANE UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

Amanda Rosemary Pahng

APPROVED: Paul J. Colombo, Ph.D. Director Jill M. Daniel, Ph.D. Jeffrey G. Tasker, Ph.D. Laura A. Schrader, Ph.D.
ABSTRACT

The hippocampus and striatum are brain structures that have dissociable roles in learning and memory. The dorsal hippocampus is necessary for spatial navigation and place learning (going to the same place in space), while the dorsal striatum is necessary for habit formation and response learning (making the same egocentric turn response). Use of a striatum-dependent learning strategy instead of a hippocampus-dependent learning strategy is related to factors that include aging, stress, drug use, and mental illness. These behaviors and lifestyle factors are related to system level changes in grey matter and activity in the hippocampus and striatum. Experiments that connect system level changes in these subcortical structures to learning strategies do not identify the underlying cellular mechanisms. The experiments in this dissertation were designed to investigate how cellular mechanisms of memory formation influence learning dependent on the dorsal hippocampus and dorsal striatum. This was accomplished by testing relationships between Ca^{2+}-mediated proteins and memory formation dependent on the hippocampus and striatum. One approach to testing relationships between Ca^{2+}-mediated proteins and memory formation was to correlate individual differences in protein levels with individual differences in place and response learning. An alternative approach to testing these relationships was to manipulate plasticity or activity in the hippocampus and measure changes in hippocampus- or striatum-dependent memory formation. The conclusions of these studies have important implications for relationships between Ca^{2+}-mediated proteins and learning, cellular evidence for how the hippocampus and striatum interact during memory formation, and phosphatase based regulation of phosphorylated CREB, which is a protein necessary for memory formation.
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[Signature]
Amanda Rosemary Pahng

APPROVED: [Signature]
Paul J. Colombo, Ph.D.
Director

[Signature]
Jill M. Daniel, Ph.D.

[Signature]
Jeffrey G. Tasker, Ph.D.

[Signature]
Laura A. Schrader, Ph.D.
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TABLE OF CONTENTS

ACKNOWLEDGMENTS ...........................................................................................................ii

LIST OF FIGURES ................................................................................................................vi

CHAPTER I. MECHANISMS OF MEMORY FORMATION AT A SYSTEMS LEVEL .......................................................... 1

A. Localization of memory and theories of learning ..................................................... 1

B. Neural circuitry of the hippocampus and striatum .............................................. 5

C. The hippocampus and striatum can function independently .......................... 12

D. The hippocampus and striatum can be interactive ......................................... 14

E. The mechanisms that mediate interactions are not understood ................... 21

CHAPTER II. CELLULAR MARKERS OF MEMORY FORMATION AND CONSOLIDATION ..................................................... 23

A. The role of tyrosine receptor kinase B in memory formation ....................... 23

B. The role of cAMP-response element binding protein in memory formation . 27

C. The role of protein phosphatases in memory formation .............................. 32

D. Approach ........................................................................................................... 36

CHAPTER III. LEARNING-INDUCED CHANGES IN PHOSPHORYLATED TYROSINE RECEPTOR KINASE B DEMONSTRATE A COMPETITIVE
5. Conclusions.......................................................................................................................... 109

CHAPTER VII. GENERAL DISCUSSION............................................................................. 111

A. Cellular mechanisms of learning strategies in the hippocampus and striatum
................................................................. ............................................................................. 112

B. Cellular mechanisms for competitive interactions between the hippocampus
and striatum .................................................................................................................. 114

C. Phosphatase based regulation of phosphorylated CREB in multiple memory
systems.......................................................................................................................... 117

CHAPTER VIII. LIST OF REFERENCES .............................................................................. 119
LIST OF FIGURES

Figure 1. Major afferents and efferents of the hippocampal memory system ...... 6

Figure 2. The tri-synaptic circuit of the hippocampus ........................................ 8

Figure 3. Major afferent and efferents of the striatal memory system ............ 11

Figure 4. Dual solution place/response training on an elevated plus maze ....... 14

Figure 5. Experiment timeline ............................................................................. 43

Figure 6. Images of pTrkB staining ..................................................................... 46

Figure 7. Mean pTrkB protein levels in the dorsal striatum ............................. 48

Figure 8. Mean pTrkB protein levels in the dorsal hippocampus .................... 49

Figure 9. Correlations between response learning and pTrkB protein levels in the
dorsal striatum .................................................................................................... 51

Figure 10. Correlations between response learning and pTrkB protein levels in
the dorsomedial and dorsolateral striatum ....................................................... 52

Figure 11. Correlations between response learning and pTrkB protein levels in
the dorsal hippocampus .................................................................................. 54

Figure 12. Correlations between response learning and pTrkB protein levels in
CA1 .................................................................................................................... 55

Figure 13. Experiment timeline ..................................................................... 67

Figure 14. Place training in a water plus maze .............................................. 69

Figure 15. Response training in a water plus maze ......................................... 71

Figure 16. Acquisition of a place task ............................................................. 74
Figure 17. Mean Fos, pCREB, and CREB protein levels in the dorsal hippocampus .............................................................................................................................. 75
Figure 18. Correlations between place learning and Fos protein levels in the dorsal hippocampus ........................................................................................................ 76
Figure 19. Correlations between place learning and Fos protein levels in the dorsolateral striatum ........................................................................................................ 77
Figure 20. Acquisition of a response task .............................................................................................................................. 79
Figure 21. Experiment timeline .............................................................................................................................. 91
Figure 22. Spatial learning in a water maze .............................................................................................................................. 97
Figure 23. Spatial memory in a water maze .............................................................................................................................. 98
Figure 24. Spatial memory in a water maze .............................................................................................................................. 99
Figure 25. Contextual fear conditioning .............................................................................................................................. 100
Figure 26. Novel object recognition .............................................................................................................................. 101
Figure 27. Novel object location .............................................................................................................................. 102
Figure 28. Mean inhibitor-2 protein levels in the dorsal hippocampus ...................................................................................... 103
Figure 29. Mean pCREB protein levels in the dorsal hippocampus ...................................................................................... 104
Figure 30. Mean CREB protein levels in the dorsal hippocampus ...................................................................................... 105
Figure 31. Mean pCREB protein levels in the dorsal striatum ...................................................................................... 106
CHAPTER I. MECHANISMS OF MEMORY FORMATION AT A SYSTEMS LEVEL

A. Localization of memory and theories of learning

A ‘memory system’ is defined by its anatomically distinctive centralized structure that uniquely processes incoming information, and includes all of the central structure’s incoming afferents and outgoing efferents in the brain (White & McDonald, 2002). The idea being that disrupting the central brain structure takes the entire memory system offline. Subcortical structures like the hippocampus and striatum were functionally dissociated from each other by testing the effects of region-specific lesions on memory formation. Early lesion experiments led to the theory of ‘multiple memory systems’ and supported previous theories of learning strategies in rodents.

Tolman developed the theory that rats use a cognitive map to move through space after analyzing learning strategies that rats use to solve complex land mazes (Tolman, 1948). At this time, the brain structure responsible for spatial learning was unknown. Following surgery to bilaterally remove his medial temporal lobes, epileptic patient, HM, demonstrated severe memory loss that was attributed to bilateral removal of his hippocampi and surrounding subcortical structures (Scoville & Milner, 1957). In 1978, O’Keefe and Nadel expanded Tolman’s cognitive map theory by describing the hippocampus as a cognitive map that is necessary for tasks that require the integration of different stimuli and
the relationships among these stimuli (O'Keefe and Nadel, 1978; White and McDonald, 2002). These stimulus-stimulus (S-S) associations represent the spatial and temporal relationships between different stimuli in the environment, which is important for navigation through space (White and McDonald, 2002; White, 2008). A lesion of the hippocampus in rodents impaired spatial learning in a water maze, win-shift in a radial arm maze, and contextual fear conditioning (Black, Nadel, & O'Keefe, 1977; Morris, Garrud, Rawlins, & O'Keefe, 1982; Olton, Walker, & Gage, 1978; Packard, Hirsh, & White, 1989; Packard & McGaugh, 1992).

The early theory that cognitive and motor habits are the result of reinforced associations between stimuli and behavioral responses was developed by Thorndike (Thorndike, 1898) and later expanded by Hull (Hull, 1943). The striatal memory system is necessary for habit formation and processing stimulus-response (S-R) associations where motor responses are reinforced (Mishkin & Petri 1984; White & McDonald 2002). The dorsal striatum has been behaviorally subdivided through lesion experiments into the dorsolateral striatum, which mediates S-R associations, and the dorsomedial striatum, which mediates expectancy-outcome mismatches (Devan, McDonald, & White, 1999; Devan & White, 1999; Yin & Knowlton, 2004; Yin, Ostlund, Knowlton, & Balleine, 2005). In rodents, a lesion of the dorsal striatum impaired response and cued learning as well as win-stay in a radial arm maze (Colombo, Davis, & Volpe, 1989; Cook & Kesner, 1988; Packard et al., 1989; Packard & McGaugh, 1992).
The use of hippocampal or striatal strategies during learning can dictate behavior in humans and can lead to system level changes in the brain. A dual solution task is a memory task where a maze can be solved by using more than one learning strategy. At the end of dual solution training, a probe trial is given that changes the task parameters so that only one strategy remains useful. Another type of dual solution probe, which will be described in later sections, involves changing the start location in a plus maze and recording the change in turn direction of animals into different arms. Altering task parameters allows experimenters to ‘probe’ what strategy was used during training by measuring changes in performance or turn direction during a probe trial. A dual solution place/response task can be learned using either a hippocampus-dependent place strategy (going to the same place in space) or a striatum-dependent response strategy (making the same egocentric turn response). A probe trial at the end of training determines if a hippocampus- or striatum-dependent strategy was used during learning. When multiple strategies are available during learning, the strategy used can be influenced by factors that include age, stress, mental illness, and drug use. In addition to linking learning strategies to behavior and age/mental illness, differences in these factors are related to changes in grey matter and activity in hippocampus and striatum. Participants that used a place strategy during a virtual dual solution task had more grey matter in the hippocampus, while response learners had more grey matter in the caudate nucleus (Bohbot, Lerch, Thorndycraft, Iaria, & Zijdenbos, 2007).
The hippocampus is vulnerable to age-associated changes, stress, mental illness, and drug use. Both episodic memory and hippocampal grey matter have been shown to sharply decline with age (Foster, 1999; Hedden & Gabrieli, 2004). Accordingly, aged subjects tend to use a response strategy in favor of a place strategy more frequently than younger subjects (Driscoll, Hamilton, Yeo, Brooks, & Sutherland, 2005; Rodgers, Sindone, & Moffat, 2012). Older individuals often perform worse on spatial tasks than young adults, which is likely due to reliance on the striatal memory system in favor of the hippocampal memory system (Konishi et al., 2013; Moffat, Zonderman, & Resnick, 2001). Much like aged individuals, stressed animals more often use a response strategy instead of a place strategy when solving tasks (Packard & Wingard, 2004; Sadowski, Jackson, Wieczorek, & Gold, 2009; Schwabe, Dalm, Schachinger, & Oitzl, 2008). The same strategy shift has been observed with people who have been exposed to psychosocial or prenatal stress (Schwabe, Bohbot, & Wolf, 2012; Schwabe et al., 2007). Spatial memory impairments and reduced hippocampal volume were present in individuals with schizophrenia and schizophrenia-spectrum disorders (Heckers, 2001; Heckers et al., 1998; Nelson, Saykin, Flashman, & Riordan, 1998; Wilkins et al., 2013). Individuals with higher instances of cigarette use, cannabis use, and alcohol use were more likely to be response learners after probing what strategy they used on a virtual dual solution task (Bohbot, Del Balso, Conrad, Konishi, & Leyton, 2013). Differences in behavior and learning strategies can be directly related to system level changes in grey matter in the hippocampus and striatum. However, in order to identify potential therapeutic and
targeted interventions of hippocampal memory impairments and reliance on striatal learning strategies, it is necessary to identify the cellular mechanisms that lead to these system-level changes in the hippocampus and striatum, which influence behavior.

B. Neural circuitry of the hippocampus and striatum

The hippocampus memory system consists of neocortical regions, subcortical para-hippocampal regions, and the hippocampus itself (Eichenbaum, 2000) (Figure 1).
Figure 1. Major afferents and efferents of the hippocampal memory system (modified from Bird and Burgess, 2008).

The hippocampus receives neocortical inputs from the frontal, temporal, parietal, and occipital lobes through cortical association areas. Before reaching the hippocampus these neocortical inputs must pass through subcortical regions in the medial temporal lobe (Bird & Burgess, 2008). Two of the major subcortical regions are the perirhinal cortex and the parahippocampal cortex, which is also known as the postrhinal cortex (Burwell, Witter, & Amaral, 1995). The parahippocampal cortex is part of the “where” stream that processes visuospatial information from the visual, parietal, and retrosplenial cortices, while the perirhinal cortex is part of the “what” stream that receives auditory, olfactory, and visual information about objects.
and events (Eichenbaum, Sauvage, Fortin, Komorowski, & Lipton, 2012; Furtak, Wei, Agster, & Burwell, 2007; van Strien, Cappaert, & Witter, 2009). The parahippocampal cortex receives very few inputs from the frontal cortex and likewise there are very few outputs from the parahippocampal cortex to the frontal cortex. In rodents, the perirhinal cortex receives very strong olfactory sensory information. Information that is processed by the parahippocampal cortex and perirhinal cortex travels through another subcortical region called the entorhinal cortex (Bird & Burgess, 2008). The entorhinal cortex is subdivided into the lateral and medial entorhinal cortices (Furtak et al., 2007; Insausti, Herrero, & Witter, 1997). Information processed in the parahippocampal cortex inputs into the medial entorhinal cortex, while information processed in the perirhinal cortex inputs into the lateral entorhinal cortex. In addition to the entorhinal cortex, the fimbria-fornix is a major input-output structure of the hippocampus that projects efferents and receives afferents from subcortical structures (Andersen et al., 2007). There is a thin sheet of myelinated fibers, which travel over the surface of the hippocampus called the alveus. The fibers of the alveus gather together in the fimbria and continue through the fornix where they eventually split around the anterior commissure to reach subcortical structures, which include the amygdala through the septal nuclei, the nucleus accumbens (ventral striatum), and the hypothalamus (Andersen et al., 2007).
Within the hippocampus, information is processed through the “tri-synaptic circuit” or “tri-synaptic pathway” (Figure 2). Glutamatergic or excitatory information from the entorhinal cortex projects through the perforant pathway to the granule cells of the dentate gyrus (DG) (Amaral & Witter, 1989; Andersen, Bliss, & Skrede, 1971). Axonal outputs of the dentate granule cells connect to the pyramidal cells in CA3 through the mossy fiber pathway. Axons of CA3 pyramidal neurons synapse onto CA1 through the Schaffer collateral pathway. After synapsing onto CA1, information exits the hippocampus from CA1 pyramidal neurons and returns back to parahippocampal subcortical and neocortical regions. The subiculum is a structure that is also part of the hippocampal formation, and it functions as an intermediary between CA1 pyramidal neurons and the entorhinal cortex (Furtak et al., 2007). In addition to glutamatergic neurons, the hippocampus also contains GABAergic inhibitory neurons (Sweatt, 2008). GABA-A receptors are present on hippocampal pyramidal cells (CA1-4) and granule cells in DG
(Mao & Robinson, 1998; Sequier et al., 1988). The role of GABAergic cells in the hippocampus is to modulate the excitability of glutamatergic synapses through feedback loops, which work by generating inhibitory postsynaptic currents depending on the current state of cell excitability (Kullmann, 2011).

In human and non-human primates, the striatum consists of the caudate nucleus and putamen, which are separated by a massive fiber plate called the internal capsule (Graybiel & Ragsdale, 1979). In contrast, there is no separation of the caudate nucleus and putamen in rodents. The rodent dorsal striatum consists of a single caudate putamen that is much smaller than the one in primates. The fiber tracts in caudate putamen that carry neurotransmitters like dopamine, serotonin, and acetylcholine in rodents are much more dispersed than in humans and primates. The dorsal and ventral striatum are dissociated by the cortical, subcortical, and dopaminergic inputs they receive (Ghiglieri, Sgobio, Costa, Picconi, & Calabresi, 2011). The dorsal striatum is classified as the caudate-putamen, while ventral striatum is classified as the nucleus accumbens and olfactory tubercle. As mentioned previously, the dorsal striatum has been behaviorally subdivided into the dorsomedial striatum and the dorsolateral striatum through lesion experiments in rodents (Devan et al., 1999; Devan & White, 1999; Yin & Knowlton, 2004; Yin et al., 2005). The anatomical reason for this dissociation is due to cortical-striatal projections (Pisa & Cyr, 1990). Medial regions of the frontal cortex including cingulate cortex and medial prefrontal cortex innervate the dorsomedial striatum, while lateral 'sensorimotor' regions including the motor cortex of the frontal lobe and the somatosensory cortex of the
parietal lobe innervate the dorsolateral striatum (Beckstead, 1979; Donoghue & Herkenham, 1986; Pisa & Cyr, 1990). This is believed to be the reason for the behavioral dissociation of the dorsal striatum, where the dorsomedial striatum processes outcome expectancies through positive or negative feedback, and dorsolateral striatum processes appropriate motor responses based on associations with a stimulus.

