EXAMINATION OF NEOCORTICAL PLASTICITY DURING FOREBRAIN-DEPENDENT TRACE-ASSOCIATIVE LEARNING TASKS

BY

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DISSERTATION

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Abstract

Classic studies utilizing general learning and memory tasks, such as environmental enrichment and acrobatic training paradigms, have robustly demonstrated increased neocortical dendritic spine density following various types of general learning. Though these studies have been instrumental in revealing experience-induced and general learning-induced plasticity, the timing of the anatomical and molecular modifications underlying these general learning and memory tasks (as well as the specific type of learning involved with these changes) are difficult to pinpoint. To date, neocortical plasticity at different time points of a more specific learning and memory task, such as associative learning, has not been closely examined.

One associative learning task that is suitable to examine neocortical modifications during different time points of learning is trace-eyeblink conditioning. During eyeblink conditioning, subjects are presented with a neutral, conditioned stimulus (CS) (i.e., tone or whisker deflection) paired with a salient, unconditioned stimulus (US) (i.e., mild periorbital eyeshock) to elicit an unconditioned response (UR) (i.e., eyeblink). With multiple CS-US pairings, subjects learn to associate the CS with the US and exhibit a conditioned response (CR) (i.e., eyeblink) when presented with the CS. In trace conditioning, there is a stimulus free interval between the CS and the US. Acquisition for trace conditioning is forebrain-dependent because it requires an intact neocortex and hippocampus (Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995; Weiss et al., 1999a; Takehara et al., 2002; Galvez et al., 2007).

Using the trace-eyeblink conditioning paradigm with whisker stimulation as the CS (whisker-trace-eyeblink: WTEB), previous findings have demonstrated that primary somatosensory cortex (barrel cortex) is required for WTEB conditioning acquisition and retention (Galvez et al., 2007). Additionally, findings have demonstrated that this trace-
associative learning results in an expansion of the cytochrome oxidase stained representation for the conditioned whisker barrels in layer IV of primary somatosensory cortex (Galvez et al., 2006; Galvez et al., 2011; Chau et al., 2013a). Together, these findings demonstrate that WTEB conditioning is a suitable task to examine neocortical anatomical and molecular modifications at different time points of trace-associative learning. Furthermore, findings from these studies demonstrate that acquisition for this trace-association results in neocortical cytochrome oxidase plasticity; however, the underlying modifications for this trace-association are unknown.

Based upon the previously mentioned findings demonstrating experience-induced synaptic modifications, one possible cause for the increase in metabolic activity following WTEB conditioning is synaptic modification. Findings from Chapter 2 demonstrating increased synapsin I expression, a presynaptic marker, in the conditioned barrels following WTEB conditioning support that synaptic modifications occur following learning. Closer examination of learning-induced synaptic modifications in Chapter 3 demonstrating transient spine proliferation during WTEB conditioning suggests that learning results in structural plasticity, or neocortical rewiring, that is time-dependent. Furthermore, findings from Chapter 5 demonstrating a similar timeline for transient up-regulation of calcium-related and synapse-related genes during the acquisition phase strongly suggest that the changes in these calcium-related and synapse-related genes are underlying the transient learning-induced structural plasticity. Additionally, findings from Chapter 4b and Chapter 6 demonstrate learning-induced plasticity in other areas of the brain that have been shown to play pivotal roles in learning and memory, such as the amygdala. Collectively, findings from this dissertation suggest that multiple brain regions work in synchrony to establish and fine-tune new connections during learning.
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Table of Contents

Section One: Introductory Material ................................................................................................ 1

    Chapter 1: Experience-Induced Anatomical & Synaptic Plasticity........................................ 2

Section Two: Examining Learning-Induced Anatomical Correlates of Forebrain-Dependent
Trace-Associative Learning ........................................................................................................ 31

    Chapter 2: Synapsin I Analysis of S1 Following WTEB Conditioning ................................. 32
    Chapter 3: Golgi-Cox Analysis of S1 During & Following WTEB Conditioning................. 49

Section Three: Examining Learning-Induced Molecular Correlates of Forebrain-Dependent
Trace-Associative Learning ........................................................................................................ 71

    Chapter 4a: Arc/Arg3.1 Analysis of S1 Following WTEB Conditioning ............................. 72
    Chapter 4b: Arc/Arg3.1 Analysis Following One-Trial Trace-Fear Conditioning............... 86
    Chapter 5: Genome-Wide Analysis of S1 During & Following WTEB Conditioning .......... 106

Section Four: Examining Learning-Induced Mechanisms at the Systems-Level ....................... 130

    Chapter 6: Systems-Level Learning-Induced Mechanisms ............................................... 131

Section Five: Conclusion ............................................................................................................ 150

References ................................................................................................................................... 154
Section One: Introductory Material
Chapter 1: Experience-Induced Anatomical & Synaptic Plasticity
Findings from classic behavioral models of learning and memory, such as enriched rearing, acrobatic training and sensory deprivation, have demonstrated robust anatomical and synaptic modifications following exposure to a new experience. Additionally, findings from a prominent molecular model of learning and memory, long-term potentiation (LTP), have also shown anatomical plasticity consistent with findings from behavioral models of learning and memory. The findings from these studies are discussed below.

**Examples from Behavioral Models**

**Enriched Rearing / Environmental Complexity**

Experience-induced neocortical anatomical modifications were first shown in behavioral models of learning and memory using enriched rearing / environmentally complex (EC) paradigms. Typically, for these types of paradigms, subjects are placed into a complex environment where they can socialize with other subjects and interact with various toys and objects for an extended period of time (ranging anywhere from one to three months). One of the first findings from this type of paradigm, conducted during development, showed an increase in weight across all cortical regions, especially the visual cortex (Rosenzweig et al., 1962; Bennett et al., 1964). Findings from these initial studies demonstrated that experience during development modifies the anatomy of the brain, especially neocortical regions. Additional investigations, also conducted during development, examining Golgi-Cox stained neurons in various cortical regions, including the visual cortex, showed that there was significantly more branching in higher-order dendrites in EC subjects compared to controls (Greenough and Volkmar, 1973; Greenough et al., 1973). Further investigations demonstrated that EC subjects had greater spine density in the visual cortex than controls (Globus et al., 1973; Diamond et al., 1975; Turner and Greenough, 1985; Kolb et al., 2003), suggesting that experience can induce...
synaptogenesis. In addition to developmental analyses, EC paradigms conducted for three months during adulthood found similar anatomical changes, such as increased cortical weight (Rosenzweig et al., 1964) and increased dendritic branching in higher-order dendrites of EC subjects compared to controls (Green et al., 1983). These analyses suggest that these anatomical dendritic spine modifications can occur into adulthood. Together, findings from these studies suggest that extensive periods of enriched experience during either development or adulthood can induce anatomical dendritic spine modifications and synaptic proliferation in primary neocortical regions of the brain.