All of the neocortex projects to the dorsal striatum, and these corticostriatal connections make up the vast majority of the striatum's afferents (Graybiel & Ragsdale, 1979). Additional striatal afferents include connections from the thalamus and limbic midbrain, comprised of the hippocampus, amygdala, anterior thalamic nuclei, septum, and fornix. The mesocortical pathway transmits dopamine from the limbic forebrain to the neocortex, while the nigrostriatal pathway transmits dopamine directly to the striatum. In addition, the limbic forebrain connects to the dorsal striatum through the ventral striatum and nucleus accumbens, which is the ventromedial and anterior part of the striatum (Graybiel & Ragsdale, 1979; Nauta, Smith, Faull, & Domesick, 1978).
Major afferents of the dorsal striatum include the substantia nigra and globus pallidus or pallidum, which project back to cerebral cortical structures (Gerfen, Baimbridge, & Thibault, 1987; Graybiel & Ragsdale, 1979) (Figure 3). The hippocampus projects to the ventral striatum (Groenewegen, Vermeulen-Van der Zee, te Kortschot, & Witter, 1987) and the dorsomedial striatum (McGeorge & Faull, 1989) in rodents. In contrast, there is no evidence that the striatum projects directly to the hippocampus. As will be demonstrated in later sections, however, there is evidence of bidirectional interactions between the hippocampus and striatum during memory formation.

There are no glutamatergic neurons in the striatum and almost all cell types in the striatum are GABAergic (Kreitzer & Malenka, 2008; Wilson, 2007).
The majority of striatal neurons are principle GABAergic medium spiny neurons. There are less striatal interneurons than principle neurons, but much like in the hippocampus, interneurons play an important role in modulating cell excitability (Ghiglieri et al., 2011; Wilson, 2007). Striatal interneurons receive all of the excitatory glutamatergic signals from external sources (Wilson, 2007). For example, large aspiny cholinergic neurons in the striatum receive excitatory glutamatergic inputs from the cortex and the thalamus (Lapper & Bolam, 1992; Thomas, Smith, Levey, & Hersch, 2000).

C. The hippocampus and striatum can function independently

Double dissociation experiments led to the theory of multiple memory systems where the hippocampus and striatum were functionally dissociated from each other by testing the effects of lesions on the memory tasks dependent on the hippocampus or striatum. These double dissociation experiments and single lesion experiments indicate that the hippocampus can operate independently from the striatum (Kesner, Bolland, & Dakis, 1993; McDonald & White, 1993, 1994; Morris et al., 1982; Packard et al., 1989; Packard & McGaugh, 1992; Packard & Teather, 1997). Packard (1989) completed a double dissociation experiment by lesioning the fornix and caudate, and testing rats on win-stay and win-shift tasks in an 8-arm radial arm maze. For the hippocampus-dependent win-shift task, rats were placed in the center of the maze with all of the arms open. When a rat chose an arm, a door closed the rat in the arm until the rat ate the food. This was repeated until all 8 arms were chosen. The number of errors was based
on the number of revisits to previously baited arms. For the striatum-dependent win-stay task all of the arms were lit, so when a rat went into an arm and ate the food the arm was rebaited and the arm remained lit. However, the second time a rat went into an arm and ate the food the light turned off and the arm remained unbaited. The number of errors was based on the number of visits to unlit arms. Rats with fornix lesions were impaired on the hippocampus-dependent win-shift task, but spared on the striatum-dependent win-stay task. In contrast, rats with caudate lesions were impaired on the win-stay task, but spared on the win-shift task. These experiments are categorized as somatic intervention experiments because they test the effects of manipulating hippocampal or striatal function on memory formation.

In contrast, behavioral intervention experiments demonstrate dissociations between the hippocampus and striatum by measuring changes in plasticity after rodents are trained on tasks dependent on the hippocampus or striatum. A dual solution place/response task can be learned using either a place strategy (going to the same place in space) or a response strategy (making the same egocentric turn response). In an example of this task, animals are placed in the south arm of an elevated plus maze and are trained to find a food reward in the west arm (Figure 4). During the probe trial, the start location is moved to the north arm so animals are classified as ‘response learners’ when they go to the east arm (same left turn). In contrast, animals are classified as ‘place learners’ when they go to the west arm (same arm location).
When rats were trained on a dual solution place/response task where learning strategy is determined by a probe, phosphorylated cAMP response element-binding protein (pCREB) and Fos protein levels increased immediately after training in the hippocampus and striatum irrespective of learning strategy (Colombo, Brightwell, & Countryman, 2003). However one hour after training, increased pCREB and Fos were only sustained in the hippocampus of place learners and in the striatum of response learners. These data suggest that sustained region-specific increases in pCREB and Fos are important for memory formation and support independence between the hippocampus and striatum.

**D. The hippocampus and striatum can be interactive**

The finding that acquisition of striatum-dependent memory tasks was not only spared following fimbria-fornix lesions, but enhanced in some cases, led to the
theory that the dorsal hippocampus can interact competitively with the dorsal striatum (McDonald & White, 1993; Packard et al., 1989). An interaction between the hippocampus and striatum is operationally defined as competitive when impairment to one memory system enhances memory dependent on the non-manipulated region. In contrast, an interaction between the hippocampus and striatum is operationally defined as cooperative when impairment to one memory system also impairs memory dependent on the non-manipulated region. Conceptually, the dorsal hippocampus and dorsal striatum both receive sensory information about a particular learning situation through cerebral cortical and thalamic inputs, but this information is represented differently in each memory system based on their unique processing styles (S-S vs. S-R associations). Outgoing information from each memory system influences behavioral responses in a particular learning situation, and depending on the task demands the outgoing information from each memory system can lead to contradictory behavioral responses (competition) or similar behavioral responses (cooperation) (McDonald and White 2002; White, 2008). In a plus maze task where correct arm choices are reinforced with a food reward or escape, hippocampal processing biases rats to solve the task using a place strategy, while the striatal processing biases rats to solve the task using a response strategy. Since only one type of behavioral response is possible (place or response), one structure is more likely to dictate behavioral output during memory formation. In this case, competition is likely to occur (Kathirvelu & Colombo, 2013). Unlike cued or spatial tasks in a water maze, there is only one type of behavioral response for contextual fear
conditioning, and using a striatal or hippocampal strategy will always result in freezing. Without the possibility of contradictory behavioral responses, competition is not likely and instead cooperation is expected (Kathirvelu & Colombo, 2013).

There are numerous experiments, which demonstrate that the hippocampus and striatum can act competitively with each other in rodents and humans. (Chang & Gold, 2003; Colombo & Gallagher, 1998; Dagnas, Guillou, Prevot, & Mons, 2013; Kathirvelu & Colombo, 2013; Lee, Duman, & Pittenger, 2008; Martel, Millard, Jaffard, & Guillou, 2006; Matthews & Best, 1995; McDonald, Ko, & Hong, 2002; McDonald & White, 1993, 1995; McIntyre, Pal, Marriott, & Gold, 2002; Moody, Bookheimer, Vanek, & Knowlton, 2004; Moussa, Poucet, Amalric, & Sargolini, 2011; Packard et al., 1989; Poldrack et al., 2001; Pych, Kim, & Gold, 2006; Schroeder, Wingard, & Packard, 2002). The primary evidence that the hippocampus can interact competitively with the striatum comes from experiments where hippocampal function was impaired by lesions or drug/genetic manipulations, after which memory for tasks known to require the intact striatum were facilitated compared to controls (Chang & Gold, 2003; Lee et al., 2008; Matthews & Best, 1995; McDonald et al., 2002; McDonald & White, 1993; Packard et al., 1989; Schroeder et al., 2002). Fimbria-fornix lesions, which impair hippocampal connectivity to other systems, facilitated memory on a striatum-dependent win-stay task, suggesting that the hippocampus can interact competitively with the dorsal striatum (McDonald & White, 1993; Packard et al., 1989). Lesioning the fimbria-fornix or hippocampus prevents the hippocampal
memory system from producing strong outgoing information that is capable of contradicting striatal output, thus giving the striatum a distinct advantage in dictating behavioral responses. There are also reports that the striatum can compete with the hippocampus, specifically, that impairing the dorsal striatum enhanced spatial memory (Lee et al., 2008; Mitchell & Hall, 1988; Moussa et al., 2011). For example, mice with dorsal striatum lesions or mutant mice expressing KCREB in the striatum were impaired on a cued water maze task, but enhanced on a spatial water maze task (Lee et al., 2008). There are a few examples of competition where enhancing the function of the hippocampus (Dagnas et al., 2013) or striatum (Kathirvelu & Colombo, 2013; Pych et al., 2006), impairs memory dependent on the opposite region.

There is also evidence of competition between the medial temporal lobes and caudate nucleus in functional magnetic resonance imaging (fMRI) studies (Albouy et al., 2008; Moody et al., 2004; Poldrack et al., 2001; Poldrack & Packard, 2003). In humans, the basal ganglia consist of the striatum (caudate and putamen) and the pallidum (globus pallidus) (Graybiel & Ragsdale, 1979). Because Parkinson’s disease is known to greatly affect dopaminergic neurons and motor systems like the basal ganglia, it is a useful method of using a caudate nucleus ‘lesion’ to test interactions in humans. Individuals with Parkinson’s disease that performed a habit-learning task demonstrated less activation in the caudate nucleus than individuals without the disease (Moody et al., 2004). Also during an explicit memory task, individuals with Parkinson’s disease demonstrated more activation in the medial temporal lobes and the prefrontal
cortex than controls. This suggests that impaired caudate nucleus function is related to an increase in medial temporal lobe activation during the memory task.

Competitive interactions have also been demonstrated with behavioral intervention experiments in rodents (Colombo & Gallagher, 1998; McIntyre et al., 2002). McIntyre (2002) measured acetylcholine release in the hippocampus while rats were trained on an amygdala-dependent conditioned place preference task using in vivo microdialysis. The amygdala is a brain region that is necessary for emotional learning and memory (Kluver & Bucy 1939; Weiskrantz 1956). The magnitude of increase in hippocampal acetylcholine was negatively correlated with good performance on a conditioned place preference task. Rats that performed the worst on the conditioned place preference task had the highest levels of acetylcholine in the hippocampus, suggesting that the hippocampus and amygdala compete for behavioral output. Colombo and Gallagher (1998) found a negative correlation between choline acetyltransferase activity in the dorsolateral striatum and memory formation on a working memory task in a water radial arm maze. Rats with the highest choline acetyltransferase activity in the dorsolateral striatum made the most working memory errors, indicating impaired hippocampus-dependent memory. In addition to demonstrating that the hippocampus and striatum can compete under normal learning conditions, these results provide evidence that individual performance can be directly linked to differences in the cellular mechanisms of memory formation in the hippocampus and striatum.
There is also evidence of cooperative interactions between the hippocampus and striatum in rodents (Gengler, Mallot, & Holscher, 2005; Kathirvelu & Colombo, 2013; McDonald & White, 1995; McIntyre, Marriott, & Gold, 2003; Miyoshi et al., 2012; Moussa et al., 2011) and humans (Albouy et al., 2008; Brown, Ross, Tobyne, & Stern, 2012; Poldrack & Rodriguez, 2004; Sadeh, Shohamy, Levy, Reggev, & Maril, 2011; Voermans et al., 2004). With cooperative interactions, impairing the striatum impairs memory dependent on the hippocampus, while enhancing the striatum enhances memory dependent on the hippocampus. The same pattern occurs with impairment or enhancement of the hippocampus during striatum-dependent memory formation. Lentiviral overexpression of CREB in the dorsolateral striatum enhanced long-term memory for cue learning (striatum-dependent) and contextual fear conditioning (hippocampus-dependent), suggesting cooperation between the striatum and hippocampus during memory formation (Kathirvelu & Colombo, 2013). Enhanced contextual fear memory supports the theory that when outgoing information from different regions (hippocampus and striatum) produce similar behavioral responses (freezing) then the interaction is cooperative. In addition to striatal enhancement, striatal impairment with excitotoxic lesions of the dorsomedial striatum impaired learning on a hippocampus-dependent continuous alternation task, suggesting cooperation between the hippocampus and striatum (Moussa et al., 2011). Cooperation can also be demonstrated when impairing both the hippocampus and striatum leads to a greater memory impairment than if the regions were lesioned individually. Miyoshi and colleagues showed that the hippocampus and dorsolateral striatum cooperate
during navigation in a water maze, because rats that received combined
hippocampus + dorsolateral striatum lesions performed worse on cued and place
learning tasks than rats, which received individual lesions of the hippocampus or
dorsolateral striatum (Miyoshi et al., 2012). This suggests that both memory
systems contribute in different ways during learning, and cooperate in order to
efficiently complete a task.

In addition to the finding that the hippocampus and amygdala compete
during conditioned place preference training when hippocampal acetylcholine
was negatively correlated with task acquisition (McIntyre et al., 2002), a
cooperative interaction between these two memory systems was found using the
same microdialysis technique (McIntyre et al., 2003). In this experiment,
microdialysis measurements of acetylcholine in the amygdala were taken during
a hippocampus-dependent spontaneous alternation task. Acetylcholine release in
the amygdala was positively correlated with acquisition of a spontaneous
alternation task. Rats that performed the best on the spontaneous alternation
task had the highest levels of acetylcholine in the amygdala, suggesting that the
hippocampus and amygdala cooperate during behavioral output (McIntyre et al.,
2003).

Most of the evidence for cooperative interactions in humans comes from
fMRI experiments where there are high levels of coactivity in the caudate nucleus
and medial temporal lobes while solving memory tasks. Successful memory of
encoded items during a episodic memory task was correlated with greater activity
in both the hippocampus and striatum (Sadeh et al., 2011). This correlation of
greater coactivity between the hippocampus and striatum only occurred with items that were remembered during test, but not for items that were forgotten. The strength of the correlation was predictive of how successful participants were at the task. These findings suggest that the hippocampus and striatum cooperate for successful completion of the episodic memory task. In another experiment, successful acquisition of a contextually-dependent route navigation task coincided with increases in hippocampal, striatal, and orbitofrontal cortex activity (Brown et al., 2012).

E. The mechanisms that mediate interactions are not understood

As demonstrated in the review of the literature above, the evidence for interactions between hippocampus and striatum is abundant and well documented. However, the underlying mechanisms for competitive and cooperative interactions are still unknown (Packard & Goodman, 2013). Almost all of these experiments test how the hippocampus and striatum interact at either a behavioral level or at a cellular level, but not both. For example, a typical example of a somatic intervention experiment would involve impairing hippocampal function through a lesion or a drug infusion, then testing memory formation of a striatum-dependent task. This example only demonstrates competition or cooperation at a behavioral level of analysis by measuring if rodents demonstrated impairment or enhancement on the task, but does not reveal a possible cellular mechanism for this effect on memory. In another example, a typical example of a behavioral intervention experiment would involve
training rodents on hippocampus-dependent task and measuring changes in striatal plasticity following learning. This only demonstrates competition or cooperation at a cellular level of analysis by measuring if there is a decrease or increase in protein levels in the striatum, but does not reveal if this change in plasticity would affect striatum-dependent memory formation.

There seems to be very little progression in the field to investigate how interactions work at both a behavioral and cellular level. In a noted exception of this, rats that received injections of dopamine antagonist, Sulpiride, in the dorsal striatum were impaired on a continuous alteration T-maze task and had lower frequencies of theta rhythms in the hippocampus during the task (Gengler et al., 2005). This demonstrates cooperation between the hippocampus and striatum where a somatic manipulation in the striatum can change electrical activity in the hippocampus. In another experiment, histone deacetylase inhibition in the hippocampus made mice more likely to use a place strategy in favor of a response strategy during a dual solution task (Dagnas et al., 2013). Dual solution training increased pCREB in the striatum of vehicle-treated mice, but not mice that received hippocampal infusions of a histone deacetylase inhibitor. This explains why these mice were less likely to use a response strategy than controls. Conducting experiments, which test interactions at both a systems level (memory enhancement or impairment) and at a cellular level (increases and decreases in protein levels), is a promising strategy for understanding the underlying neural mechanisms of interactions.
CHAPTER II. CELLULAR MARKERS OF MEMORY FORMATION AND CONSOLIDATION

A. The role of tyrosine receptor kinase B in memory formation

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that regulates cell survival (Barde, Edgar, & Thoenen, 1982; Leibrock et al., 1989) and synaptic plasticity (Kang, Jia, Suh, Tang, & Schuman, 1996; Lo, 1995; Thoenen, 1995). BDNF initiates downstream signaling by binding to its high affinity receptor, tyrosine receptor kinase B (TrkB). BDNF binding induces TrkB dimerization and phosphorylation of the tyrosine residues in the intracellular kinase domain of the receptor, leading to phosphorylation of TrkB (pTrkB) (Cunha, Brambilla, & Thomas, 2010). Phosphorylation of tyrosine residue 515 recruits Src homology adaptor protein (Shc) and activates the Ras-Mitogen-activated protein kinase (Ras-MAPK) pathway and the Phosphatidylinositol 3-kinase (PI3K) pathway, while phosphorylation of tyrosine residue 816 recruits adaptor protein phospholipase Cγ (PLCγ) and activates the Ca²⁺/calmodulin-dependent protein kinase (CaMK) pathway (Gruart, Sciarretta, Valenzuela-Harrington, Delgado-Garcia, & Minichiello, 2007; Liliana Minichiello, 2009). Downstream TrkB signaling of these pathways leads to activation of extracellular-regulated protein kinase (ERK) and CREB (Patapoutian & Reichardt, 2001; Segal, 2003) as well as CaMKII and CaMKIV (Liliana Minichiello, 2009).
BDNF and TrkB have important roles in memory formation in the hippocampus and amygdala, but less is known about the role of TrkB signaling in striatum-dependent memory formation. Several experiments have shown that levels of BDNF and pTrkB increase following learning on region-dependent memory tasks. Training rodents on hippocampus-dependent tasks such as inhibitory avoidance, spatial water maze, contextual fear conditioning, and win-shift radial arm maze increased levels of BDNF (Alonso et al., 2002; Bekinschtein et al., 2007; Bekinschtein et al., 2008; Falkenberg et al., 1992; Hall, Thomas, & Everitt, 2000; Kesslak, So, Choi, Cotman, & Gomez-Pinilla, 1998; Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000; Mizuno et al., 2003) and pTrkB (Mizuno et al., 2003) in the hippocampus. Rodents that were training on cued fear conditioning tasks had increased levels of BDNF and pTrkB in the amygdala (Ou & Gean, 2006; Rattiner, Davis, French, & Ressler, 2004). Rats trained on a light-shock version of cued fear conditioning had increased levels of pTrkB in the amygdala 2 hours after training (Rattiner et al., 2004). Rats trained on an auditory version of cued fear conditioning had increased levels of pTrkB in the amygdala 30 minutes and 1 hour after training with pTrkB levels going back to baseline levels by 2 hours (Ou & Gean, 2006). In the hippocampus, pTrkB levels significantly increased immediately and 30 minutes after spatial learning in a radial arm maze (Mizuno et al., 2003). There is no evidence that learning-induced changes in pTrkB in the hippocampus and amygdala generalize to the changes in dorsal striatum.
Blocking TrkB signaling by preventing BDNF binding through antisense or transgenic knockout mice, impaired memory dependent on the hippocampus and amygdala (Alonso et al., 2002; Bekinschtein et al., 2007; Bekinschtein et al., 2008; Chhatwal, Stanek-Rattiner, Davis, & Ressler, 2006; Heldt, Stanek, Chhatwal, & Ressler, 2007; Liu et al., 2008; Liliana Minichiello et al., 1999; Mizuno et al., 2000; Mizuno et al., 2003; Mu, Li, Yao, & Zhou, 1999; Musumeci et al., 2009; Ou, Yeh, & Gean, 2010; Rattiner et al., 2004; Saarelainen, Pussinen, Koponen, & Alhonen, 2000). Point mutations of tyrosine residues 816 (Y816) and 515 (Y515) on the TrkB receptor in transgenic mice have dissociable effects on memory (Gruart et al., 2007; Minichiello et al., 2002; Musumeci et al., 2009). A point mutation of Y816 impaired transgenic mice on contextual fear conditioning and a working memory task in a radial arm maze, but Y515 transgenic mice performed the same as controls on both memory tasks and were only impaired on a cued fear conditioning task (Musumeci et al., 2009).

K252a is a potent Trk antagonist, which inhibits tyrosine protein kinase activity (Tapley, Lamballe, & Barbacid, 1992). Infusion of K252a into the basolateral amygdala immediately before and after cued fear conditioning impaired fear memory during a 48-hour test (Rattiner et al., 2004). Infusion of K252a into the dorsal striatum blocked the effects of exogenous BDNF infusion, which facilitated performance on an operant set-shifting task (D'Amore, Tracy, & Parikh, 2013). However, because K252a is also a non-specific inhibitor of serine/threonine kinases (Kase et al., 1987; Ruegg & Burgess, 1989) it is not possible to demonstrate that K252a blocked task facilitation by only impairing
TrkB signaling and not other serine/threonine kinases. An alternative method that has been used to impair TrkB signaling is infusion of a competitive binding antagonist, TrkB-Fc. TrkB-Fc is a fusion protein, combining the Fc region of human IgG1 with the ligand-binding domain of the TrkB receptor, which scavenges BDNF to inhibit TrkB signaling (Shelton et al., 1995). TrkB-Fc binds to BDNF with the same affinity as the endogenous TrkB receptor so it functions as a competitive antagonist. Infusion of TrkB-Fc into the dentate gyrus increased social defeat related avoidance in rats that normally did not demonstrate avoidance after social defeat (Duclot & Kabbaj, 2013). Infusing TrkB-Fc in the ventromedial prefrontal cortex immediately after extinction training prevented extinction of conditioned place aversion in rats twenty-four hours after training (Wang et al., 2012). Finally, infusion of TrkB-Fc into the amygdala thirty minutes prior to cued fear conditioning impaired long-term fear memory (Ou et al., 2010; Ou & Gean, 2006).

There is also evidence of memory enhancement in the hippocampus from increasing BDNF levels and facilitating TrkB signaling (Alonso et al., 2002; Koponen et al., 2004; Martinez-Moreno, Rodriguez-Duran, & Escobar, 2011). However, there is contrasting evidence that overexpressing BDNF can cause memory impairments and increase susceptibility to seizures and anxiety (Croll et al., 1999; Cunha et al., 2009; Papaleo et al., 2011). One possible explanation for this discrepancy is that the effect of BDNF expression on memory functions as an inverted U-shaped curve, where too little BDNF impairs memory but so does too much BDNF. This explanation of BDNF’s bidirectional effects on memory
suggests that there is an optimal range of BDNF expression for memory enhancement. In one experiment that supports this explanation, BDNF was overexpressed using a recombinant adeno-associated virus (rAAV). Half of the rats infused with rAAV-BDNF were impaired on spatial learning in a water maze compared to controls, while the remaining rats infused with rAAV-BDNF were enhanced on the task (Pietropaolo, Paterna, Bueler, Feldon, & Yee, 2007). Results from a BDNF ELISA assay demonstrated that the impaired rats had significantly higher levels of BDNF than the rats with enhanced memory on the spatial memory task, but both groups had greater levels of BDNF than controls. BDNF is also an upstream activator cAMP-response element binding protein (CREB), which is a protein necessary for memory formation. Reciprocally, phosphorylation of CREB activates transcription of BDNF (Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998).

B. The role of cAMP-response element binding protein in memory formation

Cyclic AMP-response element binding protein (CREB) is a transcription factor that regulates expression of genes necessary for the maintenance of memory (Impey et al., 2004; Lonze & Ginty, 2002; Shaywitz & Greenberg, 1999). CREB contains a basic leucine zipper (bZIP) domain that allows it to form a homodimer or a heterodimer with other CREB family members such as CREM and ATF-1 (Busch & Sassone-Corsi, 1990; Shaywitz & Greenberg, 1999). The bZIP domain of the CREB C-terminus region allows CREB (Trans element) to bind to a CRE promoter (Cis element) on CREB target genes. CREB activation
and regulation of target gene transcription depends on phosphorylation of serine 133 in the kinase-inducible transactivation domain (KID), which is located in the N-terminal region of CREB (Gonzalez, Menzel, Leonard, Fischer, & Montminy, 1991). Once CREB is phosphorylated at serine 133 (pCREB), CREB-binding protein (CBP) attaches to CREB’s KID domain through its own KID interaction domain (KIX) (Chrivia et al., 1993). CBP as well as p300 and P/CAF are all histone acetyl transferases that function as co-activators to loosen chromatin and recruit the RNA polymerase II complex (Byrne and Roberts, 2009). RNA polymerase II binds to the upstream ‘TATA box’ promoter region on targeted immediate early genes like BDNF, c-fos, and arc to begin transcription (Nakajima et al., 1997). Upstream activators of CREB such as PKA, CaMKII, CaMKIV, and RSK are responsible for phosphorylation of CREB, while CBP is activated and phosphorylated through CaMKIV (Sweatt, 2008).

CREB and the target genes it regulates have important roles in long-term memory in rodents. Increased CREB and pCREB have been reported following training on memory tasks that are dependent on the integrity of specific brain regions. These include place and response training in a plus maze (Colombo et al., 2003), spatial learning in a radial arm maze and water maze (Mizuno et al., 2002; Porte, Buhot, & Mons, 2008), socially transmitted food preference (Countryman, Orlowski, Brightwell, Oskowitz, & Colombo, 2005), contextual fear conditioning (Stanciu, Radulovic, & Spiess, 2001), and inhibitory avoidance (Bernabeu et al., 1997; Cammarota et al., 2000). Decreasing CREB protein levels through region-specific injections of CREB antisense impaired spatial
memory (Florian, Mons, & Roullet, 2006; Guzowski & McGaugh, 1997) and amygdala-dependent conditioned taste aversion (Lampecht, Hazvi, & Dudai, 1997). Dominant-negative mutant mouse models such as CREB αδ and KCREB have been used to disrupt CREB and impair spatial memory (Bourtchuladze et al., 1994; Kogan et al., 1997; Lee et al., 2008; Pittenger et al., 2002), fear conditioning (Bourtchuladze et al., 1994; Kogan et al., 1997), socially transmitted food preference (Kogan et al., 1997), and cued learning (Lee et al., 2008). Targeted disruptions of CREB alpha and delta isoforms in CREB αδ mice and a mutated K287L change within the DNA-binding domain of KCREB mice prevents CREB from binding to CRE sites (Pandey, Mittal, & Silva, 2000; Shaywitz & Greenberg, 1999; Walton, Rehfuss, Chrivia, Lochner, & Goodman, 1992). Another form of mutant CREB used in conditional knockout mice models involves changing serine 133 to an alanine. mCREB or CREBS133A mutations allow CREB to bind to CRE promotor sites, but prevent phosphorylation of CREB (Gonzalez & Montminy, 1989). Temporally-regulated and tamoxifen-inducible CREBS133A mice demonstrated impaired cued and contextual fear memory (Kida et al., 2002).

These studies demonstrate the importance of CREB activation in memory formation. However, it is not always the case that dominant-negative CREB mutant mice are impaired on memory tasks dependent on the hippocampus (Balschun et al., 2003; Gass et al., 1998). CREB αδ mice were not impaired on contextual fear conditioning or spatial learning in a water maze, which was most likely due to normal expression of the beta isoform of CREB (Gass et al., 1998).
An incomplete knockdown of all three CREB isoforms (alpha, beta, delta) in the hippocampus did not impair spatial learning in a water maze, fear conditioning, or hippocampal LTP (Balschun et al., 2003). As is suggested in the study, it is possible that incomplete knockdown of all three CREB isoforms or compensation by other CREB family members such as CREM prevented impairments in spatial memory and hippocampal LTP. Despite these findings, there is substantial evidence that CREB is important for memory formation in the hippocampus.

Delivery of dominant-negative mCREB with viral-mediated gene transfer has the advantage of allowing regional and temporal specificity through stereotaxically targeting a brain region of interest prior to behavioral testing. The various viral vectors used to deliver mCREB and wild-type CREB can have acute effects that last for several days like herpes simplex virus (HSV), alphaviruses (Semliki forest and Sindbis virus), and adenovirus or they can have long-term effects that last for several months like lentivirus and adeno-associated virus (Barco & Marie, 2011). Expression of mCREB with HSV in the hippocampus impaired long-term memory, but not short-term memory for social transmission of food preference (Brightwell, Smith, Countryman, Neve, & Colombo, 2005). Infusing HSV-mCREB into the dorsolateral striatum impaired long-term memory for response training (Brightwell, Smith, Neve, & Colombo, 2008). In addition, expression of mCREB with adenovirus in the perirhinal cortex and expression of mCREB with HSV in the olfactory bulb impaired novel object recognition and odor preference learning, respectively (Warburton et al., 2005; Yuan, Harley, Darby-King, Neve, & McLean, 2003). Long-term and stable expression of
mCREB in the hippocampus using a lentiviral vector impaired memory for place learning in a water maze and contextual fear conditioning (Kathirvelu, East, Hill, Smith, & Colombo, 2012). In addition to memory impairments, rats infused with lentiviral mCREB demonstrated a decreased ratio of pCREB/CREB protein levels in the dorsal hippocampus compared to controls.

While disrupting CREB activity impairs memory in rodents, overexpression of CREB has been shown to facilitate memory dependent on the striatum and hippocampus. CREBY134F and CREBDIEDML mice are two transgenic strains that have increased CREB protein levels and enhanced memory for contextual fear conditioning, social recognition, spatial water maze, and passive avoidance (Suzuki et al., 2011). A Y134F point mutation in CREBY134F mice increased CREB’s affinity for protein kinase A (PKA) therefore allowing lower levels of PKA to activate CREB, which would normally not do so in wild-type mice (Barco & Marie, 2011; Du, Asahara, Jhala, Wagner, & Montminy, 2000). CREBDIEDML mice have a six amino acid mutation in the CREB’s KID domain that allows CREB to interact with CBP without phosphorylation of serine 133 being necessary (Cardinaux et al., 2000; Impey et al., 2002). In addition to CREB overexpression enhancing spatial memory in mice, overexpression of CREB rescued spatial memory impairments in CREB αδ mice (Sekeres, Neve, Frankland, & Josselyn, 2010).

Overexpression of CREB with HSV and Sindbis virus in the hippocampus facilitated long-term spatial memory (Brightwell, Smith, Neve, & Colombo, 2007; Sekeres et al., 2010) and contextual fear memory (Restivo, Tafi, Ammassari-
Teule, & Marie, 2009). Overexpression of HSV-CREB in the basolateral amygdala and auditory thalamic projections to lateral amygdala enhanced expression of cued fear memory (Han et al., 2008; Josselyn et al., 2001). Finally, overexpression of CREB in the dorsolateral striatum with lentiviral vectors enhanced cued learning (Kathirvelu & Colombo, 2013). These data demonstrate that increasing CREB in hippocampus, striatum, and amygdala enhances memory dependent on those regions. The findings in this section also highlight the importance of learning-induced changes in phosphorylation-based activation of CREB during memory formation.

C. The role of protein phosphatases in memory formation

Kinases such as PKA, CAMKII, and PKC are responsible for phosphorylating target proteins like CREB, and these proteins are also dephosphorylated by protein phosphatases (Squire, 2008). Kinases phosphorylate substrates by adding a phosphate group that was transferred from ATP, while phosphatases dephosphorylate substrates by removing a phosphate group through hydrolysis (Krebs & Beavo, 1979). Protein phosphatases can be classified as protein-tyrosine phosphatases or protein serine/threonine phosphatases (PSPs). PSPs are responsible for the vast majority of dephosphorylation in mammals. The four main types of these phosphatases are PP1, PP2A, PP2B (calcineurin), and PP2C (Cohen, 1989; Hunter, 1995; Mansuy & Shenolikar, 2006). Both exogenous and endogenous inhibitors of phosphatase activity have been used to test the role of protein phosphatases in learning and
memory. Inhibiting protein phosphatase 1 (PP1) activity has been shown to increase phosphorylation of CREB (Alberts, Montminy, Shenolikar, & Feramisco, 1994; Bito, Deisseroth, & Tsien, 1996) and enhance memory (Genoux et al., 2002).

Most exogenous inhibitors of PSP activity are immunosuppressant drugs like cyclosporin A and FK506 or microbial toxins like okadaic acid. Cyclosporin A and FK-506 are T-cell immunosuppressants that inhibit Ca\(^{2+}\)-calmodulin-dependent calcineurin activity (Schreiber, 1992; Yu, Luo, Bu, Zhang, & Wei, 2006). In a previous experiment, rats were trained on a fear conditioning task where a light was paired with a footshock. In addition, rats were given a 24-hour test where no shock was administered to test for extinction of fear memory. Control mice exhibited normal extinction of the fear memory, but extinction was blocked in mice that received bilateral infusions of cyclosporin A or FK-506 into the amygdala (Lin et al., 2003). Initial fear conditioning increased levels of phosphorylated Akt (pAkt), but these levels of pAkt decreased during the extinction test in controls. In contrast, rats infused with FK-506 prior to the extinction test had similar levels of pAkt during extinction as controls during training (Lin et al., 2003). Protein kinase, Akt, is a downstream target of the PI3K pathway, so its activation is downstream of the TrkB receptor and upstream of CREB (Cunha et al., 2010). This demonstrates that inhibiting phosphatase activity impairs extinction by preventing dephosphorylation of Akt.

Okadaic acid is a tumor promotor, which also functions as a strong inhibitor of PP1 and PP2A by blocking dephosphorylation of proteins regulated
Intracerebroventricular bilateral injections of okadaic acid have been shown to impair spatial memory (Kamat, Tota, Saxena, Shukla, & Nath, 2010), while bilateral infusions of okadaic acid into the amygdala enhanced conditioned taste aversion and increased pCREB levels in the amygdala (Oberbeck, McCormack, & Houpt, 2010). The effects of okadaic acid infusions on memory are inconsistent, and this suggests that more specific methods of modifying PP1 or PP2A activity may yield more consistent findings.

In tissue extracts, phosphatase activity was increased following treatment with ethanol, but the addition of partially purified rabbit tissue enzymes prevented this increase in phosphatase activity and led to a 70% inhibition of phosphatase activity (Brandt, Lee, & Killilea, 1975; Huang & Glinsmann, 1976). These enzymes, which caused phosphatase inhibition, were identified as inhibitor 1 (I-1) and inhibitor-2 (I-2). I-1 and I-2 are two endogenous regulatory proteins that can modify the activity of PP1 (Hunter, 1995; Munton, Vizi, & Mansuy, 2004). In addition to phosphorylating CREB at serine 133, PKA phosphorylates I-1 into a state that inhibits PP1 activity, while calcineurin is responsible for dephosphorylating I-1 at the same site (Hunter, 1995; Waddell, 2003). I-1 is only able to inhibit PP1 activity if it is phosphorylated, so when calcineurin is inactivated, calcineurin cannot dephosphorylate I-1. This causes I-1 to be constitutively active, which causes PP1 to be continuously inhibited.

Genoux and colleagues created a tetO promotor mouse model where I-1 was constitutively active and PP1 was continuously inhibited following ingestion
of doxycycline. Blocking PP1 with constitutively active I-1 accelerated learning on a novel object recognition task and prolonged memory (Genoux et al., 2002). Typically training distributed over multiple sessions is better for learning than massed training trials. Control mice that were given distributed training on the novel object recognition task demonstrated better memory than controls that were given massed training. I-1 mutants that were given massed training on the object recognition task performed the same as controls that were given distributed training, suggesting that blocking PP1 can promote memory formation.