Findings from EC paradigms have demonstrated that neuronal properties can change following an extended period of enrichment; however, the specific type of learning and when these anatomical and synaptic modifications occur as a result of that learning is hard to determine with these types of paradigms. More specifically, multiple types of learning, such as associative and procedural learning from interacting with their environment, are occurring during EC paradigms making it difficult to pinpoint the exact experience that produces some or all of the observed anatomical spine modifications. Furthermore, with EC paradigms, it is difficult to determine whether learning or activity (both physical and via sensory stimulation) produces these neocortical anatomical changes. Acrobatic training tasks, paradigms that are specific to motor learning, have helped further demonstrate that anatomical modifications result from learning, and not just sensory stimulation or activation.

**Acrobatic Training**

In acrobatic training paradigms, subjects learn to traverse through a series of complex obstacles that enhance their motor skills. In typical acrobatic training studies, subjects are trained with one of the following conditions: acrobatic conditioning (subjects learn to traverse
through a series of complex obstacles), motor control conditioning (subjects traverse through an obstacle-free runway or have free access to a running wheel) or inactive control (subjects receive no motor training). Findings from these acrobatic training paradigms have demonstrated an increase in the number of synapses per neuron in the motor cortex of acrobatic conditioned subjects compared to motor control conditioned and inactive control subjects (Kleim et al., 1996). These analyses demonstrated that motor learning, and not just motor activity, induces neocortical synaptogenesis. Additional investigations using electron microscopy also found that compared to motor and inactive controls, subjects that were acrobatically trained exhibited more synapses between the parallel fiber and Purkinje cells in the cerebellar cortex, a brain region heavily involved with motor coordination and balance (Kleim et al., 1998; Federmeier et al., 2002). Together, these findings demonstrate that motor skill learning induces synaptic changes in the motor cortex and cerebellum, and that these changes are not due to motor activity alone. Furthermore, findings from these studies, similar to findings from EC studies, suggest that learning induces neocortical synaptic modifications.

Though acrobatic training paradigms involve a more specific type of learning compared to EC paradigms, the learning involved with acrobatic training paradigms still includes a variety of motor skills, such as balance and coordination. Additionally, these paradigms involve a variety of sensory systems. Furthermore, these paradigms predominately focus on examination of procedural learning. A category of paradigms that are more specific than acrobatic training and allow for a more precise observation of neocortical plasticity are sensory deprivation paradigms.
**Sensory Deprivation**

Sensory deprivation paradigms are more specific than acrobatic training paradigms due to their dependence on direct manipulation of a specific sensory modality. One common sensory deprivation paradigm is whisker deprivation. With whisker deprivation, whiskers are removed or trimmed for varying lengths of time to induce plasticity in primary somatosensory cortex. Note that an advantage of using the rodent whisker system is that sensory information from individual whiskers are sent to a specific region in layer IV of primary somatosensory cortex (whisker barrel cortex) in a 1:1 configuration, resulting in a somatotopic map of the whisker pad (Woolsey and Van der Loos, 1970).

Findings from whisker deprivation studies conducted during development have demonstrated robust changes to the neocortical barrel representation in layer IV of primary somatosensory cortex. For example, removal of some or all whiskers during development results in alteration of cytochrome oxidase stained neocortical barrel representation in layer IV of primary somatosensory cortex (Wong-Riley and Welt, 1980). Additional whisker deprivation studies conducted with young mice around one month in age have demonstrated increased neocortical dendritic spine turnover in primary somatosensory cortex within forty-eight hours of whisker deprivation (Trachtenberg et al., 2002), demonstrating dendritic spine modifications following active sensory manipulation. Together, these studies demonstrate that whisker deprivation during development induces neocortical anatomical modifications.

Though many of these observed whisker deprivation induced neocortical changes were conducted during development, similar to most EC analyses, whisker deprivation studies conducted during adulthood have also demonstrated that the barrel cortex remains plastic into adulthood. Although deprivation during adulthood does not modify the neocortical barrel
representation in layer IV of primary somatosensory cortex as observed during development (Wong-Riley and Welt, 1980), changes to metabolic activity and dendritic spines within neocortical barrels have been observed. For example, two months of bilateral removal of all but one whisker during adulthood increase spine density in layer IV and decreased spine density in layer III of spared barrels (Kossut, 1998). Furthermore, two months of whisker deprivation in adults also decreases spine elimination rates in deprived primary somatosensory cortex compared to controls (Zuo et al., 2005). Together, findings from these relatively long sensory deprivation studies support that experience via active peripheral sensory manipulations during adulthood produces dendritic spine modifications in primary somatosensory cortex. Currently, no one has examined whether similar dendritic spine modifications occur in adulthood following a brief period of sensory deprivation, consistent with that reported during many learning paradigms.
Anatomical Plasticity in S1 following 5-Days of Whisker Deprivation in Adult Mice*

Introduction

Sensory deprivation, where input from one sensory system is either reduced or eliminated, is one of the most widely used paradigms for exploring experience-dependent neocortical plasticity. One of the more commonly used experience-dependent paradigms in rodents is whisker deprivation. With whisker deprivation, whiskers from the animal’s snout are removed or trimmed for varying lengths of time to induce plasticity in primary somatosensory neocortex. Many studies examining short periods of whisker deprivation during early developmental time points have demonstrated various forms of synaptic and anatomical plasticity (for review, see Holtmaat and Svoboda, 2009). However, there have been few studies examining similar types of plasticity, especially anatomical plasticity, in layer IV of primary somatosensory neocortex following the same short period of whisker deprivation during adulthood. Most adult experience-dependent studies have examined neocortical synaptic and anatomical plasticity following extensive periods of deprivation or stimulation. For example, Polley and colleagues (2004) demonstrated that one-month of rearing adult rodents in a naturalistic environment, where they had extensive opportunities for whisker stimulation, resulted in an augmented metabolic whisker representation in layer IV of primary somatosensory neocortex compared to standard laboratory cage controls. Furthermore, continuous whisker stimulation for twenty-four hours during adulthood has been shown to increase inhibitory synapses in layer IV of respective stimulated whisker barrels (Knott et al., 2002). Similarly, findings from sensory deprivation paradigms have shown increased spine density in layer IV and decreased spine density in layer III of spared barrels following two-months of bilateral removal of all but one whisker (Kossut, 1998). Together, these and other similar analyses strongly suggest that an extended period of sensory stimulation or deprivation during adulthood results in synaptic and anatomical plasticity
in layer IV of primary somatosensory neocortex. However, there have been limited studies examining similar types of neocortical plasticity, especially anatomical plasticity, following brief periods of whisker deprivation during adulthood.

Findings from various learning studies have suggested that brief experiences in adulthood can induce synaptic modifications. For example, associative learning tasks involving the whisker system that take less than five days to acquire have demonstrated metabolic expansion of the conditioned whisker barrel representation in layer IV of primary somatosensory neocortex (Siucinska and Kossut, 1996, 2004; Galvez et al., 2006). Analyses using a similar associative learning task have further demonstrated increased synapsin I expression in layer IV of conditioned barrels in primary somatosensory neocortex (Chau et al., 2013a), suggesting learning-induced synaptic modifications in conditioned neocortical barrels. Synapsin I is a presynaptic phosphoprotein involved with neurotransmitter release (Cesca et al., 2010) and has been shown to correlate with synapse number (Lohmann et al., 1978; Moore and Bernstein, 1989; Chin et al., 1995; Perlini et al., 2011). Collectively, these studies demonstrate that associative learning tasks, especially those involving the whisker system, during adulthood can rapidly induce neocortical metabolic and synaptic plasticity. Findings from these studies further suggest that experience-dependent paradigms, such as whisker deprivation, should also rapidly induce similar types of plasticity in layer IV of primary somatosensory neocortex. In support of this hypothesis, recent analyses found that a brief period of whisker trimming during adulthood resulted in shorter thalamic projections to layer IV of primary somatosensory neocortex (Oberlaender et al., 2012), suggesting that whisker deprivation can rapidly induce layer IV anatomical plasticity in adulthood. However, anatomical properties in layer IV of primary somatosensory neocortex following a brief period of whisker deprivation during adulthood have
not been extensively explored. The present study utilized synapsin I expression and Golgi-Cox staining in unilateral whisker deprived mice to determine if a brief period of whisker deprivation during adulthood results in synaptic and anatomical modification in layers IV and II/III of primary somatosensory neocortex.

Methods

Subjects

Twenty-three adult (three-months in age) C57BL/6J mice were individually housed under a 12 h light/dark cycle with lights on at 7:00AM, and had access to food and water ad libitum.

Whisker Deprivation

Mice were randomly assigned to one of two groups: whisker-deprived or control. Whisker-deprived mice were gently restrained while all whiskers on one-side of their snout were trimmed to less than 1 mm (Figure 1A). Control mice were also restrained, but no whiskers were trimmed. Instead, their whiskers were gently brushed with scissors to mimic the stimulation whisker-deprived mice received during whisker trimming. These procedures were repeated every other day for five days (Figure 1B). All procedures were performed in accordance with guidelines approved by the University of Illinois at Urbana-Champaign’s Institutional Animal Care and Use Committee.

Note, neocortical whisker barrels contralateral to the deprived side of the whisker-deprived mice (i.e., those receiving input from the whiskers that were removed) are referred to as deprived while neocortical whisker barrels ipsilateral to the deprived side of whisker-deprived mice (i.e., those receiving input from the whiskers that not removed) are referred to as spared. Whisker barrels of control mice are referred to as controls.
**Immunohistochemistry**

Mice (whisker-deprived: n= 7, controls: n= 5) were given an overdose of sodium pentobarbital following whisker deprivation and transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 2% paraformaldehyde in PBS. The neocortex was then dissected, flattened, placed in 4% paraformaldehyde overnight at 4°C and transferred into 30% sucrose, where it remained until sectioned. The tissue was sectioned 30 µm tangentially to the cortical surface and collected in a cryoprotective solution (30% sucrose and 30% ethylene glycol in 0.1M PBS). Every third section was stained for cytochrome oxidase (CO) and sections

**Cytochrome Oxidase.** Every third section was incubated in a cytochrome oxidase (CO) solution (0.03% cytochrome c (Sigma-Aldrich), 0.05% 3,3’-diaminobenzidine (DAB) and 4% sucrose in 0.1M PBS) for 3 h at 37°C, rinsed several times with 0.1M PBS, mounted onto slides, dehydrated and coverslipped (Figure 2A).

**Synapsin I.** Sections adjacent to CO-stained tissue exhibiting cortical barrels in layer IV of primary somatosensory cortex were washed several times in PBS and treated with 0.6% H2O2. Sections were then blocked in PBS-X (2% normal donkey serum and 0.5% triton-x in PBS) for 1 h and incubated overnight at 4°C in Anti-Synapsin I (1:500; Sigma-Aldrich). The sections were then washed several times in PBS-X, incubated for 2 h in biotinylated anti-rabbit (1:250; Vector Laboratories), washed several times in PBS-X and incubated in an avidin-biotin complex (ABC) solution (Vectastain) for 1 h. Following several washes in 0.1M PBS, the peroxidase reaction was visualized with a standard diaminobenzidine solution (0.05% DAB, 0.7% nickel ammonium sulphate and 0.01% H2O2). To minimize the immunohistochemical reactivity variability of synapsin I, tissue from both whisker-deprived and control groups were reacted together.

Sections were then washed several times in PBS, mounted onto slides and coverslipped (Figure
An Olympus BX50 microscope with a Zeiss AxioCam ICc 1 camera was used for synapsin-positive puncta visualization.

**Analysis**

For CO analysis of barrel length, digital images were acquired on an Olympus BX50 microscope with a Zeiss AxioCam ICc 1 camera at 2x magnification. Once acquired, ImageJ (NIH, Version 1.45s) was used to measure C row barrel length as previously described (Galvez et al., 2006; Galvez et al., 2007; Chau et al., 2013a). Briefly, a digital image of the barrels was visualized in ImageJ and a rectangle size of 800 x 20 µm² was placed on top of the first two barrels in row C. A histogram of the mean gray values across the rectangle was then generated using ImageJ. The lowest point between these elevated regions was used to delineate boundaries between barrels. Barrel length was measured as the distance between each of these boundaries for the first two barrels in row C. Due to sampling variability in the sections obtained, only the first two barrels in row C were examined.