Initially, I-2 was thought to act in the exact same manner as I-1 when it came to inhibiting PP1 activity, but several studies have demonstrated that I-2 functions as an activator of PP1 (Hou et al., 2013; Nigavekar, Tan, & Cannon, 2002; Oliver & Shenolikar, 1998; Tung, Wang, & Chan, 1995). Glc7 is the yeast homolog of PP1 and Glc8 is the yeast homolog of I-2. Yeast cells that grow in culture go through a stationary phase where there is little to no yeast growth, which is related to increased Glc7 (PP1) activity. However, deletion of Glc8 (I-2) prevented the stationary phase from occurring. This shows that Glc8 (I-2) can activate Glc7 (PP1). After deleting Glc8 (I-2), Glc7 (PP1) became constitutively inhibited and did not undergo the stationary phase (Nigavekar et al., 2002). Knocking down I-2 in cortical neurons, via lentivirus-expressed RNAi, led to a fourfold increase in PP1 threonine 320 phosphorylation, which indicates decreased PP1 activity (Hou et al., 2013). An increase in PP1 threonine 320 phosphorylation resulted in decreased in PP1 activity because phosphorylation of
threonine 320 inhibits the ability of PP1 to bind to its substrates. These findings suggest that inactivating I-2 could enhance memory by preventing dephosphorylation of proteins important for memory formation. However, the role of I-2 in memory formation and phosphorylation of CREB has not been tested in vivo.

D. Approach

The experiments in this dissertation were designed to investigate how cellular mechanisms of memory formation influence learning dependent on the hippocampus and striatum. The mechanisms of interest tested in this dissertation were changes in neurotrophins, transcription factors, GABAergic inhibition, and phosphatase activity. Ca\textsuperscript{2+}-mediated protein levels in the hippocampus and striatum were measured after learning dependent of these brain regions. These proteins included Fos, phosphorylated TrkB, and phosphorylated CREB. Relationships between proteins levels and learning on tasks dependent on the hippocampus and striatum were tested using both behavioral and somatic interventions. In addition, both group and individual differences in protein levels were measured after memory formation.

Behavioral and somatic interventions:

Behavioral intervention experiments measure cellular changes in plasticity after animals are trained on memory tasks dependent on the hippocampus or striatum. A behavioral intervention was used to measure changes in pTrkB in the
dorsal hippocampus and dorsal striatum after rats were trained on a striatum-dependent response task in a water plus maze (Chapter III). Levels of pTrkB in the dorsal hippocampus were measured to test a competitive interaction between the hippocampus and striatum during memory formation. Levels of pTrkB in the dorsal striatum were measured to test learning-induced changes in pTrkB after response learning. An alternative experimental approach involves using somatic interventions to test system level changes in learning after manipulating cellular mechanisms of memory formation. A somatic intervention was used to increase GABAergic signaling in hippocampus during acquisition of a place and response task in a water plus maze (Chapter IV). In order to test relationships between proteins levels and learning, levels of pCREB and Fos were measured in the hippocampus and striatum after training. Another somatic intervention was used to knock down phosphatase regulatory protein, inhibitor-2, during memory formation of tasks dependent on the hippocampus (Chapter V). In order to test the regulatory role of inhibitor-2 on protein phosphorylation, levels of pCREB were measured in the hippocampus and striatum after testing.

**Group and individual differences:**

Investigation of group differences in Ca\(^{2+}\)-mediated proteins involved testing mean increases and decreases in protein levels, while investigation of individual differences in Ca\(^{2+}\)-mediated proteins involved testing correlations between individual protein levels and acquisition of memory tasks. Group differences in levels of pTrkB (Chapter III), Fos (Chapter IV) and pCREB
(Chapter IV, Chapter V) were measured after hippocampus- or striatum-dependent memory formation. This tests how differences in learning at a systems level are related to changes in cellular plasticity. There is a range of how much protein levels increase after learning, which varies among animals. Because of this individual variability, an alternative approach to testing relationships between Ca\(^{2+}\)-mediated proteins and memory formation was to correlate individual differences in hippocampal and striatal protein levels with individual differences in learning (Chapter III & Chapter IV). This tests how individual variability at a cellular level is related to acquisition of memory tasks dependent on the hippocampus and striatum.
CHAPTER III. LEARNING-INDUCED CHANGES IN PHOSPHORYLATED TYROSINE RECEPTOR KINASE B DEMONSTRATE A COMPETITIVE INTERACTION BETWEEN THE HIPPOCAMPUS AND STRIATUM FOLLOWING RESPONSE LEARNING

1. Introduction

The hippocampus and striatum have distinct roles in learning and memory. The dorsal hippocampus is necessary for place learning and the formation of cognitive maps to navigate through space (O'Keefe and Nadel, 1978; Tolman, 1948; White and McDonald, 2002), while the dorsal striatum is necessary for response learning and the formation of motor habits (Hull, 1943; Mishkin & Petri 1984; White & McDonald 2002). The dorsal striatum has been behaviorally and anatomically dissociated into the dorsomedial and dorsolateral striatum. The dorsomedial striatum processes expectancy-outcome mismatches, while the dorsolateral striatum processes stimulus-response associations (Devan et al., 1999; Devan & White, 1999; Yin & Knowlton, 2004; Yin et al., 2005).

In addition to having distinct roles in learning and memory, the hippocampus and striatum have been shown to competitively interact with each other. There is evidence of bi-directional competition between the hippocampus and striatum during memory formation. Inactivation of the hippocampus during memory formation with temporary or permanent lesions enhanced acquisition of striatum-dependent memory tasks (Chang, 2003; Matthews & Best, 1995;
McDonald & White, 1993; Packard et al., 1989; Schroeder et al., 2002).

Inactivation of the striatum with permanent lesions and transgenic inhibition of CREB also reportedly enhanced acquisition of hippocampus-dependent memory tasks (Lee et al., 2008; Mitchell & Hall, 1988). The cellular mechanisms that regulate competitive interactions between memory systems are not understood (Packard & Goodman, 2013). Therefore the current study was designed to test if response learning suppresses plasticity in the dorsal hippocampus.

BDNF is a neurotrophin that regulates synaptic plasticity by binding to the TrkB receptor (Kang et al., 1996; Lo, 1995; Thoenen, 1995). BDNF binding leads to phosphorylation of the TrkB receptor and activation of downstream proteins necessary for memory formation (Minichiello, 2009; Patapoutian & Reichardt, 2001; Segal, 2003). Training rodents on hippocampus- and amygdala-dependent memory tasks increased levels of phosphorylated TrkB (pTrkB) in the hippocampus (Mizuno et al., 2003) and amygdala (Ou & Gean, 2006; Rattiner et al., 2004). TrkB signaling has an important role in memory formation in the hippocampus and amygdala, but learning-induced increases in pTrkB in the dorsal striatum have not been examined.

The present study was designed to test the hypothesis that pTrkB protein levels are significantly lower in the dorsal hippocampus of response-trained rats compared to swim controls, due to a competitive interaction between the hippocampus and striatum. Additionally, this study tested the hypothesis that response training elevates pTrkB in the dorsal striatum. Rats were trained on a response task to find a hidden platform in a water plus maze to a criterion of 9
out of 10 correct responses. Swim controls were matched for time in the water and number of trials without a hidden platform. Trained rats and controls were sacrificed either 30 minutes or 2 hours after the end of response training. Immunohistochemistry was used to measure pTrkB protein levels in the dorsal hippocampus and dorsal striatum of response-trained rats and swim controls.

2. Methods

**Subjects:**

Thirty-two adult male Long-Evans hooded rats (3-4 months of age) were in-house bred and purchased from Charles River (Raleigh, NC). The experimental design was as follows: 2 time points (30 minutes; 2 hours) x 2 training conditions (trained; control) x 8 rats per condition = 32 rats total. All rats were group-housed in plastic cages with sawdust bedding in a temperature- and humidity-controlled room on a 12-hour light/dark cycle (lights on at 7:00 am). All training occurred during the light cycle. Rats were given *ad libitum* access to food and water throughout the duration of training.

**Response task:**

**Apparatus:**

The water version of the plus maze is as described previously (Brightwell et al., 2008; Kathirvelu & Colombo, 2013). The apparatus consists of four Plexiglas channels at ninety degree angles, with each channel measuring 50 cm x 21 cm x 31 cm (length x width x height). The plus maze was placed in the
middle of a circular pool measuring 1.83 m x 0.58 m (diameter x height). Non-toxic white tempera paint (250 ml) was added to make the water opaque, and the temperature of the water was maintained at 27 °C. A white removable platform measuring 12 cm x 20.5 cm (length x width) could be attached at the distal end of the arms, 2 cm below the surface of the water. A black curtain containing no salient cues surrounded the pool.

**Training:**

Rats were trained on a response task in a water plus maze to a criterion of 9 out of 10 correct choices to find the hidden platform (range, 10-22 trials; average, 13.125). Rats were given a trial to determine if they initially turn left or right, and were subsequently trained to the opposite turn direction of their initial preference. A Plexiglas guillotine door always blocked the arm opposite of the start arm so that rats could only make left or right turns. For the remainder of the trials, they were released in a pseudorandom order from all four-start locations, and an escape platform was located at the end of either the left or right arm. If rats were unable to find the platform within 60 seconds, then the experimenter led them to the platform. After reaching the platform, rats remained on it for 15 seconds before they were returned to a holding cage for a 15-second intertrial interval. A full body entry into the incorrect arm was scored as an error. Swim controls were placed in the water plus maze without the hidden platform, and were yoked to the trained rats by total number of trials and latency within each
trial. One swim control was excluded from the experiment because the rat climbed on top of the maze and did not swim.

Figure 5. Experiment timeline. Learning-induced changes in pTrkB in the hippocampus and striatum after response training in a water plus maze.

**Immunohistochemistry:**

Either 30 minutes or 2 hours after the end of training (Figure 5), animals were given an intraperitoneal injection of a ketamine-xylazine solution (1.65 mg/kg) and were transcardially perfused with 2% sodium nitrate in saline followed by 4% paraformaldehyde in 0.1 M PBS. The brains were removed and placed in vials containing 4% paraformaldehyde for further post-fixing overnight at 4 °C. After 24 hours, the brains were transferred to a 20% sucrose/phosphate solution. Coronal brain sections (50 μm) were taken throughout the striatum (1.68 mm to -0.36 mm relative to bregma) and the hippocampus (-2.64 mm to -3.48 mm relative to bregma).
mm relative to bregma) using a freezing microtome. Free floating tissue sections were placed in cryoprotectant and stored at -20 °C until immunohistochemistry was performed. Using 24 mm net-wells (Corning, Corning, NY), tissue sections were washed eight times in 0.05 M PBS for 40 minutes. Afterwards, the sections were washed in a peroxidase blocking solution (1% NGS/ 0.02% TX/ 1% H202 in 0.05 M PBS) for 10 minutes followed by a non-specific binding solution (2% NGS/ 0.4% TX in 0.05 M PBS) for 15 minutes. The sections were incubated for 48 hours at 4 °C in anti-TrkB phospho Y515 antibody (1:500; abcam, Cambridge, MA) prepared in 1% NGS/ 0.4% TX in 0.05 M PBS.

The tissue sections were washed four times in 0.05 M PBS for 1 hour before incubating at room temperature for 1 hour in goat anti-rabbit polyclonal secondary antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) prepared in 1% NGS/ 0.2% TX in 0.05 M PBS. After three 5-minute 0.05 M PBS washes, the sections were treated with an avidin-biotinylated horseradish peroxidase complex (ABC) in PBS (Elite Kit, Vector Laboratories) for 45 minutes at room temperature. Sections were washed four times with 0.05 M PBS for an additional hour before staining was visualized using diaminobenzidine (Sigma-Aldrich). The reaction was stopped after 8 minutes by three washes of 0.01 M PBS over 30 minutes. All sections were mounted on slides, allowed to dry for 48 hours, and then coverslipped.

Quantification of pTrkB staining:
Slide mounted tissue sections were imaged using an Olympus BX51 light microscope connected to a digital camera interfaced with Picture Frame imaging software. All images were taken with a 2x objective. MCID Analysis 7.0 software was used to quantify pTrkB staining in the images. For each image, the average optical density of the corpus callosum was calculated by measuring the optical density of samples confined within the corpus callosum (area of the circular sampling tool = 532.33 \( \mu \text{m}^2 \)). This was done to account for variations in background staining among sections and to set a threshold that optimally differentiates pTrkB-labeled cells from non-specific background staining. To set a threshold for each region, the highest and lowest contrast (difference between staining of interest and background staining) images were quantified and the final threshold was chosen based on highest threshold possible to quantify the lowest contrast images. For the dorsomedial and dorsolateral striatum, the threshold used for each image was the average optical density of the corpus callosum multiplied by 1.7. Large circles were used to sample the total target area and optical density of pTrkB staining above threshold in the dorsomedial and dorsolateral striatum (area of the sampling tool = 5253.28 \( \mu \text{m}^2 \)). For the dorsal hippocampus, the thresholds used for each image were the average optical density of the corpus callosum multiplied by 1.72 (CA1), 1.7 (CA3), 1.62 (dorsal blade of dentate gyrus), and 1.73 (ventral blade of dentate gyrus). A circle tool was used to sample the total target area and optical density of pTrkB staining above threshold in principal cell fields of the hippocampus (area of the sampling tool = 79.13 \( \mu \text{m}^2 \)). Optical density and total target area were used to quantify the
amount of pTrkB protein in each region. Immunostaining was most evident in the
cell bodies of the different regions, and less evident in the processes (Figure 6).

**Figure 6.** Images of pTrkB staining. (A) pTrkB staining in the dorsal hippocampus
(2x objective). (B) pTrkB staining in the dorsal striatum (2x objective). (C) pTrkB
staining in CA3 of the dorsal hippocampus (20x objective). (D) pTrkB staining in
the dorsolateral striatum (20x objective).

**Statistical Analyses:**

All data were analyzed using SPSS software. The dependent variables were pTrkB protein levels expressed as optical density and total target area. The
independent variables were the group (trained and control), time point (30
minutes and 2 hours), and region (subregions of the dorsal striatum and cell
fields of the dorsal hippocampus). One-way ANOVAs were used to analyze differences in pTrkB protein levels between groups, time points, and regions. A posteriori comparisons were performed using Fisher’s LSD test. Pearson’s r correlations were used to analyze the relationships between pTrkB protein levels and the total number of trials to reach a criterion of 9 out 10 correct responses. Significance levels for most tests were set at p<0.05. In order to lower the chance of making a Type I error, a Bonferroni correction was applied to the multiple correlations run for each hypothesis of correlations between total trials and pTrkB protein levels (α / number of correlations).

3. Results

Response training decreases pTrkB protein levels in the dorsal hippocampus relative to controls: For the optical density measure of pTrkB staining, there were no differences in protein levels between the dorsomedial and dorsolateral striatum [F(1,30)=0.647, p=0.425], so these subregions were pooled for subsequent analyses. There were also no differences in protein levels between principle cell fields of dorsal hippocampus (CA1, CA3, DG) [F(1,30)=2.574, p=0.058], so these cell fields were also pooled when analyzing group differences. Following response training in a water maze, a factorial ANOVA did not reveal a significant effect of group (response-trained rats, swim controls) [F(1,30)=1.847, p=0.180], time point (30 minutes, 2 hours) [F(1,30)=1.492, p=0.227], or group x time point
interaction [F(1,30)=0.157, p=0.694] for pTrkB protein levels in the dorsal striatum (Figure 7).

![Dorsal Striatum Graph](image)

**Figure 7.** Mean pTrkB protein levels in the dorsal striatum. There was no difference in levels of pTrkB in the dorsal striatum of trained rats compared to controls (p=0.694).

However in the dorsal hippocampus, there was a significant main effect of group [F(1,30)=12.666, p=0.001], a significant group x time point interaction [F(1,30)=9.972, p=0.002] for pTrkB protein levels, but no main effect of time point
Post hoc analyses revealed that there was significantly less pTrkB in the dorsal hippocampus of response-trained rats than controls 30 minutes after training (p<0.001), but not 2 hours after training (p=0.756) (Figure 8).

![Dorsal Hippocampus](image)

**Figure 8.** Mean pTrkB protein levels in the dorsal hippocampus. There was significantly less pTrkB in the dorsal hippocampus of trained rats compared to controls at 30 minutes (p<0.001).

Interestingly, there were no group differences in pTrkB protein levels in either region using the total target area measure. This suggests that group
differences in pTrkB after response learning are best measured by the optical density measure of staining, and not by the area measure. Overall, there was a significant decrease in pTrkB protein levels in the dorsal hippocampus of response-trained rats compared to controls, but no group difference in pTrkB levels in the dorsal striatum.

Individual differences in pTrkB protein levels in the striatum and hippocampus are related to acquisition of a response task:

To determine if individual differences in pTrkB are related to response learning, correlations were tested between the total number of trials to reach criterion and pTrkB protein levels in the dorsal striatum and dorsal hippocampus. For these correlations, a Bonferroni correction was applied and the level of alpha was adjusted to $p<0.0125$. For the total target area measure of pTrkB staining, there was a positive correlation between total number of trials and pTrkB protein levels in the dorsal striatum of response-trained rats [$r=0.873$, $p=0.005$] (Figure 9.a), but not swim controls [$r=0.060$, $p=0.888$] (Figure 9.b).
Figure 9. Correlations between response learning and pTrkB protein levels in the dorsal striatum. There was a significant positive correlation between total number of trials and pTrkB in the dorsal striatum in trained rats [(A) p=0.005], but not swim controls [(B) p=0.888)].

For subregions of the dorsal striatum, there were positive correlations between total number of trials and pTrkB protein levels in the dorsomedial striatum of response-trained rats \([r=0.839, p=0.009]\) (Figure 10.a), but not swim controls \([r=0.010, p=0.981]\) (Figure 10.b), and in the dorsolateral striatum of response trained rats \([r=0.823, p=0.012]\) (Figure 10.c), but not swim controls \([r=0.213, p=0.614]\) (Figure 10.d).
Figure 10. Correlations between response learning and pTrkB protein levels in the dorsomedial and dorsolateral striatum. There were significant positive correlations between total number of trials and pTrkB in subregions of the striatum in trained rats [(A) p=0.009; (C) p=0.012], but not swim controls [(B) p=0.981; (D) p=0.614]].

There were no significant correlations between pTrkB and total trials for the optical density measure of pTrkB staining in the dorsal striatum. These data demonstrate that there were relationships between individual performance on a response task and pTrkB in subregions of the striatum, which was due to
learning and not locomotor activity. Unexpectedly, however, higher levels of pTrkB in the dorsal striatum were related to slower acquisition of a response task.

There were positive correlations between total number of trials and pTrkB protein levels in CA1 and overall in the dorsal hippocampus of response-trained rats, but not swim controls at the alpha level of p<0.05. These positive correlations were in the expected direction, but the correlations were no longer significant after applying a Bonferroni correction at the alpha level of p<0.0125. However, there was an indication of a positive correlation between total number of trials and pTrkB protein levels in the dorsal hippocampus of response-trained rats \([r=0.714, p=0.047]\) (Figure 11.a), but no indication of a correlation in swim controls \([r=0.253, p=0.545]\) (Figure 11.b).
Correlations between response learning and pTrkB protein levels in the dorsal hippocampus. There was an indication of a positive correlation between the total number of trials and pTrkB in the dorsal hippocampus of trained rats [(A) \( p=0.047 \)] that was not significant after applying a Bonferroni correction. In contrast, there was no indication of a positive correlation in swim controls [(B) \( p=0.545 \)].

In cell fields of the dorsal hippocampus, there was an indication of a positive correlation between total number of trials and pTrkB protein levels in CA1 of response-trained rats \( [r=0.714, p=0.037] \) (Figure 12.a), but no indication of a correlation in swim controls \( [r=0.366, p=0.373] \) (Figure 12.b). There were no significant correlations between pTrkB and total trials for the optical density measure.
Figure 12. Correlations between response learning and pTrkB protein levels in CA1. There was an indication of a positive correlation between the total number of trials and pTrkB in CA1 of trained rats [(A) \(p = 0.037\)] that was not significant after applying a Bonferroni correction. In contrast, there was no indication of a positive correlation in swim controls [(B) \(p = 0.373\)].

These data indicate that there were relationships between individual performance on a response task and pTrkB protein levels in the dorsal hippocampus, which was due to learning and not locomotor activity. However, because these correlations were significant at the alpha level of \(p < 0.05\) and not at the adjusted alpha level of \(p < 0.0125\) for multiple correlations, the conclusion that higher levels of hippocampal pTrkB dorsal hippocampus were associated with slower acquisition of a response task cannot be made.

4. Discussion
In order to study the cellular mechanisms of interactions between memory systems, the hypothesis that response training decreases levels of pTrkB in the dorsal hippocampus due to competition was tested. This study also tested the hypothesis that response learning elevates levels of pTrkB in the dorsal striatum compared to swim controls. The results of the present study partially support these hypotheses. The results show that response learning led to decreased phosphorylation of TrkB in the dorsal hippocampus. In addition, there was a non-significant indication that pTrkB levels in cell fields of the hippocampus were negatively associated with acquisition of a response task. The results show that pTrkB protein levels in the dorsomedial and dorsolateral striatum were negatively correlated with acquisition of a response task, despite the lack of an overall change in striatal pTrkB in trained rats. These findings provide new evidence that the hippocampus and striatum compete during memory formation, and identify suppression of TrkB phosphorylation in the dorsal hippocampus during response learning as a potential cellular mechanism for competition.

The decrease in pTrkB levels in the dorsal hippocampus of response-trained rats compared to controls supports the hypothesis of this study, and is evidence that the hippocampus and striatum interact in a competitive manner during memory formation. This evidence of a competitive interaction suggests that a decrease in pTrkB protein levels in hippocampus is a potentially important step in acquisition of a response task. In addition, there was a non-significant indication that pTrkB levels in the dorsal hippocampus were negatively associated with acquisition of a response task among individual animals, but no
indication of this relationship in swim controls. These data are consistent with previous reports of competition that will be discussed in later paragraphs, and provide new evidence of a cellular mechanism for competitive interactions.

The finding that response-trained rats did not have elevated levels of pTrkB in the dorsal striatum compared to swim controls was unexpected. Based on the current findings it is possible that response learning does not increase pTrkB levels in the dorsal striatum, however, it is not possible to rule out other contributing factors that might have caused this non-significant finding. Higher levels of pTrkB in the dorsomedial and dorsolateral striatum were expected to be related to better performance on a response task, but instead the opposite was found. A possible explanation for this finding is based on how each striatal subregion individually processes sensory information. The dorsomedial striatum processes goal directed behavior and expectancy-outcome mismatches. In the example of a response task, the ‘expectation’ is that consistently turning the same direction will result in the ‘outcome’ of locating the hidden platform. If turning a consistent direction does not result in locating the hidden platform, then that is an expectancy-outcome mismatch. For positive correlations between pTrkB levels and response learning in the dorsomedial striatum, rats with more trials and more pTrkB had more opportunities for expectancy-outcome mismatches. The dorsolateral striatum processes motor habits and the reinforcement of stimulus-response associations. During a response task, the ‘stimulus’ of reaching the choice point at the end of the start arm results in a behavioral ‘response’ to turn a certain direction. The stimulus-response
association was reinforced when the rat’s consistent response to turn left or right led them to find the platform. For positive correlations between pTrkB and response learning in the dorsolateral striatum, rats with more trials and more pTrkB had more opportunities for correct stimulus-response associations. Alternatively, individual differences in basal pTrkB levels in the dorsomedial and dorsolateral striatum may contribute to differences in response learning. These relationships are not due to locomotor activity, because there were no relationships between total number of trials and pTrkB levels in swim controls. There is strong evidence that different memory systems are functionally specialized at a behavioral level. Mapping the processing styles of the dorsomedial and dorsolateral striatum on to individual pTrkB protein levels instead of task performance fits with the current data. However, it is important to point out that this theory does not fit with the conventional thinking that proteins including pCREB and Fos increase after learning in a similar manner in both the hippocampus and striatum.

Other reports in which inhibition of the hippocampus with temporary and permanent lesions enhanced acquisition of a response task (Chang, 2003; Matthews & Best, 1995; Schroeder et al., 2002) and cued learning (Lee et al., 2008) have provided the primary evidence of competitive interactions. However, these experiments do not identify the cellular mechanism, which causes inactivation of the hippocampus to enhance striatum-dependent learning. Other experiments have less detailed accounts of potential cellular mechanisms for interactions. For example, infusing glucose into the dorsal striatum impaired
acquisition of a place task (Pych et al., 2006). A possible, but untested
explanation for this previous finding is that enhancing striatal plasticity through
infusion of glucose decreased proteins levels in the hippocampus and impaired
place learning. In the current study, the finding that response learning decreases
pTrkB levels in the dorsal hippocampus provides support for this explanation.

There was an indication of positive correlations between response
learning and levels of pTrkB in CA1 and overall in the dorsal hippocampus, but
no indication of this relationship in swim controls. This finding is consistent with
other examples of competition where performance on memory tasks was
correlated with protein levels in brain regions not contributing to memory
formation (Colombo & Gallagher, 1998; McIntyre et al., 2002). In a previous
study, rats that performed the worst on an amygdala-dependent conditioned
place preference task had the highest levels of acetylcholine in the hippocampus
(McIntyre et al., 2002). Additionally, rats with the highest choline
acetyltransferase activity in the dorsolateral striatum made the most working
memory errors, a measure of hippocampus-dependent memory (Colombo &
Gallagher, 1998). The current data indicate that rats that were the worst at a
response task (i.e. had the most trials to reach criterion) had the highest levels of
pTrkB in the dorsal hippocampus. Overall, the present study identifies
suppression of pTrkB as a potential cellular mechanism for competition in the
intact and normally functioning brain.

In addition to demonstrating that the hippocampus and striatum can
compete during response learning, these results provide evidence that individual
performance can be linked to differences in pTrkB protein levels in the hippocampus. Interestingly, the group decrease in pTrkB levels in the hippocampus of response-trained rats was demonstrated by a decrease in the optical density of staining, while the correlations between response learning and pTrkB were demonstrated by variation in the total target area measure of staining. The optical density of staining reveals changes in the amount of protein within cells, while the area measure reveals the number of cells that express a protein above a certain threshold. This suggests that group differences may be most strongly associated with the levels of phosphorylation among cells engaged in memory processing, whereas relationships between performance and pTrkB among individuals may be related to increased recruitment of cells in a memory circuit, indicated by levels of staining above a threshold. Studies have shown that memories are encoded in a sparse arrangement of cells (Perez-Orive et al., 2002) and that only a small percentage of cells need to be recruited during memory formation (Han et al., 2007). These correlational data suggest that the arrangement of cells recruited into these cell assemblies can vary among individuals during acquisition of a response task. Also, importantly, this variation is related to differences in acquisition on a response task and is not caused by differences in locomotor activity.

5. Conclusions

This study tested the hypothesis that response training increases pTrkB levels in the dorsal striatum, but decreases pTrkB levels in the dorsal
hippocampus. The results demonstrate that response learning suppresses phosphorylation of the TrkB receptor in the dorsal hippocampus. However, there was no evidence that response learning increased pTrkB levels in the dorsal striatum. It is possible, but not conclusive that individual differences in pTrkB levels in subregions of the striatum are related to the behavioral processing styles of the dorsomedial (stimulus-response associations) and dorsolateral striatum (expectancy-outcome mismatches) rather than task performance. In addition to decreased pTrkB protein levels in the dorsal hippocampus of response-trained rats, there was an indication that rats that performed the worst on the response task had the highest levels of pTrkB in the dorsal hippocampus, suggesting competition.

Most importantly, these data provide new evidence that suppression of pTrkB in the hippocampus after response learning is a potential cellular mechanism for competition. Because of the strong evidence of bidirectional competition, it is possible that suppression of protein levels in the striatum could be increased or removed entirely after manipulating hippocampal function. This change in the ability of the hippocampus to suppress striatal plasticity is likely to impair or enhance striatum-dependent memory, respectively. The best way to test this cellular mechanism of competition is to inactivate the hippocampus and test the effects on striatum-dependent memory formation and protein levels in the striatum. This will reveal evidence of how competition at a behavioral level (effect on memory formation) relates to cellular changes in the striatum during learning.
CHAPTER IV. TESTING THE NEURONAL MECHANISMS OF COMPETITIVE INTERACTIONS CAUSED BY HIPPOCAMPUS-SPECIFIC INACTIVATION

1. Introduction

Evidence that the hippocampus can interact competitively with the striatum primarily comes from experiments where pharmacological and inactivating manipulations in the hippocampus enhance striatum-dependent memory formation. Permanent radio-frequency and electrolytic lesions of the striatum enhanced place learning in a radial arm maze and spatial learning in a water maze, respectively (Lee et al., 2008; Matthews & Best, 1995). Temporary inactivations of the hippocampus through pharmacological manipulations have demonstrated that the hippocampus and striatum can competitively interact. Immediate post-training infusions of sodium channel blocker, bupivacaine, enhanced memory on a response task and impaired memory on a place version of the task (Schroeder et al., 2002). When infusions of bupivacaine were given two hours post-training there was no effect on memory on either a response or place task. This demonstrates that there is a time-dependent effect for temporary manipulations that must occur during memory consolidation in order to effect memory. Additionally, pre-training manipulations of the hippocampus with another sodium channel blocker, lidocaine, impaired acquisition of a place task, but enhanced acquisition of a response task on elevated land maze (Chang & Gold, 2003). It is important to point out that there is also evidence that
pharmacological, genetic manipulations, and lesions of the hippocampus have no effect on striatum-dependent memory formation (Brightwell et al., 2008; Packard & McGaugh, 1992; Packard & Teather, 1997). Two previously proposed explanations for findings of independence instead of competition include a lack of extramaze cues to engage the hippocampus during memory formation (Chang & Gold, 2004; Packard & Goodman, 2013) and a lack of parametric space to demonstrate enhanced striatum-dependent memory (Brightwell et al., 2008; Matthews & Best, 1995). Altogether these findings demonstrate that the hippocampus and striatum can competitively interact during memory formation.

These findings demonstrate behavioral evidence for competition between the hippocampus and striatum, but do not identify the underlying cellular mechanisms, which regulate competition and enhance striatum-dependent memory. At present, these cellular mechanisms underlying interactions between the hippocampus and striatum are not understood (Packard & Goodman, 2013). In examples of competitive interactions, a possible explanation for enhanced striatum-dependent memory formation is that impairing the hippocampus increases protein levels in the striatum. There is evidence that plasticity in striatum is negatively correlated with performance on a hippocampus-dependent working memory task in a radial arm maze (Colombo & Gallagher, 1998). Specifically, rats with the highest choline acetyltransferase activity in the dorsolateral striatum made the most working memory errors, indicating impaired performance on the hippocampus-dependent task (Colombo & Gallagher, 1998). In an experiment where a histone deacetylase inhibitor, Trichostatin A, biased mice to use a place
strategy in favor of a response strategy, strategy selection was revealed by changes in striatal plasticity (Dagnas et al., 2013). Mice that received infusions of Trichostatin A in the dorsal hippocampus had less pCREB in the striatum than vehicle controls and were more likely to use a place strategy. In this example, lower levels of pCREB in the striatum of Trichostatin A-infused mice most likely explains why they were less likely to use a response strategy than the vehicle controls.

An increase in transcription factor, CREB, and immediate early gene, c-fos, in the dorsal striatum could explain why striatum-dependent memory is enhanced after inactivating the hippocampus. Previous research demonstrated that the formation of memory for place and response learning is related to sustained increases in pCREB and Fos protein levels in the hippocampus and striatum, respectively (Colombo et al., 2003). Increased levels of CREB and pCREB in the hippocampus have also been reported following spatial learning in a water maze (Mizuno et al., 2002; Porte et al., 2008), social transmission of food preference (Countryman et al., 2005), contextual fear conditioning (Stanciu et al., 2001), and inhibitory avoidance training (Bernabeu et al., 1997; Cammarota et al., 2000). Additionally, levels of Fos increased in the hippocampus after inhibitory avoidance training (Cammarota et al., 2000), contextual fear conditioning (Stanciu et al., 2001), and spatial learning in a radial arm maze (He, Yamada, & Nabeshima, 2002).

The present study was designed to test the central hypothesis that competition induced by pre-training, localized inhibition of the dorsal
hippocampus causes regional shifts in the levels of Fos and pCREB in the dorsolateral striatum and dorsal hippocampus. GABAergic inhibitory receptors are present throughout the cell fields of the hippocampus (Mao & Robinson, 1998; Sequier et al., 1988). Accordingly, infusion of selective GABA-A agonist, muscimol, is a useful method for causing inhibitory effects in the hippocampus. The first part of this study tests the hypothesis that inhibiting the hippocampus with muscimol impairs place learning and decreases levels of Fos and pCREB in the dorsal hippocampus. The purpose of this part of the study was to verify that the hippocampus can be inhibited by muscimol, and that this inhibition can be measured by both behavioral and cellular changes in hippocampus-dependent memory formation. The second part of this study tests competition between the hippocampus and striatum by testing the hypothesis that inhibiting the hippocampus with muscimol enhances response learning by increasing levels of Fos and pCREB in the dorsolateral striatum. Rats were implanted with bilateral guide cannulae in the dorsal hippocampus and given nine days to recover and habituate to the infusion procedure. Twenty minutes prior to training, rats received bilateral infusions of either muscimol or saline into the dorsal hippocampus. Rats were trained for a fixed number of trials on either a place or response task in a water plus maze. The number of correct arm choices and errors were recorded during training. Brain tissue was collected one hour after the end of training. Western blotting was used to measure changes in protein levels in the dorsal hippocampus and dorsolateral striatum of rats infused with muscimol or saline.
2. Methods

Animals:

A total of thirty-four adult male Long-Evans hooded rats (3-4 months of age) were purchased from Charles River (Raleigh, NC) for this study. The rats were used as follows: 2 memory tasks (place; response) x 2 drug infusions (muscimol; saline) x 8-9 rats per condition = 34 rats total. All rats were group-housed in plastic cages with sawdust bedding in a temperature- and humidity-controlled room on a 12-hour light/dark cycle (lights on at 7:00 am) until cannulation surgery, after which they were single-housed. All training occurred during the light cycle. Rats were given *ad libitum* access to food and water throughout the duration of training.

Surgical Procedure:

Cannulation surgery was performed on all rats in stereotaxic frame under a continuous flow of gaseous mixture containing isoflurane and oxygen. The scalp was incised along the midline of the head and retracted. Holes were drilled through the skull bilaterally over the target coordinates and three screw locations. Twenty-eight gauge bilateral guide cannulae (Plastics One) were implanted 1 mm above the dorsal hippocampus (AP: -3.1mm; ML: +/-1.5mm; DV: -2mm) of rats based on (Schroeder et al., 2002). Each guide cannula was fixed to the skull with dental acrylic and screws were attached at three different coordinates: (1) AP: 1.9mm; ML: -1.5mm (2) AP: -7.1mm; ML: +3mm (3) AP: -7.1mm; ML: -3mm. All rats were given intramuscular injections of 0.1 ml (0.006 mg) buprenorphine.
as a postoperative analgesic. After the surgery, rats were placed under a heat lamp until ambulatory. Rats were given approximately 9 days to recover before infusions and training. During the last 4 days of this recovery period, all rats were handled 4 times for 5 minutes each. During this handling period, the dummy cannulae (Plastics One) as well as the dust caps (Plastics One) were replaced and sterilized 3 times before training. After each replacement of the dummy cannulae, rats were placed in a plastic bucket measuring 30 cm x 36 cm (diameter x height) and the guide cannulae were connected to the infusion pump for 2 ½ minutes. This was done to habituate rats to the infusion procedure and reduce stress on training day (Figure 13).