To examine synapsin I expression, adjacent CO stained tissue was used as a guide to locate the barrel cortex in synapsin I stained tissue at 2x magnification with a Zeiss AxioCam ICc 1 camera attached to an Olympus BX50 microscope. Once the region and barrel of interest were localized, a picture of synapsin I positive expression was taken in the center of the barrel hollow at 60x magnification. The digital image was then visualized on ImageJ and a counting frame of 159 x 118 µ² was placed into the center of the image. Synapsin I positive puncta between 0-100 µ² were counted using the Analyze Particle feature on ImageJ. Synapsin I expression from three barrels of each mouse were examined, averaged and combined into one score for each mouse.
Golgi-Cox

Following five-days of whisker deprivation, mice (whisker-deprived: n = 6, controls: n = 5) were transcardially perfused with PBS and their brains were processed for Golgi-Cox staining (Galvez et al., 2003). Briefly, brains were placed in a standard Golgi-Cox solution until adequately impregnated (25-35 days). Once impregnated, the brains were embedded in 10% celloidin and sectioned at 175 µm. Primary somatosensory cortex (barrel field) was then located based upon coronal landmarks (Franklin and Paxinos, 2007). For analyses in layer II/III, at least six forked apical pyramidal neurons per animal were examined. These neurons have a very distinct Y-shaped apical dendrite and are only located in layers II/III (Figure 4A). Additionally, these neurons have been previously shown to exhibit synaptic plasticity following enriched rearing (Uylings et al., 1978), a type of experience-dependent paradigm. Note that spine density and dendritic analyses for apical and basilar dendrites were analyzed separately. For analyses in layer IV, at least six spiny stellate neurons per animal were examined (Figure 5A). Spine density was determined by counting the number of spines on second and third order branches at least 10 µm in length. For dendritic branch analyses, the bifurcation ratio was determined up to the fourth order branches. Briefly, for each branch order, the number of bifurcating branches was divided by the sum of bifurcating and normal ending for that branch order. For the neuronal bifurcation ratio (NR), the total number of bifurcating branches (all branch orders) was divided by the sum of all bifurcating and normal ending branches (all branch orders).

Statistics

Analyses of CO, synapsin I expression and spine density of Golgi-Cox stained neurons were conducted with a one-way ANOVA. Bifurcation ratio analyses were conducted with a two-way ANOVA. When appropriate, follow-up post hoc analyses used the Fisher's LSD criterion
for significance. All statistical analyses were conducted with SigmaPlot (Systat Software), and all post-hoc analyses exhibiting significant differences had an adjusted p-value below 0.05.

Results

**Cytochrome Oxidase Expression**

Analysis of barrel length in primary somatosensory cortex demonstrated that there was no significant difference between neocortical whisker barrels contralateral to the deprived side of whisker-deprived mice (deprived), neocortical whisker barrels ipsilateral to the deprived side of whisker-deprived mice (spared) and whisker barrels of control mice (control) (C1 barrel: F(2,17)= 0.75, p > 0.05; C2 barrel: F(2,17)= 0.16, p > 0.05; Figure 2B). Note there was no significant difference between the two hemispheres of the control mice, so the values were combined for these analyses. These data suggest that sensory deprivation does not alter the size of the cytochrome oxidase whisker representation.

**Synapsin I Expression**

Analyses of synapsin I expression in layer IV of primary somatosensory cortex demonstrated a significant difference between deprived whisker barrels, spared whisker barrels, and control barrels (F(2,20)= 8.49, p < 0.05; Figure 3B). Note that there was no significant difference between the two hemispheres of the control mice, so the values were combined for these analyses. Follow-up post hoc analyses indicated that the spared whisker barrels of whisker-deprived mice exhibited the greatest synapsin I expression (M= 176.76; SD= 60.24) followed by whisker barrels of control mice (M= 130.26; SD = 34.14) and deprived whisker barrels of whisker-deprived mice (M= 83.33; SD= 28.52). These data suggest that synapsin I expression is greatest in spared whisker barrels followed by control whisker barrels and deprived whisker barrels.
**Golgi Analyses**

**Layers II/III**

At least thirteen neurons were examined in each group. A one-way ANOVA demonstrated a significant difference in spine density of forked apical pyramidal neurons between deprived whisker barrels, spared whisker barrels and control barrels on apical dendrites \( (F_{(2,63)} = 5.04, p < 0.05) \). No differences were detected in spine density between the two hemispheres in the control barrels, so they were combined into one group for these analyses. Follow-up post hoc analyses indicated that the spared whisker barrels of whisker-deprived mice \( (M= 1.27; SD= 0.68) \) exhibited significantly greater spine density than the deprived whisker barrels of whisker-deprived mice \( (M= 0.73; SD= 0.37) \) and whisker barrels of control mice \( (M= 1.10; SD= 0.68; Figure 4B) \). These data suggest that spared whisker barrels exhibited greater spine density than deprived whisker barrels and control whisker barrels in layers II/III. There was no significant difference in spine density on basilar dendrites between deprived whisker barrels, spared whisker barrels or control barrels. Further analyses examining dendritic bifurcation did not detect any significant difference at any branch order or across the entire neuron (Figure 4C).

**Layer IV**

At least seventy-two neurons were examined in each group. A one-way ANOVA demonstrated a significant difference in spine density of spiny stellate neurons between deprived whisker barrels, spared whisker barrels and control barrels \( (F_{(2,272)}= 4.29, p < 0.05) \). No differences were detected in spine density between the two hemispheres in the control barrels, so they were combined into one group for these analyses. No significant differences between the second and third order spine densities across all groups were detected, so these were also
combined into their respective groups. Follow-up post hoc analyses indicated that the deprived whisker barrels of whisker-deprived mice (M= 0.44; SD= 0.27) exhibited a significantly smaller mean spine density than spared whisker barrels of whisker-deprived mice (M= 0.58; SD= 0.32) and control mice (M= 0.55; SD= 0.31; Figure 5B). These data suggest that deprived whisker barrels exhibited less spine density than spared and control whisker barrels. Further analyses using a two-way ANOVA examining dendritic bifurcation demonstrated a significant difference in branching ($F_{(3, 429)} = 89.72, p < 0.05$). Follow up post hoc analyses using Fisher's LSD criterion for significance and corrected for multiple comparisons indicated a significant increase in dendritic branching of spiny stellate neurons in the spared whisker barrel of whisker-deprived mice on third order branches compared to deprived whisker barrels of whisker-deprived mice and control whisker barrels. No other branch orders exhibited a significant difference (Figure 5C).

**Discussion**

Many studies using experience-dependent paradigms have demonstrated neocortical synaptic and anatomical plasticity during adulthood following prolonged periods of sensory deprivation (Kossut, 1998) or stimulation (Knott et al., 2002). Recent studies looking at axonal length of thalamocortical projections demonstrated axonal modifications following a brief period of sensory deprivation during adulthood (Wimmer et al., 2010; Oberlaender et al., 2012), suggesting that anatomical plasticity can also occur in adulthood following brief periods of sensory deprivation. Additionally, associative learning tasks that take less than five days to acquire have shown changes in metabolic activity (Siucinska and Kossut, 1996; Galvez et al., 2006; Chau et al., 2013a) and synaptic properties (Chau et al., 2013a) in layer IV of primary somatosensory cortex. These studies further suggest that neocortical synaptic plasticity can
occur relatively quickly in layer IV of primary somatosensory cortex during adulthood. However, to our knowledge, there have been limited explorations of neocortical plasticity, especially anatomical plasticity in layer IV of primary somatosensory cortex following brief periods of whisker deprivation during adulthood. To examine neocortical synaptic and anatomical plasticity following a brief period of sensory deprivation during adulthood, the following study whisker-deprived adult mice for five days and examined synapsin I expression and Golgi-Cox stained neurons in primary somatosensory cortex.

Our synapsin I expression analyses revealed that five days of whisker deprivation during adulthood elevates synapsin I expression in the spared whisker barrels of whisker-deprived mice compared to the deprived whisker barrels of whisker-deprived mice and whisker barrels of control mice (Figure 3B). These findings suggest that a brief period of sensory deprivation increases the number of synapses in spared whisker barrels compared to deprived and control whisker barrels.

Though it is possible that this increase in synapsin I expression is due to elevated use of the spared whiskers, prior whisker deprivation behavioral analyses suggest that this is unlikely. Analyses have shown that partial unilateral or bilateral whisker deprivation does not alter performance on a cliff detection task, grooming, burrowing or rearing behavior, suggesting that whisker-deprived mice do not selectively increase the use of their non-trimmed whiskers (Glazewski et al., 2007). These analyses further suggest that the increased synapsin I expression in spared whisker barrels are not due to increased use.

Together, our synapsin I analyses revealed that five days of whisker deprivation during adulthood decreased synapsin I expression in the deprived whisker barrels of whisker-deprived mice compared to the spared whisker barrels of whisker-deprived mice and control mice,
suggesting that a brief period of whisker deprivation decreases synapses in the deprived whisker barrels. Collectively, findings from our synapsin I analyses suggest spine elimination in the deprived whisker barrels and spine proliferation in the spared whisker barrels of whisker-deprived mice. To explore the anatomical correlates of the altered synapsin I expression, the present study then examined Golgi-Cox stained neurons in primary somatosensory cortex.

Anatomical analyses in layer IV demonstrated that spine density of spine stellate neurons in the deprived whisker barrels was significantly reduced compared to the spared whisker barrels of whisker-deprived mice and the whisker barrels of control mice (Figure 5B). Previous studies have established that spiny stellate neurons in layer IV of primary somatosensory cortex are excitatory neurons that receive most of their input from the ventral posterior medial (VPM) nucleus of the thalamus (Viaene et al., 2011). These findings suggest that increased spine density in layer IV spiny stellate neurons is due to increased VPM axonal input. Although only spiny stellate neurons were examined, both synapsin I and spine density analyses demonstrated similar results suggesting that the observed structural plasticity of spiny stellate neurons is contributing to the observed modification of synapsin I expression.

Anatomical analyses in layers II/III of primary somatosensory cortex demonstrated a significant increase in spine density of forked apical pyramidal neurons in the spared whisker barrels compared to the deprived whisker barrels of whisker-deprived mice (Figure 4B). Previous studies have reported that layer IV spiny stellate neurons project to and produce EPSPs in these apical pyramidal neurons in layer II/III of the barrel cortex (Feldmeyer et al., 2002). These findings suggest that the observed synaptic changes on forked apical pyramidal neurons are at least partially due to increased axonal innervations from layer IV spiny stellate neurons. Although only one cell type was examined in layers II/III, these findings along with prior studies
(Kossut, 1998) further support that a brief period of sensory deprivation can modulate spine properties on neurons in layers II/III during adulthood. Collectively, analyses from this study demonstrate anatomical plasticity of excitatory neurons in the barrel cortex following a brief period of sensory deprivation during adulthood. Additional studies are needed to determine whether other types of neurons are also modified during adulthood following a brief period of whisker deprivation.

Findings from the present study demonstrate that a brief period of sensory deprivation during adulthood induces neocortical synaptic and anatomical modifications. To our knowledge, this is the first finding demonstrating rapid sensory deprivation induced anatomical plasticity in layer IV of primary somatosensory cortex during adulthood. These findings are consistent with various learning and memory tasks examining neocortical plasticity. For example, approximately five days of associative learning during adulthood has been shown to increase synapsin I expression in layer IV of primary somatosensory neocortex (Chau et al., 2013a). These findings are also consistent with analyses of glucose metabolism and 2DG activation following whisker removal. Dietrich et al (1985) demonstrated that glucose metabolism increased five days following whisker deprivation in the spared hemisphere. Additionally, a similar form of whisker deprivation where only a single whisker or row of whiskers are removed demonstrated increased 2DG activation for the spared whisker(s) as early as seven days following sensory deprivation (Levin and Dunn-Meynell, 1991; Siucinska and Kossut, 1994; Kossut, 1998). Furthermore, trimming all but two whiskers for as little as three days results in significantly more responsive cells in spared whisker barrels compared to controls (Diamond et al., 1993). Although these studies do not directly investigate synaptic changes, the increased
glucose metabolism, 2DG activation and cell response are consistent with the findings from this study demonstrating rapid changes in spine density following five days of whisker deprivation.

Unlike our dendritic spine analyses, examination of the size of the whisker cytochrome oxidase representation did not reveal a deprivation-induced change. These findings are consistent with previous deprivation analyses (Wong-Riley and Welt, 1980); however, they are not consistent with associative learning studies. Various whisker associative learning studies have demonstrated that acquisition results in an expansion of the whisker metabolic representation for the conditioned whiskers in layer IV of primary somatosensory neocortex (Siucinska and Kossut, 1996, 2004; Galvez et al., 2006; Chau et al., 2013a). However, as previously mentioned, our cytochrome oxidase analyses following whisker deprivation did not detect a significant difference in the size of the whisker barrel representation. These data suggest that the mechanisms underlying learning-induced metabolic plasticity are different from the mechanisms engaged with whisker deprivation. Further analyses will be needed to determine the specific experiences that will lead to this form of neocortical plasticity in adulthood.