![Experiment Timeline](image)

*Figure 13. Experiment timeline. Infusion of muscimol or saline into the dorsal hippocampus prior to place or response training in a water plus maze.*

**Infusions:**

Twenty minutes prior to training, rats received bilateral intrahippocampal infusions (0.5 μl per side) of either 0.9% NaCl or muscimol HCl (Sigma Aldrich,
1μg/1μl) dissolved in 0.9% NaCl. Muscimol is a GABA-A agonist that can be used to temporally inactivate local neurons and not fibers of passage (Edeline, Hars, Hennevin, & Cotillon, 2002). The internal cannulae (30 gauge, Plastics One) extended 1mm below the tip of the guide cannulae and were connected to a 10 μl Hamilton syringe via polyethylene tubing. Infusions were performed in freely moving rats at a rate of 0.2 μl per minute with a 1-minute post-infusion diffusion. The drugs were coded prior to infusions to keep the experimenters blind to the treatment effects during training.

Apparatus:

The water version of the plus maze is as described previously (Brightwell et al., 2008; Kathirvelu & Colombo, 2013). In brief, the apparatus consists of four Plexiglas channels at ninety degree angles, with each channel measuring 50 cm x 21 cm x 31 cm (length x width x height). The plus maze was placed in the middle of a circular pool measuring 1.83 m x 0.58 m (diameter x height). A black curtain surrounded the pool, which contained several three-dimensional cues in each quadrant of the pool. Non-toxic white tempera paint (250 ml) was added to make the water opaque, and the temperature of the water was maintained at 25 °C. A white removable platform measuring 12 cm x 20.5 cm (length x width) could be attached at the end of the different arms, 2 cm below the surface of the water.

Place Training:
Rats were trained on a place task in a water plus maze to find a hidden platform for a total of 40 trials (Figure 14). For each trial, rats were released from three start arm locations (south, west, east) and were trained to find a hidden platform that was always located in the end of the north arm. If they were unable to find the platform within 60 seconds they were led to the platform by the experimenter. Upon reaching the platform, rats remained on the platform for 15 seconds before being returned to a holding cage for a 30-second inter trial interval. A full body entry into the incorrect arm was scored as an error. The number of correct choices out of 40 trials was recorded for analysis of place learning.

![Place Training in a Water Plus Maze – 40 trials](image)

*Figure 14. Place training in a water plus maze*

**Response Training:**

Rats were trained on a response task in a water plus maze to find a hidden platform for 50 trials (Figure 15). Rats were given a trial without a platform to determine if they initially turn left or right, and were subsequently trained to
turn to the opposite direction of their initial preference. For this trial, the arm opposite of the start arm was blocked. For the remaining 50 trials, however, all arms were open and rats were released from four-start arm locations with either the left or right arm always containing the hidden platform. If they were unable to find the platform within 60 seconds they were led to the platform by the experimenter. Upon reaching the platform, rats remained on the platform for 15 seconds before returning to a holding cage for a 30-second inter trial interval. A full body entry into the incorrect arm was scored as an error. One rat was excluded from the experiment, because the animal was unable to complete the 50 trials. The number of correct choices out of 50 trials was recorded for analysis of response learning.
Western Blotting:

One hour after the end of training, rats were killed by decapitation. The tissue preparation has been previously described (Kathirvelu et al., 2012). Tissue homogenates from the dorsal hippocampus and dorsolateral striatum of individual rats were normalized by total protein concentrations with a 1:1 mixture of 2x sample buffer: running buffer. Normalized homogenates from samples (15 µg total protein/25 µl) and standards (homogenates from samples from naïve rats in a linear range from 10 to 60 µg) were separated by gel electrophoresis. The gels were electrophoretically transferred to PVDF immobilon membranes.
Membranes were washed with 0.05 M PBS 3 x 5 min and blocked with NFDM-TWEEN-PBS 3 x 15 min, then incubated overnight at 4 °C in rabbit anti-phospho-CREB antibody (1:1000; Cell Signaling), rabbit anti-CREB antibody (1:1000; Cell Signaling), or c-Fos rabbit polyclonal antibody (1:1000; Santa Cruz Biotechnology). The membranes were washed in 0.05 M PBS and blocked with NFDM-TWEEN-PBS before incubation at room temperature for 1.5 hours in goat anti-rabbit secondary HRP antibody (1:10000; Kirkegaard & Perry Laboratories). The membranes were washed in 0.05 M PBS-TWEEN 15 x 6 min, then incubated in a chemiluminescent reagent (SuperSignal West Pico Chemiluminescent substrate, Thermo Scientific), and exposed to film.

Quantification of Protein Levels:

Images from films were taken using Flashpoint 128 Framegrabber software. Integrated optical density and target area measurements were made for both known protein standards and unknown samples using MCID Analysis 7.0 software. A standard curve was created from the known protein concentrations of the standards in order to calculate the protein concentrations of the unknown samples (Colombo, Wetsel, & Gallagher, 1997).

Statistical Analysis:

Data was analyzed using SPSS software. Repeated measures ANOVAs were used to analyze group differences between the number of correct arm choices across blocks of trials for place and response learning. One-way
ANOVAs were used to analyze group differences on individual blocks of trials and protein levels between groups. Pearson’s r correlations were used to analyze the relationship between protein levels and the number of correct arm choices for blocks of trials. Significance levels for most tests was set at $p<0.05$. In order to lower the chance of making a Type I error, a Bonferroni correction was applied to the multiple correlations run for each hypothesis of correlations between the number of correct arm choices and protein levels ($\alpha / \text{number of correlations}$).

3. Results

Inhibiting the hippocampus with muscimol impairs place learning in a water plus maze:

Twenty minutes after infusion of muscimol or saline into the dorsal hippocampus, rats were trained on a place task in a water plus maze. The 40 trials of place training were separated into four blocks of 10 trials for behavioral analyses. For the number of correct arm choices across four blocks of 10 trials, a repeated measures ANOVA revealed a significant effect of block [$F(1,15)=28.516$, $p<0.001$], a significant effect for the block x group interaction [$F(1,15)=5.000$, $p=0.004$], but no effect of group [$F(1,15)=2.636$, $p=0.125$] (Figure 16). A posteriori analysis of the block effect revealed that both rats infused with muscimol [$F(1,8)=7.777$, $p=0.001$] and rats infused with saline [$F(1,7)=25.194$, $p<0.001$] significantly improved during acquisition of a place task.
Figure 16. Acquisition of a place task. Infusion of muscimol into the dorsal hippocampus impairs place learning (p=0.004).

One-way ANOVAs were used to analyze group differences on each of the four blocks of 10 trials. There was no significant difference between groups for block 1 [F(1,15)=0.497, p=0.492], block 2 [F(1,15)=0.050, p=0.826], and block 3 [F(1,15)=1.027, p=0.327]. However, there was a significant group effect in the last 10 trials of a place task [F(1,15)=15.197, p=0.001]. This demonstrates that rats infused with muscimol in the dorsal hippocampus made significantly more errors than saline controls during acquisition of a place task.

Inhibiting the hippocampus with muscimol does not significantly decrease levels of Fos, pCREB, or CREB in the dorsal hippocampus:
A one-way ANOVA revealed no significant effect of group for protein levels of Fos $[F(1,15)=1.593, p=0.229]$, pCREB $[F(1,15)=0.773, p=0.394]$, and CREB $[F(1,15)=0.288, p=0.288]$ in the dorsal hippocampus of place-trained rats (Figure 17). There were numeric decreases in levels of Fos, pCREB, and CREB in the dorsal hippocampus of rats infused with muscimol, but these did not differ statistically from protein levels in saline controls.

*Figure 17.* Mean Fos, pCREB, and CREB protein levels in the dorsal hippocampus. Muscimol rats have numerically less Fos ($p=0.229$), pCREB ($p=0.394$), and CREB ($p=0.288$) in the dorsal hippocampus compared to saline controls.
Inhibiting the hippocampus with muscimol disrupts the relationship between place learning and the immediate early gene, *c-fos*, in the dorsal hippocampus, but not in the dorsolateral striatum:

Correlations were analyzed between the number of correct arm choices for a place task and protein levels in the dorsal hippocampus of rats infused with muscimol or saline. There was a significant positive correlation between total number of correct choices on a place task and Fos protein levels in the dorsal hippocampus of rats infused with saline \([r=0.852, p=0.015]\), but not for rats that received hippocampal infusions of muscimol \([r=-0.509, p=0.198]\) (Figure 18).

*Figure 18.* Correlations between place learning and Fos protein levels in the dorsal hippocampus. There was a significant positive correlation between total number of correct choices and Fos protein levels in the dorsal hippocampus of rats infused with saline \((A) p= 0.015\), but not rats infused with muscimol \((B) p=0.198\).
This relationship between acquisition of a place task and Fos protein levels was not present in the dorsolateral striatum of rats infused with either saline \([r=0.494, p=0.260]\) or muscimol \([r=-0.077, p=0.844]\) (Figure 19).

![Figure 19. Correlations between place learning and Fos protein levels in the dorsolateral striatum. There were no significant correlations between the total number of correct choices and Fos protein levels in the dorsolateral striatum of rats infused with saline \[(A) p=0.260]\) or rats infused with muscimol \[(B) p=0.844]\).](image)

This demonstrates that there was a relationship between place learning and Fos protein levels in the dorsal hippocampus of rats infused with saline, but not rats infused with muscimol. Rats infused with saline that did the best on the place task had the highest levels of Fos in the dorsal hippocampus. In addition, there was no relationship between place learning and Fos levels in the dorsolateral striatum for either group.
Inhibiting the hippocampus with muscimol does not disrupt the relationship between place learning and transcription factor, CREB, in the dorsal hippocampus:

There were no significant positive correlations between the total number of correct choices on a place task and pCREB protein levels in the dorsal hippocampus of rats infused with saline [r=0.323, p=0.480] or muscimol [r=-0.550, p=0.125] (data not shown). There were also no significant positive correlations between the total number of correct choices and CREB protein levels in the dorsal hippocampus of rats infused with saline [r=0.030, p=0.949] or muscimol [r=-0.089, p=0.819] (data not shown). Additionally, correlations between the total number of correct choices and protein levels were tested for four blocks of 10 trials and eight blocks of 5 trials. For these tests, a Bonferroni correction was applied and the level of alpha was adjusted to p<0.013 and p<0.006, respectively. After applying the Bonferroni correction, there were no significant correlations between the number of correct choices and levels of pCREB or CREB in rats infused with saline or muscimol.

Inhibiting the hippocampus with muscimol has no effect on response learning in a water plus maze:

Twenty minutes after infusion of muscimol or saline into the dorsal hippocampus, rats were trained on a response task in a water plus maze. The 50 trials of response training were separated into five blocks of 10 trials for behavioral analyses. For number of correct arm choices across blocks, a
repeated measures ANOVA revealed a significant effect of block 
\[ F(1,15)=14.042, \ p<0.001 \], no effect of group \[ F(1,15)=1.001, \ p=0.333 \], and no 
block x group interaction \[ F(1,15)=1.237, \ p=0.305 \] (Figure 20). A posteriori 
analysis of the block effect revealed that both rats infused with muscimol 
\[ F(1,7)=4.492, \ p=0.027 \] and rats infused with saline \[ F(1,8)=11.533, \ p=0.001 \] 
significantly improved during acquisition of a response task.

![Response Learning in a Water Maze](image)

*Figure 20. Acquisition of a response task. Infusion of muscimol into the dorsal 
hippocampus has no effect on response learning (p=0.305).*

4. Discussion

The goal of the current study was to test the currently unknown cellular 
mechanisms that mediate competitive interactions between the hippocampus 
and striatum. An additional goal of this study was to investigate the availability of
extramaze cues as a primary determinant in whether or not the hippocampus and striatum interact during memory formation. Specifically, this study tested the central hypothesis that competition induced by pre-training, localized inhibition of the dorsal hippocampus causes regional shifts in the levels of Fos and pCREB in the dorsolateral striatum and dorsal hippocampus. Inhibiting the hippocampus with muscimol impaired place learning, but did not enhance response learning. Accordingly, these data are inconsistent with the theory that the hippocampus and striatum compete during memory formation, and provide evidence that response learning in cue-rich environment is not sufficient to engage the hippocampus in a competitive manner. The findings of this study also demonstrate how levels of Fos in the hippocampus influence place learning under normal and impaired learning conditions.

Rats infused with muscimol were impaired on a place task compared to saline controls. Rats infused with muscimol made more errors and less correct arm choices than their saline counterparts during acquisition of a place task. This impairment in place learning was supported by evidence of impaired plasticity in the hippocampus after training. In rats that received intrahippocampal infusions of muscimol there was a numeric decrease in Fos, pCREB, and CREB protein levels in the dorsal hippocampus compared to saline controls. Individual performance on a place task was related to increases in Fos, but not pCREB or CREB in the dorsal hippocampus of saline controls. These data suggest that success on the place task was more likely with higher levels of Fos in the hippocampus. Alternatively, individual differences in basal Fos levels in the
dorsal hippocampus may contribute to differences in place learning. Interestingly, Fos levels in the dorsal hippocampus of rats infused with muscimol were not related to place learning. These findings provide evidence that individual performance on a place task can be directly linked to differences in Fos protein levels in the dorsal hippocampus under normal learning conditions, but not under impaired learning conditions. This relationship between place learning and Fos levels was specific to the hippocampus, because Fos levels in the dorsolateral striatum were not related to place learning in either rats infused with muscimol or saline. Overall these findings support the hypothesis that impaired place learning is revealed by regional shifts in hippocampal plasticity.

Both rats that received intrahippocampal infusions of saline and muscimol demonstrated significant improvement during acquisition of a response task. However, inhibiting the hippocampus with muscimol had no effect on response learning in the water plus maze. The findings of this study do not support the hypothesis that inhibiting the hippocampus with muscimol enhances response learning, due to a competition interaction. However, these data are consistent with previous reports of independence between the hippocampus and striatum during memory formation. Reports of competition include experiments where both permanent and temporary inactivations of the hippocampus with lesions and pharmacological manipulations enhanced performance on striatum-dependent memory tasks (Chang & Gold, 2003; Lee et al., 2008; Matthews & Best, 1995; Schroeder et al., 2002). The temporary inactivation experiments used sodium channel blockers, lidocaine (Chang & Gold, 2003) and bupivacaine (Schroeder et
al., 2002), to enhance response learning in a elevated land maze and a water plus maze, respectively. One difference between these sodium channel blockers and GABA-A agonist, muscimol, besides the method of action and duration of action, is that muscimol only inhibits local neuronal cell bodies, while lidocaine and bupivacaine block activity in cell bodies and passing axonal fibers (Edeline et al., 2002; Martin & Ghez, 1999). Although this is unlikely to make a large difference in behavior, the difference between the drugs cannot be ruled out as a possible reason for the inconsistent findings. A much simpler explanation is that the response task paradigms were not equivalent in motivational factors or the duration of training, and the current study was not a replication of either experiment.

It is still not understood under what conditions the hippocampus and striatum are independent or interactive during learning and memory. One previously proposed explanation for findings of independence instead of competition is a lack of parametric space to demonstrate enhanced striatum-dependent memory (Brightwell et al., 2008; Matthews & Best, 1995). If a memory task is too easy, then there is not enough parametric space to demonstrate task facilitation. In a previous study, there was no parametric space to demonstrate improvement on a response task during testing, because of the low difficulty of the task (Brightwell et al., 2008). In the current study, however, the difficulty of the response task was greatly increased by giving animals three possible open arm choices instead of the usual two possible open arm choices. It is important to point out that the response task used in the current study was not too difficult
either, because both groups were able to significantly improve during training. There was enough parametric space in the current study to demonstrate enhancement, but there was no evidence of enhanced response learning in rats infused with muscimol in the dorsal hippocampus. This explanation of inadequate parametric space is not supported by the data in this study, so there must be another explanation for why independence was found.

Another proposed explanation for finding independence instead of competition is a lack of extramaze cues to engage the hippocampus during memory formation (Chang & Gold, 2004; Packard & Goodman, 2013). In this case, interactions are more likely to occur when both memory systems are activated during memory formation, while independence occurs when the task parameters do not allow both memory systems to become engaged during learning. For example, there was a lack of available extramaze cues in the experiment where infusions of HSV-mCREB into the dorsal hippocampus did not enhance response learning (Brightwell et al., 2008). In a previous experiment, rats received infusions of artificial cerebrospinal fluid (aCSF) into the dorsolateral striatum and were trained on a response task in either a cue-poor environment or a cue-rich environment. Rats infused with aCSF in the cue-poor environment learned the response task significantly faster than rats infused with aCSF in a cue-rich environment (Chang & Gold, 2004). In this example, the hippocampus was more likely to be activated in the cue-rich condition therefore making the striatum-dependent response task more difficult to learn. This hypothesis is supported by an experiment where levels of acetylcholine in the hippocampus
remained elevated during response training in a cue-rich environment, but declined during response training in a cue-poor environment (Pych, Chang, Colon-Rivera, Haag, & Gold, 2005). These findings suggest, but do not test whether or not competition is more likely to occur in a cue rich environment where the hippocampus is more active during learning. In the current study, rats were trained on a response task in a cue rich environment with several three-dimensional extramaze cues located in each quadrant of the pool. The findings of the current study provide evidence that response learning in cue-rich environment is not sufficient to engage the hippocampus in a competitive manner.