As discussed, our observed increases in synapsin I expression and spine density in spared whisker barrels following a brief period of sensory deprivation during adulthood are consistent with previous associative learning and experience-dependent paradigms. To our knowledge, this is the first study to demonstrate rapid spine plasticity in adult neocortex following a brief period of sensory deprivation. Additional studies are needed to examine whether other types of plasticity are occurring rapidly in the adult primary somatosensory cortex following dramatic changes to the peripheral sensory system. Collectively, these analyses further suggest that extensive periods of either deprivation or stimulation in adulthood are not the only way to induce plasticity in layer IV of primary somatosensory cortex, as has been previously proposed.
Furthermore, the current study demonstrates that analyses following extensive periods of either deprivation or stimulation during adulthood are missing various rapid forms of plasticity that could provide better insight into understanding other forms of rapid plasticity, such as those reported from associative learning and memory paradigms.
Figure 1. Schematic of whisker deprivation paradigm. (A) Mice in the whisker-deprived group had all their whiskers on one side of their snout trimmed to less than 1 mm. To mimic the whisker stimulation whisker-deprived mice received during whisker trimming, mice in the control group also had all their whiskers on one side of their snout gently brushed with scissors. (B) To ensure that whiskers of whisker-deprived mice stayed below 1 mm, all mice received either whisker trimming (whisker-deprived) or nothing (control) every other day (upside-down triangles) for five days. All mice were collected for either immunohistochemistry or Golgi-Cox staining on the fifth day (oval).
Figure 2. No significant difference in barrel length was observed between deprived, spared and control whisker barrels. (A) Representative photomicrograph of cytochrome oxidase stained barrels in layer IV of primary somatosensory cortex. Scale bar = 200 µm. (B) There was no significant difference in the length of the cytochrome oxidase (CO) stained whisker barrel representation between deprived, spared and control whisker barrels. These findings are consistent with previous reports (Wong-Riley et al., 1980).
Figure 3. Whisker deprivation decreases synapsin I expression in deprived whisker barrels while increasing synapsin I expression in spared whisker barrels. (A) Representative photomicrograph of synapsin I stained barrels in layer IV of primary somatosensory cortex at 2x (left) and 40x magnification (right). Scale bar = 50 µm. (B) Mean synapsin I positive puncta (±SEM) following five days of whisker deprivation (whisker-deprived) or control. Spared whisker barrels exhibited significantly more synapsin I positive puncta followed by whisker barrels of control mice and deprived whisker barrels from whisker-deprived mice. All groups were significantly different from each other. * p < 0.05.
**Figure 4.** Anatomical changes in layers II/III of primary somatosensory cortex following five days of whisker deprivation. (A) Representative photomicrograph of Golgi-Cox stained forked apical pyramidal neuron (arrow) in layers II/III of primary somatosensory cortex. Scale bar = 50 µm. (B) Mean spine density (±SEM) for apical dendrites in deprived and spared whisker barrels of whisker-deprived mice and control mice. Layers II/III apical dendrites in spared whisker barrels of whisker-deprived mice had significantly greater spine density compared to deprived whisker barrels of whisker-deprived mice and control mice. * p < 0.05. (C) Mean bifurcation ratio (±SEM) of neurons in layers II/III in deprived and spared whisker barrels of whisker-deprived mice and control mice (1R = 1st branch order, 2R = 2nd branch order, 3R = 3rd branch order, 4R = 4th branch order and NR = all branch orders).
Figure 5. Anatomical changes in layer IV of primary somatosensory cortex following five days of whisker deprivation. (A) Representative photomicrograph of Golgi-Cox stained stellate neuron (arrow) in layer IV of primary somatosensory cortex. Scale bar = 50 µm. (B) Mean spine density (±SEM) of layer IV neurons in deprived and spared whisker barrels of whisker-deprived mice and control mice. Layer IV neurons in deprived whisker barrels of whisker-deprived mice had a significantly smaller spine density compared to spared whisker barrels of whisker-deprived mice and control mice. * p < 0.05. (C) Mean bifurcation ratio (±SEM) of layer IV neurons in deprived and spared whisker barrels of whisker-deprived mice and control mice (1R = 1st branch order, 2R = 2nd branch order, 3R = 3rd branch order, 4R = 4th branch order and NR = all branch orders). * p < 0.05.
Summary

Together, the findings presented from studies using behavioral models of learning and memory demonstrate robust neocortical anatomical dendritic spine modifications, such as increased dendritic material and spine density, following experience-dependent paradigms. However, as previously discussed, more than one type of learning occurs during EC paradigms making it difficult to determine which experience results in the observed anatomical spine modifications. Additionally, the timeline of the observed neocortical dendritic spine modifications are also difficult to pinpoint with EC paradigms since subjects are in the EC paradigm for an extended period of time. Although findings from slightly more experience-specific paradigms, such as acrobatic training, helped with narrowing down what the animals are learning (motor skill learning), these paradigms still involved more than one type of motor skill learning. Furthermore, these paradigms predominately focus on examination of procedural learning tasks. Sensory deprivation paradigms are even more specific since researchers are actively manipulating the subject’s sensory experience; however, findings from these sensory deprivation paradigms are due to dramatic peripheral manipulations to a sensory system, and are not necessarily in the same category of learning as EC and acrobatic training paradigms.

Examples from a Molecular Model

One of the most widely investigated molecular models of learning and memory is long-term potentiation (LTP). LTP was first discovered by Bliss and Lomo (1973) when they found that high-frequency stimulation (HFS) of the perforant path in the hippocampus resulted in changes to firing rates of granule cells in the dentate gyrus. Findings from their study (and many others since) demonstrating that LTP increases synaptic strength suggest that LTP could be a molecular model for learning and memory.
Many subsequent analyses have established that calcium is essential for LTP induction (Dunwiddie and Lynch, 1979). More specifically, Lynch and colleagues (1983) demonstrated that hippocampal LTP induction was blocked by inhibiting intracellular calcium with ethylene glycol tetraacetic acid (EGTA). Furthermore, later studies demonstrated that calcium released from internal stores results in anatomical dendritic spine changes (Korkotian and Segal, 1999). Together, findings from these studies emphasize the importance of calcium with LTP induction, and further suggest that calcium plays a role in the underlying mechanisms contributing to the observed anatomical dendritic spine changes following LTP induction discussed below.

**Long-Term Potentiation (LTP)**

Many findings from studies examining LTP have demonstrated that LTP induces anatomical spine modifications. For example, studies have shown increased dendritic spine head width two minutes after LTP induction and up to twenty-three hours after stimulation in hippocampal slices (Van Harreveld and Fifkova, 1975; Fifkova and Van Harreveld, 1977; Fifkova and Anderson, 1981; Chang and Greenough, 1984). Additionally, LTP induction in awake animals has been shown to increase spine density in the hippocampus (Medvedev et al., 2012). Together, these studies strongly suggest that LTP induces anatomical spine modifications. Similar to the findings presented from behavioral models of learning and memory, these LTP analyses further demonstrate that a molecular model of learning and memory also results in anatomical modifications of dendritic spines.

**Conclusion**

Together, the findings presented from studies using behavioral and molecular models of learning and memory demonstrate robust anatomical dendritic spine modifications. However, as previously discussed with the behavioral analyses, the type of learning associated with the
anatomical spine modifications are often hard to determine. To date, there have been little investigations exploring neocortical anatomical dendritic modifications following a more specific non-procedural learning and memory task, such as forebrain-dependent associative learning.
Section Two: Examining Learning-Induced Anatomical Correlates of Forebrain-Dependent Trace-Associative Learning
Chapter 2: Synapsin I Analysis of S1 Following WTEB Conditioning*

Introduction

The brain’s ability to alter neuronal connections with experience has been extensively investigated using various behavioral paradigms. For example, auditory discrimination training modulates the preferred frequency in primary auditory cortex (Bakin & Weinberger, 1990; Disterhoft & Stuart, 1976; Kitzes, Farley, & Starr, 1978; Kraus & Disterhoft, 1982; Recanzone, Schreiner, & Merzenich, 1993; Weinberger, Hopkins, & Diamond, 1984). Furthermore, tactile tasks such as roughness discrimination or enriched environment exploration have been shown to increase the whisker neocortical representation in layer IV while decreasing the representation in adjacent layers in primary somatosensory cortex (Guic, Carrasco, Rodriguez, Robles, & Merzenich, 2008; Polley, Kvasnak, & Frostig, 2004; Polley, Rickert, & Frostig, 2005), demonstrating learning-induced changes in neocortical representations of sensory stimuli. Furthermore, acquisition of whisker-trace-eyeblink (WTEB) conditioning expands the neocortical representation for the conditioned whiskers (Galvez, Cua, & Disterhoft, 2011; Galvez, Weiss, Weible, & Disterhoft, 2006). Together, these findings suggest that associative learning induces synaptic modification. However, to our knowledge, there have been no close examinations of neocortical synaptic modification following forebrain-dependent trace associative learning.

To examine neocortical synaptic modification following forebrain-dependent trace associative learning, the present study utilized the trace-eyeblink conditioning paradigm. During eyeblink conditioning, subjects are presented with a neutral, conditioned stimulus (CS) (i.e., tone, light, or whisker deflection) paired with a salient, unconditioned stimulus (US) (i.e., air-puff to the eye or a mild periorbital eyeshock) to elicit an unconditioned response (UR) (i.e., eyeblink). With multiple CS-US pairings, subjects learn to associate the CS with the US and exhibit a
conditioned response (CR) (i.e., eyeblink) when presented with the CS. Trace conditioning is when there is a separation in time between the CS and the US. Acquisition for this form of conditioning is forebrain-dependent because it requires neuronal activation in both the neocortex and the hippocampus (Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995; Weiss et al., 1999a; Takehara et al., 2002; Galvez et al., 2007).

Utilizing the trace-eyeblink conditioning paradigm with whisker stimulation as the CS (whisker-trace-eyeblink: WTEB), we have previously demonstrated that learning results in an expansion of the cytochrome oxidase staining representation, an indicator of metabolic activity, for the conditioned whisker barrels in layer IV of primary somatosensory cortex (Galvez et al., 2006; Galvez et al., 2011). These findings demonstrated that acquisition for the trace association results in neocortical cytochrome oxidase plasticity; however, the cause for this plasticity is unknown. A likely explanation for these findings, that has not been explored, is that trace conditioning induces neocortical synaptic proliferation.

The present study utilized neocortical synapsin I expression to examine learning-induced synaptic proliferation. Synapsin I is part of a phosphoprotein family involved with regulating the release of neurotransmitters at the synapse (Cesca et al., 2010). Increases in synapsin I expression have been associated with synaptogenesis. More specifically, synapsin I expression correlates with synaptogenesis during development (Lohmann et al., 1978; Moore and Bernstein, 1989). Furthermore, knockdown of synapsin I has been shown to correlate with decreases in synaptogenesis (Chin et al., 1995; Perlini et al., 2011). Synapsin I expression is also augmented in the hippocampus following spatial learning (Gomez-Pinilla, So, & Kesslak, 2001), paralleling findings correlating hippocampal synaptogenesis (Ramirez-Amaya et al., 1999; Ramirez-Amaya et al., 2001) and synaptic modification (Miranda et al., 2006) with water maze training. Together,
these findings suggest that synapsin I is a suitable marker for synapse quantity. The following study utilized WTEB conditioning and synapsin I expression to determine if acquisition for a forebrain-dependent trace association induces neocortical synaptic plasticity.

**Methods**

**Subjects**

Eighteen three-month old male C57BL/6J mice were individually housed under a 12 h light/dark cycle with lights on at 7:00AM and had access to food and water *ad libitum*.

**Surgery**

Mice were surgically implanted with a headpiece necessary for WTEB conditioning (Galvez et al., 2009). Mice were anesthetized with a ketamine (1 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.) cocktail. Once anesthetized, a plastic strip connector containing two Teflon-coated stainless steel wires and one ground wire was fitted to the head. The Teflon-coated wires were surgically implanted underneath the skin and emerged around the right periorbital region. The headpiece was secured to the skull with dental acrylic. Mice had at least five days to recover before any behavioral training. All procedures were performed in accordance with guidelines approved by the University of Illinois at Urbana-Champaign’s Institutional Animal Care and Use Committee.

**Behavioral Task**

Training chambers were standard laboratory cages placed inside a sound-attenuated chamber. Mice were connected to a tether via their headpiece and allowed to move freely in the training chamber for 1 h during habituation. Following habituation, mice were randomly assigned to either unpaired conditioning (n = 6) or trace-paired conditioning (n = 8). A computer running routines written on LabView software delivered all stimuli (whisker stimulation and
mild periorbital eyeshock) and acquired behavioral data. Unpaired conditioned mice randomly received either a whisker stimulation or periorbital shock each session with a 22 s mean ITI (varied randomly between 15 to 30 s) (Figure 6A); note, unpaired conditioned mice received the same amount of whisker stimulation and periorbital shock presentation as trace-paired conditioned mice. The unpaired conditioned group (stimulation-control) is consistent with pseudo-conditioning groups used in some studies. Trace-paired conditioned mice received 250 ms of whisker stimulation delivered via a custom-made whisker stimulator (Galvez et al., 2009), 250 ms of stimulus-free (trace) interval followed by 100 ms of periorbital shock (0.1 to 1 mA periorbital square wave shock, 60 Hz, 0.5 ms pulses) (Figure 6B). Note, hippocampal lesions have been shown to impair acquisition with a 250 ms trace interval in mice (Tseng et al., 2004). Trace-paired conditioned mice were given 30 trials per session with a 45-s mean intertrial interval (ITI) ranging from 30 to 60 s. An optic sensor placed in front of the right eye was used to monitor eyelid closure. Using information from the optic sensor, a CR was defined as a 4 standard deviation change in voltage from baseline occurring within 35 ms of CS onset (Figure 6C) (Moyer et al., 1990; Tseng et al., 2004; Weiss and Disterhoft, 2011). All unpaired conditioned and trace-paired conditioned mice received one session of conditioning per day. Trace-paired conditioned mice were trained until a learning criterion of four-CRs during five-consecutive trials was met. Unpaired conditioned mice were randomly yoked to six trace-paired conditioned mice. Naïve mice (n = 4) did not undergo surgery or eyeblink conditioning but were collected at the same time as all of the other mice.