There are other factors like stress, which influence the relative activation of the hippocampal and striatal memory systems during memory formation (Packard & Goodman, 2013). Stress leads to a preferential engagement of the striatum during learning (Packard & Wingard, 2004; Sadowski et al., 2009; Schwabe et al., 2008). In addition, stress has an impairing effect on hippocampus-dependent memory that can be reversed by lesioning the amygdala (Kim, Lee, Han, & Packard, 2001). In the current study, it is possible that stress related to aversive water maze training or pre-training drug infusions or a combination of the two could have given an advantage to the striatum during response learning, so that the extramaze cues did not make response training more difficult for saline-infused controls. If the spatial cues did not influence response learning in controls due to stress, then inactivating the hippocampus would have no effect on response learning in rats infused with muscimol. Future
research should investigate the contributions of both stress and extramaze cues in interactions between the hippocampus and striatum.

5. Conclusions

This study tested the hypothesis that localized inhibition of the dorsal hippocampus with muscimol impairs place learning, but enhances response learning in a water plus maze. This study also tested the hypothesis that impairment of place learning would be revealed by dysregulation of Fos and pCREB levels in the hippocampus, while enhancement of response learning would be revealed by increased Fos and pCREB levels in the dorsolateral striatum. Inhibiting the hippocampus with muscimol impaired place learning and disrupted Fos protein levels in the dorsal hippocampus. Inconsistent with a competitive interaction between the hippocampus and striatum, inhibiting the hippocampus with muscimol did not enhance response learning. These data provide evidence that response learning in a cue-rich environment is not sufficient to engage the hippocampus in a competitive manner during memory formation, and other factors such as stress level should be considered in experiments testing competitive interactions.

The relationship between place learning and Fos levels one hour after training was specific to the dorsal hippocampus and not to the dorsolateral striatum. This finding is consistent with the previous finding that levels of Fos and pCREB were only sustained in the dorsal hippocampus of place learners, but not in the dorsal striatum of place learners (Colombo et al., 2003). This indicates that
sustained increases in protein levels are necessary in the region contributing to
formation of a memory, but not in a region that does not contribute to memory
formation. Investigations of potential mechanisms that control these region-
specific increases in plasticity have important implications for memory formation.
CHAPTER V. INFUSION OF LENTIVIRAL INHIBITOR-2 SHORT HAIRPIN RNA INTO THE DORSAL HIPPOCAMPUS ENHANCES CONTEXTUAL FEAR CONDITIONING AND SPATIAL MEMORY IN A WATER MAZE

1. Introduction

Protein phosphatases are responsible for dephosphorylating proteins important for memory like CREB. A region-specific decrease in phosphatase activity is a possible mechanism for sustained activation of CREB during memory formation. Protein phosphatases may contribute to memory formation by preventing sustained increases in pCREB in regions not necessary for acquisition of a memory task (Colombo, 2004). Protein phosphatase 1 (PP1) is a serine/threonine phosphatase that has a suppressive effect on memory. Decreasing PP1 activity using a tetO promotor mouse model system enhanced memory for novel object recognition (Genoux et al., 2002). Memory enhancement following inhibition of PP1 was likely due to increased levels of phosphorylation in proteins important for memory formation. For example, bilateral infusions of okadaic acid, an exogenous PP1 and PP2A inhibitor, into the amygdala enhanced conditioned taste aversion and increased pCREB levels in the amygdala (Oberbeck et al., 2010). There is evidence that PP1 is the primary phosphatase responsible for dephosphorylating CREB at serine 133. Inhibiting PP1 activity with okadaic acid increased pCREB levels in cultured hippocampal neurons (Bito et al., 1996). Also, inhibiting PP1 with the regulatory
protein, inhibitor-1, prolonged increases in CREB phosphorylation in 3t3 fibroblasts following cAMP stimulation (Alberts et al., 1994).

Inhibitor-2 (I-2) is an endogenous regulatory protein that can modify the activity of PP1, but contrary to its name, there is evidence that I-2 can function as an activator of PP1 (Hou et al., 2013; Nigavekar et al., 2002; Oliver & Shenolikar, 1998; Tung et al., 1995). Knocking down I-2 with a lentiviral vector decreased PP1 activity in primary cortical neurons by increasing phosphorylation of PP1 at threonine 320 (Hou et al., 2013). This suggests that I-2 is an endogenous activator of PP1, which is likely to increase dephosphorylation of CREB and suppress memory formation. However, the role of I-2 in memory formation has not been examined.

The present study was designed to test the hypothesis that localized gene silencing of I-2 in the dorsal hippocampus enhances memory for hippocampus-dependent tasks in rats. This experiment also tests the hypothesis that knocking down I-2 increases levels of phosphorylated CREB in the dorsal hippocampus. Rats received bilateral infusions of lentiviral I-2shRNA into the dorsal hippocampus, which has been shown to decrease I-2 levels and PP1 activity in cultured cortical neurons (Hou et al., 2013). Two weeks after surgery, rats were tested on hippocampus-dependent memory tasks including place learning in a water maze, contextual fear conditioning, novel object recognition, and novel object location. Brain tissue from the dorsal hippocampus and dorsal striatum was collected twenty-four hours after the end of behavioral testing. Western blotting was used to measure changes in protein levels in the dorsal
hippocampus and dorsal striatum of rats infused with lenti-I-2shRNA as well as controls.

2. Methods

Animals:

Ten adult male Long-Evans hooded rats (3-4 months of age) were purchased from Charles River (Raleigh, NC). All rats were group-housed in plastic cages with sawdust bedding in a temperature- and humidity-controlled room on a 12-hour light/dark cycle (lights on at 7:00 am). All training occurred during the light cycle. Rats were given ad libitum access to food and water throughout the duration of training.

Lentivirus vector-mediated gene transfer:

Houhui Xia, Ph.D., and Hongtian Yang (Neuroscience Center, LSU Health Sciences Center) supplied the lentiviral vectors. Lentiviral I-2shRNA is a construct that expresses genomic shRNA that corresponds to the 3’ untranslated region of I-2 mRNA and knocks down I-2 protein levels. The control lentiviral vector expresses GFP and has no effect on I-2 protein levels. The estimated titer of both viruses was $10^9$ infectious units/ml, and the viruses were dissolved in phosphate buffered saline.

Surgical procedure:
Stereotaxic surgery was performed on all rats in a stereotaxic frame under a continuous flow of gaseous mixture containing isoflurane and oxygen. The scalp was incised along the midline of the head and retracted. Holes were drilled through the skull bilaterally over the target coordinates. Immediately prior to infusion into the dorsal hippocampus, 5 microliters of 20% mannitol (Sigma-Aldrich) was mixed with 10 microliters of lentiviral I-2shRNA or 10 microliters of lentiviral-GFP to facilitate diffusion. Three microliters of the lentivirus and mannitol mixture was infused bilaterally into the dorsal hippocampus at two sites (site 1: AP = -3.1, ML = +/-1.4, DV = -3.8; site 2: AP = -4.4, ML = +/- 3.3, DV= -3.3). Surgical shams underwent the same surgical procedure as the lentiviral-infused rats except nothing was infused into the dorsal hippocampus. Once the needles were removed, the drill holes were filled with bone wax and the incision was closed with wound clips. The rats were given intramuscular injections of 0.1 ml (0.006 mg) buprenorphine as a postoperative analgesic. After surgery, rats were placed under a heat lamp until ambulatory. The number of rats per condition were as follows: lenti-I-2shRNA=5, surgical shams=2, lenti-GFP=3. All rats were given two weeks to recover from surgery and for the lentivirus to reach maximum expression before behavioral training and testing (Figure 21).
Figure 21. Experiment timeline. Infusion of lenti-I-2shRNA in the dorsal hippocampus of rats prior to training and testing on hippocampus-dependent memory tasks. CFC= Contextual fear conditioning; NOR= Novel object recognition; NOL= Novel object location.

Apparatus:

Water maze:

Training and testing on the spatial water maze task was conducted in a white circular pool (diameter 1.83m x height 0.58m) surrounded by a black curtain containing several three-dimensional cues in each quadrant of the pool. The water was tinted white with ~250 ml of white tempera paint and maintained at 27°C. A circular, retractable platform with a 15 cm diameter was located two centimeters below the surface of the water when fully extended. When retracted, the platform remained near the bottom of the pool where rats were unable to reach it. All behavioral measures of performance were recorded with a computer tracking system (HVS image 2100 tracking system).

Contextual fear conditioning chamber:

A chamber measuring 20.3 x 25.4 x 20.3 cm (L x W x H) was used to train and test rats on a contextual fear conditioning task (Avoidance Monitor LM100,
Kinder Scientific). A video camera was located in the lid of the chamber to record behavior and an adjustable house light was located near the top of the chamber. The walls of the chamber were white and had a smooth surface. The floor was a removable stainless steel grid, which was able to deliver a shock.

**Open field:**

An open field measuring 91.4 x 91.4 x 45.7 cm (L x W x H) was used to train and test rats on novel object recognition and novel object location tasks. A video camera was suspended from the ceiling of the room above the open field to record behavior. The walls of the open field were made of a black semi-reflective surface. Several 4 x 4 squares were outlined by yellow tape on the floor of the open field under clear Plexiglas.

**Behavioral training/testing:**

**Spatial water maze:**

Two weeks following hippocampal infusion, rats began spatial learning in a water maze. Spatial memory was assessed using a place task where rats were pseudorandomly started from one of four start locations and trained to find a hidden platform in the water maze. Rats were given 3 trials per day for a total of 4 days. The trial ended when rats reached the platform or when 90 seconds had elapsed and rats was led to the platform by the experimenter. Rats remained on the platform for 15 seconds and were placed in a carrying cage for an additional 15 seconds between trials. The 6th and 12th trials (last trial on days 2 and 4) were
probe trials to measure spatial bias where the platform was retracted for the first 30 seconds of the trial.

**Contextual fear conditioning:**

For contextual fear conditioning, rats were trained with a one-shock paradigm (1.5 mA, 2s) that they received 5 minutes after placement in the contextual fear conditioning chamber. Rats were taken out of the chamber 30 seconds after the shock was administered and they were tested 24 hours after training. During testing, the rats remained in the box for 8 minutes during which no shock was administered and freezing (the absence of movement except for breathing) was measured. The chamber was cleaned with ethanol between each animal.

**Novel object recognition/location:**

Prior to training, rats were habituated in an open field for 10 minutes a day for a total of 3 days. Twenty-four hours after habituation, rats were placed in a box with two identical objects (17 cm from each wall) for 6 minutes. A 24-hour delay was imposed between training and testing. Both the objects and open field were cleaned with ethanol between rats.

*Novel object recognition-* During testing, one object was replaced with a novel object and rats were placed in the open field for 5 minutes. The following discrimination ratio was used to assess novel object recognition: 
\[
\frac{(\text{time with the novel object} - \text{time with the familiar object})}{\text{(total time with both objects)}}
\]

*Novel object location-* During testing, one object was moved 17 cm away from its previous location and rats were placed in the open field for 5 minutes.
The following discrimination ratio was used to assess novel object location: 
\[
\frac{(\text{time with the relocated object} - \text{time with the stationary object})}{(\text{total time with both objects})}
\].

**Western blotting:**

Twenty-four hours after the end of behavioral training, all rats were killed by decapitation. The tissue preparation process has been previously described (Kathirvelu et al., 2012). Tissue homogenates from the dorsal hippocampus and dorsal striatum of individual rats were normalized by total protein concentrations with a 1:1 mixture of 2x sample buffer: running buffer. Normalized homogenates from samples (15 µg total protein/25 µl for pCREB and CREB; 40 µg total protein/25 µl for I-2) and standards (homogenates from samples from naïve rats in a linear range from 5 to 45 µg for pCREB and CREB; 20 to 60 µg for I-2) were separated by gel electrophoresis. The gels were electrophoretically transferred to PVDF immobilon membranes (Millipore). Membranes were washed with 0.05 M PBS 3 x 5 min and blocked with NFDM-TWEEN-PBS 3 x 15 min, then incubated overnight at 4 °C in rabbit anti-phospho-CREB antibody (1:1000; Cell Signaling), rabbit anti-CREB antibody (1:1000; Cell Signaling), or goat anti-mouse Inhibitor-2 antibody (1:100; R&D systems). The membranes were washed in 0.05 M PBS and blocked with NFDM-TWEEN-PBS before incubation at room temperature for 1.5 hours in goat anti-rabbit secondary HRP (1:10000; Kirkegaard & Perry Laboratories) for the CREB and pCREB primary antibodies or in mouse anti-goat IgG HRP (1:5000; R&D systems) for the I-2 primary antibody. The membranes
were washed in 0.05 M PBS-TWEEN 15 x 6 min, then incubated in a chemiluminescent reagent (SuperSignal West Pico Chemiluminescent substrate, Thermo Scientific), and exposed to film.

**Quantification of protein levels:**

Images of the western blot films were taken using Flashpoint 128 Framegrabber software. Integrated optical density and target area measurements were made for both known protein standards and unknown samples using MCID Analysis 7.0 software. A standard curve was created from the known protein concentrations of the standards in order to calculate the protein concentrations of the unknown samples (Colombo et al., 1997).

**Statistical analysis:**

All data were analyzed using SPSS software. For the spatial memory task, repeated measures ANOVAs were used to analyze group differences in latency to find the platform across days, percent time in target quadrant across probes, and proximity to the platform across probes. *A posteriori* comparisons were performed using Fisher’s LSD test. One-way ANOVAs were used to analyze group differences in freezing for contextual fear conditioning, time spent with the novel or relocated objects, and protein levels. One-sample t-tests were used to determine if the groups performed significantly different from chance during the novel object recognition and novel object location tests. Significance levels for all tests were set at p<0.05. There were no significant differences between lenti-
GFP-controls and surgical shams on any of the behavioral measures or the western blotting results, so these data were pooled into one control group for all subsequent analyses.

3. Results

Infusion of I-2shRNA into the dorsal hippocampus enhances spatial memory in a water maze:

For latency to find the hidden platform across days, a repeated measures ANOVA revealed a significant effect of day [F(1,8)=4.397, p=0.013], no significant effect of group [F(1,8)=0.827, p=0.390], and no day x group interaction [F(1,8)=4.615, p=0.116] (Figure 22). A posteriori analysis of the day effect revealed that rats infused with lenti-I-2shRNA demonstrated a significant decrease in latency to find the platform during acquisition of a place task [F(1,4)=7.704, p=0.004], whereas controls did not [F(1,4)=0.576, p=0.642].
**Figure 22.** Spatial learning in a water maze. Rats infused with lenti-I-2shRNA demonstrated a decrease in latency to find the platform ($p=0.004$), while controls did not ($p=0.642$).

During the last trial on days 2 and 4 of a place task, all rats were given a probe trial to measure spatial bias during which the platform was retracted for 30 seconds. Spatial bias was measured as percentage of time during the 30-second probe that rats spent swimming in the quadrant of the pool containing the retracted platform (the target quadrant). For the probes, a repeated measures ANOVA revealed a significant effect of group [$F(1,8)=9.064$, $p=0.020$], no significant effect of day [$F(1,8)=1.268$, $p=0.301$], and no day x group interaction [$F(1,8)=0.243$, $p=0.637$] (Figure 23). This demonstrates that rats infused with lenti-I-2shRNA spent significantly more time in the target quadrant and had a greater spatial bias for the location of the hidden platform than controls.
**Figure 23.** Spatial memory in a water maze. Lenti-I-2shRNA rats spent significantly more time in the target quadrant than controls during the probe trials (p=0.020).

For proximity to the platform during the probes, a repeated measures ANOVA revealed a significant effect of group [F(1,8)=5.731, p=0.048], no significant effect of day [F(1,8)=3.651, p=0.098], and no day x group interaction [F(1,8)=0.003, p=0.960] (Figure 24). This demonstrates that rats infused with lenti-I-2shRNA swam significantly closer to the location of the retracted platform than controls during the probes of spatial memory.
Figure 24. Spatial memory in a water maze. Lenti-I-2shRNA rats swam significantly closer to the platform than controls during the probe trials (p=0.048).

Infusion of I-2shRNA into the dorsal hippocampus enhances fear memory:

After place learning in a water maze, rats were given one session of contextual fear conditioning and were tested for fear memory 24 hours later. During the first two minutes of the fear memory test, there was a significant difference in percent freezing between the lenti-I-2shRNA rats and controls [F(1,8)=6.989, p=0.030] (Figure 25).
Figure 25. Contextual fear conditioning. Lenti-I2shRNA rats demonstrated significantly more freezing than controls (p=0.030).

This demonstrates that rats infused with lenti-I-2shRNA had a stronger contextual fear memory than controls during the first two minutes of testing. It has been previously demonstrated that fear memory is strongly revealed during the initial two minutes of testing (Kathirvelu et al., 2012). These behavioral data are consistent with the results of spatial water maze testing, and support the role of I-2 as a negative regulator of memory formation.

Infusion of I-2shRNA into the dorsal hippocampus does not enhance novel object recognition or novel object location.
For the 24-hour novel object recognition test, there was not a significant difference between treatment groups in time spent with the novel object versus time spent with the familiar object [F(1,8)=1.236, p=0.309] (Figure 26). One-sample t-tests revealed that neither rats infused with lenti-l-2shRNA [t(5)=0.223, p=0.838] nor controls [t(5)=-1.304, p=0.289] discriminated between objects during the 24-hour test.

Figure 26. Novel object recognition. Lenti-l-2shRNA rats and controls spent an equivalent amount of time with the novel and familiar objects (p=0.309).

For the 24-hour novel object location test, there was not a significant difference between groups in time spent with the relocated object versus time spent with the stationary object [F(1,8)=1.900, p=0.205] (Figure 25). One-sample t-tests revealed that neither rats infused with lenti-l-2shRNA [t(5)=-0.970, p=0.367] nor controls [t(5)=1.236, p=0.268] discriminated between objects during the 24-hour test.
p=0.387] nor controls [t(5)=1.043, p=0.356] discriminated between locations during the 24-hour test.

Figure 27. Novel object location. Lenti-I-2shRNA rats and controls spent an equivalent amount of time with the relocated and stationary objects (p=0.205).

Infusion of I-2shRNA into in the dorsal hippocampus increases pCREB in the dorsal hippocampus, but not in the dorsal striatum:

Western blotting revealed no significant difference between lenti-I-2shRNA rats and controls for I-2 protein levels in the dorsal hippocampus [F(1,7)=0.080, p=0.787] (Figure 28).
Figure 28. Mean inhibitor-2 protein levels in the dorsal hippocampus. Lenti-I-2shRNA rats and controls had equivalent levels of I-2 in the dorsal hippocampus (p=0.787).

However, there were significantly greater levels of pCREB in the lenti-I-2-shRNA group compared to controls in the dorsal hippocampus [F(1,7)=10.509, p=0.014] (Figure 29).
Figure 29. Mean pCREB protein levels in the dorsal hippocampus. Lenti-I-2shRNA rats had significantly more pCREB than controls in the dorsal hippocampus (p=0.014).

Increased pCREB protein levels in the dorsal hippocampus of lenti-I-2shRNA rats was not due to an elevation of total CREB protein levels, because the amount of total CREB in the dorsal hippocampus was statistically equivalent between groups [F(1,7)=0.545, p=0.484] (Figure 30).
Figure 30. Mean CREB protein levels in the dorsal hippocampus. Lenti-I-2shRNA rats and controls had equivalent levels of total CREB in the dorsal hippocampus (p=0.484).

Western blotting revealed that there was no difference in pCREB levels between lenti-I-2shRNA rats and controls in the dorsal striatum [F(1,7)=0.007, p=0.935] suggesting that the effect of lenti-I-2shRNA infusion on pCREB was restricted to the targeted region (Figure 31). These results indicate that expression of I-2shRNA increases pCREB protein levels in the dorsal hippocampus and is independent of pCREB protein levels in the dorsal striatum.
Figure 31. Mean pCREB protein levels in the dorsal striatum. Lenti-I-2shRNA rats and controls had equivalent levels of pCREB in the dorsal striatum (p=0.935).

4. Discussion

The results of this study support the hypothesis that localized gene silencing of I-2 in the dorsal hippocampus enhances memory for hippocampus-dependent memory tasks and increases pCREB protein levels in the dorsal hippocampus. Infusion of I-2shRNA into the dorsal hippocampus enhanced spatial memory in the water maze and context-dependent fear memory, but did not enhance memory for novel object recognition and location. There was a significant increase in pCREB protein levels in the dorsal hippocampus despite
equivalent levels of I-2 in the dorsal hippocampus. These data provide evidence that I-2 is a suppressor of hippocampus-dependent memory formation, and importantly, that the mechanism of this effect is most likely due to CREB dephosphorylation.

Rats infused with lenti-I-2shRNA demonstrated enhanced spatial memory in a water maze compared to controls. In addition, rats infused with lenti-I-2shRNA exhibited a much stronger fear memory than controls during the contextual fear memory test. Infusion of lenti-I-2shRNA did not enhance memory for novel object recognition and novel object location. In order to test memory enhancement, relatively weak training paradigms were used so that controls would not perform at levels too high to demonstrate enhancement. Accordingly, the most likely explanation for the current findings is that novel object recognition and location training was too weak for rats to form a memory trace, thus memory enhancement was not possible. It is also plausible, but unlikely that memory enhancement due to expression of I-2shRNA does not generalize to these hippocampus-dependent memory tasks. The findings of the current study, suggest that I-2 is a suppressor of memory, because knocking down I-2 in the hippocampus enhanced memory for aversively motivated hippocampus-dependent tasks.

This lentivirus has previously been shown to decrease I-2 (Hou et al., 2013) and pCREB (unpublished result) in cultured cortical neurons, but the effect of lentiviral I-2 knockdown in dissected tissue homogenates has not been tested. The finding that infusion of I-2 shRNA did not decrease I-2 levels in the dorsal
hippocampus was unexpected. It has previously been shown that increases in 
CREB in a small percentage neurons (~15%) can lead to robust memory 
enhancement following infusion with HSV-CREB in the amygdala (Josselyn, 
2010; Josselyn et al., 2001). So it is possible that infection of lenti-I-2shRNA in 
only a small subset of neurons in the hippocampus was sufficient to cause a 
robust effect on memory.

Lentiviral expression of I-2shRNA increased pCREB protein levels in the 
dorsal hippocampus, but not the dorsal striatum. There was no difference in 
CREB protein levels between groups in the dorsal hippocampus. This 
demonstrates that the increase in pCREB was not due to more total CREB in the 
rats infused with lenti-I-2shRNA. The equivalent levels of pCREB in the dorsal 
striatum demonstrate that the increase in pCREB in rats infused with lenti-I-2shRNA was specific to the dorsal hippocampus. These data also indicate that 
increases in pCREB in the hippocampus do not have a suppressive effect on 
striatal pCREB twenty-four hours after testing, which would indicate a competitive 
interaction between the hippocampus and striatum. The enhancement of spatial 
memory and fear memory as well as increased pCREB levels in the 
hippocampus of normal functioning animals following lenti-I-2shRNA infusion 
lends new support to previous reports that I-2 works as an activator of PP1 in 
vitro (Hou et al., 2013; Nigavekar et al., 2002; Oliver & Shenolikar, 1998; Tung et 
al., 1995). Knocking down I-2 in cortical neurons down-regulates PP1 activity 
(Hou et al., 2013) and blocking PP1 has been shown to increase phosphorylation 
of CREB in cell culture (Alberts et al., 1994; Bito et al., 1996). PP1 has previously
been described as a ‘molecule of forgetfulness’, which suppresses memory formation and long-term maintenance of memory (Genoux et al., 2002; Silva & Josselyn, 2002). The finding that infusion of I-2shRNA increased levels of pCREB supports the role of I-2 as an activator of PP1 and explains these findings. When I-2 is knocked down in vivo, I-2 cannot activate PP1, so PP1 is prevented from dephosphorylating pCREB at serine 133. Sustained phosphorylation of CREB at serine 133 likely leads to enhanced memory formation and a decreased likelihood of memories to extinguish. Place learning led to sustained increases in hippocampal pCREB and Fos (Colombo et al., 2003) and this current study demonstrates that maintaining increases in hippocampal pCREB with I-2shRNA enhances hippocampus-dependent memory, most likely by down-regulating PP1 activity.

5. Conclusions

This study tested the hypothesis that localized gene silencing of I-2 in the dorsal hippocampus enhances memory for hippocampus-dependent tasks and increases pCREB protein levels in the dorsal hippocampus. Infusion of lenti-I-2shRNA in the dorsal hippocampus enhanced spatial memory, contextual fear memory, and increased pCREB in the dorsal hippocampus. These data demonstrate that I-2 is a suppressor of memory formation and provide new evidence consistent with the theory that I-2 functions as an activator of PP1. The findings of the current study identify inhibitor-2 regulation of PP1 activity as a potential mechanism for controlling sustained increases in pCREB during
memory formation. During learning, it is possible that inhibitor-2 functions to increase PP1 activity in regions not necessary for formation of a memory, so that levels of pCREB remain elevated in the brain region contributing to memory formation.
CHAPTER VII. GENERAL DISCUSSION

The hippocampus and striatum have dissociable roles in learning and memory. The hippocampus is necessary for spatial navigation and place learning, while the striatum is necessary for habit formation and response learning. In a previous study, a virtual dual solution place/response task was used to probe which strategy individuals predominately use during learning (Bohbot et al., 2007). Individuals who used a place strategy on the dual solution task had increased grey matter in the hippocampus, while response learners had increased grey matter in the caudate. This demonstrates that use of a learning strategy is related to system level changes in the hippocampus and striatum, but does not identify the underlying cellular mechanisms that influence behavior. The experiments in this dissertation were designed to investigate how cellular mechanisms of memory formation influence behavior in the hippocampus and striatum during learning. This was accomplished by testing relationships between Ca^{2+}-mediated proteins and learning on tasks dependent on the hippocampus and striatum. As indicated in the paragraphs that follow, the results of the current studies have important implications for cellular mechanisms of memory formation, cellular mechanisms for competitive interactions among systems specialized for memory, and phosphatase-based regulation of CREB activity in multiple memory systems.
A. Cellular mechanisms of learning strategies in the hippocampus and striatum

An overall finding of this dissertation is that differences in memory formation are related to changes in proteins necessary for learning in the hippocampus and striatum. Acquisition of a response task in a water plus maze was related to phosphorylation of the TrkB receptor in the dorsomedial and dorsolateral striatum (Chapter III). All rats learned to the same level, but some rats took longer to learn the task than others. Rats that took the longest to learn the response task had the highest levels of pTrkB in subregions of the dorsal striatum. This finding suggests that individual differences in pTrkB in the dorsal striatum may be related to striatal processing styles (stimulus-response and expectancy-outcome) rather than the speed of task acquisition. Performance on a place task in a water plus maze was related to levels of Fos in the dorsal hippocampus of rats that received intrahippocampal infusions of saline (Chapter IV). Animals with the best performance on the place task made more correct arm choices and fewer errors when searching for the hidden platform. For rats infused with saline, success on the place task was more likely with greater levels of Fos in the dorsal hippocampus. Interestingly, these relationships between learning and plasticity were not found in non-learning and impaired learning conditions. In the subregions of the dorsal striatum, there was no relationship between pTrkB levels and total trials in swim controls that were not trained on the response task. In the dorsal hippocampus, there was no relationship between
Fos levels and correct choices in rats that received intra-hippocampal infusions of GABA-A agonist, muscimol, and were impaired on the place task.

Acquiring a memory task does not cause a uniform increase in protein levels among animals. There is variability in protein levels measured after training as well as differences in acquisition of memory tasks among individual animals. The findings of this dissertation demonstrate that this variability in protein levels and these differences in learning are related, and suggest that these relationships are involved in the formation of memory traces. There is strong evidence that variation in protein levels and excitability within individual neurons prior to learning determines which cells are preferentially recruited into a memory trace (Han et al., 2007; Yiu et al., 2014). There is also evidence that selectively deleting these neurons after learning blocks memory recall during testing (Han et al., 2009). Accordingly, it is possible that variation in protein levels within these same individual neurons after learning determines the strength of the memory. The current findings demonstrate that differences in task performance in non-learning and impaired-learning conditions are not related to variability in protein levels. This suggests that changes in protein levels in these conditions are uninvolved in formation of a memory trace. It is likely that individual increases in proteins levels with repeated use of hippocampus-dependent or striatum-dependent learning strategies strengthens the neuronal circuits involved in the formation of memory traces and leads to system level changes in these structures such as increased grey matter.
The findings in this dissertation also have implications for changes in regions not necessary for acquisition of a particular memory task. There was no relationship between Fos protein levels in the dorsolateral striatum and place learning, suggesting independence between the hippocampus and striatum during memory formation when measuring striatal Fos (Chapter IV). However, response learning was negatively associated with pTrkB protein levels in the dorsal hippocampus (Chapter III). These data indicate that rats that took the longest to learn the response task had the highest levels of pTrkB in cell fields of the hippocampus, supporting a competitive interaction between the hippocampus and striatum. This conclusion suggests that use of hippocampus-dependent or striatum-dependent learning strategies, which are likely to lead to system level changes in one brain structure, impair changes in the opposite structure. These data also highlight the tendency in the literature for evidence of both independence and interactions during memory formation. Exploration of conditions that influence the relative engagement of the hippocampus and striatum during memory formation will be discussed in the next section, as will the potential contribution of these conditions to whether or not the hippocampus and striatum function independently or are interactive during memory formation.

**B. Cellular mechanisms for competitive interactions between the hippocampus and striatum**

There are numerous reports supporting the theory that the hippocampus and striatum interact in a competitive manner during memory formation.
However, the underlying mechanisms for interactions between the hippocampus and striatum are still unknown (Packard & Goodman, 2013). An important conclusion from this dissertation is that suppression of plasticity in the dorsal hippocampus after response learning is likely a cellular mechanism for competition. This finding provides cellular evidence for how the hippocampus and striatum compete during memory formation. A previous report demonstrated that infusing glucose into the dorsal striatum impaired acquisition of a place task (Pych et al., 2006), but the cellular mechanism for impaired place learning was not identified. Based on the finding that response learning suppresses of hippocampal pTrkB, it is likely that infusion of glucose into the striatum impaired place learning by decreasing protein levels in the hippocampus after learning. Interestingly, response learning led to suppression of pTrkB in the dorsal hippocampus (Chapter III), but enhancement of hippocampal function through infusion of lentiviral-I-2shRNA did not cause suppression of plasticity in the dorsal striatum (Chapter V). In the lentiviral I-2shRNA study, levels of pCREB in the hippocampus and striatum were measured past the point where any learning-induced increases in protein levels could be measured. These findings indicate that the advantaged memory system only suppresses plasticity in the opposite region during a specific time window after learning. This time window of suppression is most likely during memory formation and not after consolidation of the memory.

The same mechanism of opposing action on plasticity in the memory system not necessary for task acquisition explains enhancement of striatum-
dependent memory following inactivation of the hippocampus in previous studies. Based on the current findings it is likely that impairing the hippocampus increases excitability and protein levels in the striatum, which subsequently facilitates striatum-dependent memory. This hypothesis was tested in the study where the hippocampus was impaired with muscimol during response learning (Chapter IV). The muscimol inactivation study was an attempt to establish a re-testable paradigm for investigating competitive interactions at both a cellular and behavioral level of analysis. However, independence between the hippocampus and striatum during response learning was found instead. Sherry and Schacter (1987) proposed the theory that multiple memory systems evolved to process incompatible information, and are therefore functionally specialized for a particular processing style as a result. This allows parallel processing of sensory information and independence between the hippocampus and striatum during memory formation (White & McDonald, 2002). There are different factors that can influence the relative activation of hippocampus and striatum during learning. Altering these factors is likely to influence which memory system controls behavioral output, and whether or not the hippocampus and striatum are competitive (Packard & Goodman, 2013). It has been previously proposed that insufficient extramaze cues may prevent activation of the hippocampus during response learning, and therefore prevent competition between the hippocampus and striatum (Chang & Gold, 2004; Packard & Goodman, 2013; Pych et al., 2005). A cue-rich environment was used in the muscimol inactivation study, so a finding of this dissertation is that response learning in a cue-rich environment is
not sufficient to engage the hippocampus in a competitive manner. Instead, these findings suggest that increased stress during response learning may lead to preferential engagement of the striatum, possibly diminishing the distracting effect of extramaze cues and leading to independence between the memory systems. Future experiments would need to explore conditions, including stress level, which influence the relative activation of the hippocampus and striatum in order to establish a re-testable paradigm for demonstrating competitive interactions.

C. Phosphatase based regulation of phosphorylated CREB in multiple memory systems

This dissertation provides new evidence that I-2 is a suppressor of CREB phosphorylation and memory formation, most likely by increasing PP1 activity (Chapter V). In addition, inhibitor-2 regulation of PP1 activity was identified as a probable mechanism for controlling sustained increases in pCREB during memory formation. Place learning led to sustained increases in pCREB in the dorsal hippocampus, but not the dorsal striatum (Colombo et al., 2003). During place learning, it is possible that inhibitor-2 functions to increase PP1 activity in striatum, so that levels of pCREB remain elevated in the hippocampus, but return to baseline levels in the striatum. These data indicate that phosphatase based regulation of phosphorylated CREB in multiple memory systems contributes to memory formation.
Targeted knockdown of I-2 has important implications for therapeutic interventions of hippocampal memory impairments associated with aging. Both episodic memory and hippocampal grey matter have been shown to sharply decline with age (Foster, 1999; Hedden & Gabrieli, 2004). In addition, there is evidence of protein phosphatase dysregulation in aged animals. In a previous study, levels of pCREB in the hippocampus of young rats significantly increased after fear conditioning compared to basal levels (Monti, Berteotti, & Contestabile, 2005). In aged animals, however, there was not a significant increase in pCREB levels after learning. Levels of protein phosphatase 2B, calcineurin, decreased in young rats after fear conditioning, but did not decrease in aged rats after learning. This suggests that dysregulation of phosphatase activity could be a cause of age-related memory impairments and pCREB dysregulation. Future research should investigate if knocking down I-2 in aged animals could prevent or reverse age-related memory impairments.
CHAPTER VIII. LIST OF REFERENCES


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BIOGRAPHY

Amanda Rosemary Pahng was born on April 8\textsuperscript{th}, 1987 in Atlanta, Georgia to Tom Hill and Jan Riley. Amanda attended North Springs Chapter High School in Atlanta where she was enrolled in the Math & Science magnet program. After graduation, Amanda attended the University of Georgia in Athens where she graduated \textit{magna cum laude} in May 2009 with a Bachelor of Science in Psychology and a Minor in Statistics. In fall of 2009, Amanda entered the Neuroscience Ph.D. program at Tulane University. She worked under the direction of Dr. Paul Colombo to investigate the cellular mechanisms of memory formation in the hippocampus and striatum. In summer of 2015, Amanda accepted a NIAAA-funded T32 post-doctoral position in the Department of Physiology at Louisiana State University under the direction of Dr. Scott Edwards to investigate neurobiological changes associated with drug and alcohol abuse.