**Histology**

Subjects were given an overdose of sodium pentobarbital 1 h following the last conditioning session and transcardially perfused with 0.1M phosphate buffered saline (PBS)
followed by 2% paraformaldehyde in PBS. The neocortex was then dissected, flattened, placed in 4% paraformaldehyde overnight at 4°C and transferred into 30% sucrose where it remained at 4°C until sectioned. The tissue was sectioned 30 µm tangentially to the cortical surface and collected in a cryoprotective solution.

To visualize the neocortical barrels receiving input from each individual whisker, this study took advantage of the highly organized whisker-to-neocortical projection. In the vibrissae system, tactile information from individual whisker deflections is sent to the neocortex via a tri-synaptic pathway (facial nerve: medullary barrelettes: thalamic barreloids: somatosensory barrel cortex) (Woolsey and Van der Loos, 1970). Layer IV somatosensory cortical barrels receive tactile input from the large facial whiskers in a one-to-one orientation such that the whisker pad is a topographically-organized map of the whisker barrel cortex (Woolsey and Van der Loos, 1970). To localize this whisker barrel pattern, every third section was stained for cytochrome oxidase (CO) and adjacent sections were then co-stained for synapsin I and NeuN.

**Cytochrome Oxidase.** Every third neocortical section was incubated in a cytochrome oxidase (CO) solution (0.03% cytochrome c (Sigma-Aldrich), 0.05% 3,3’-diaminobenzidine (DAB) and 4% sucrose in 0.1M PBS) for 3 h at 37°C and rinsed several times with 0.1M PBS (Wong-Riley and Welt, 1980). CO staining has been used extensively by various laboratories to visualize cortical barrels (Wong-Riley and Welt, 1980; Frostig, 2006; Galvez et al., 2006). The staining results in darkly stained ovals in primary somatosensory cortex corresponding to the individual whisker projections from the whisker pad (Figure 9A).

**Synapsin I.** Sections adjacent to the CO-stained tissue exhibiting cortical barrels in layer IV of somatosensory neocortex were washed several times in 0.1M PBS, blocked in PBS-X (2% normal donkey serum and 0.5% Triton-X in PBS) for 1 h at room temperature and incubated
overnight at 4°C in a primary antibody cocktail containing Anti-Synapsin I (1:500; Sigma-Aldrich) and Anti-NeuN (1:100; EMD Millipore) in PBS-X. The sections were then washed several times in PBS-X and incubated for 2 h at room temperature in a secondary antibody cocktail containing DyLight 405 Anti-Rabbit IgG (1:1000; Jackson ImmunoResearch) and AlexaFluor® 488 Anti-Mouse IgG (1:50; Jackson ImmunoResearch) in PBS-X. Sections were then washed several times in 0.1M PBS, mounted onto slides, covered with ProLong® Gold antifade reagent (Invitrogen) and coverslipped. A multiphoton confocal microscope (Zeiss LSM 710 NLO) was used for visualization (Figure 10A).

**Data Analysis**

For CO analysis of barrel size, digital images were acquired on an Olympus BX50 microscope with a Zeiss AxioCam ICc 1 camera at 2.5x magnification. Once acquired, ImageJ Software (NIH, Version 1.45s) was used to measure C row barrel length as previously described (see Galvez et al., 2007; Galvez et al., 2006; Figure 9A). Briefly, a digital image of the barrels was visualized in ImageJ and a rectangle size of 350 x 40 µm² was placed on top of the first two-barrels in row C. A histogram of the mean gray values across the rectangle was then generated using ImageJ (Figure 9B). The lowest point between these elevated regions was used to delineate boundaries between barrels. Barrel length was measured as the distance between each of these boundaries for the first two barrels in row C. Due to sampling variability in the sections obtained, only the first two barrels were examined.

To examine synapsin I, the barrel cortex was first localized using NeuN at 2.5x magnification with a Zeiss LSM 710 NLO confocal microscope (Figure 10A). Once the region and barrel of interest were localized, a picture of synapsin I positive puncta was taken at 63x magnification. The digital image was then visualized on ImageJ and a counting frame of 133
µm^2 was randomly placed in the center of the barrel hollow. Synapsin I positive puncta between 0-100 µm^2 were counted using the Analyze Particle feature on ImageJ (Figure 10B).

**Statistics**

Behavioral analysis was conducted with a two-way repeated measures ANOVA. The metabolic activity of the barrels and the synapsin I expression analyses were conducted with a two-way ANOVA, followed by a Dunnett test comparing results of all conditioning groups to the naïve group. When appropriate, follow-up post hoc analyses used the Tukey criterion for significance.

**Results**

**Behavioral Analysis**

A two-way repeated measures ANOVA demonstrated a significant main effect between trace-paired and unpaired conditioned mice (F(1,11)= 7.90, p < 0.05) and day of training (F(3,11)= 3.21, p < 0.05; Figure 7). Trace-paired conditioned mice took an average of 3.75 days (SD= 0.89) to reach criterion. Post hoc analyses indicated that mean percent CRs were significantly higher for trace-paired conditioned mice (M= 51.10, SD= 22.26) compared to unpaired conditioned mice (M= 5.38, SD= 8.70) on the day of criterion. Furthermore, post hoc analyses also indicated that trace-paired conditioned mice, unlike unpaired conditioned mice, demonstrated a significant increase in percent CR across training sessions, suggesting that trace-paired mice learned the whisker-stimulation-eyeshock-association.

Additional one-way repeated ANOVAs of CR parameters demonstrated adaptive properties such as a decrease in CR onset (F(3,18)= 5.62, p < 0.05; Figure 8A), decrease in CR peak time (F(3,18)= 10.06, p < 0.05; Figure 8B), increase in CR duration (F(3,18)= 5.39, p < 0.05; Figure 8C) and increase in CR area (F(3,18)= 6.69, p < 0.05; Figure 8D). Together, these analyses
further suggest that trace-paired conditioned mice learned the trace-association.

**Metabolic Activity**

Analyses of barrel length demonstrated that there was a significant difference between trace-paired and unpaired conditioning ($F_{(1,32)}= 5.22, p < 0.05$), and between stimulated and non-stimulated whiskers ($F_{(1,32)}= 5.39, p < 0.05$). Post hoc analyses using the Tukey criterion for significance indicated that the mean barrel size was significantly greater in stimulated barrels of trace-paired conditioned mice ($M= 201.74, SD= 32.00$) compared to non-stimulated barrels of trace-paired conditioned mice ($M= 168.55, SD= 15.32$) and both stimulated ($M= 168.91, SD= 25.25$) and non-stimulated barrels of unpaired conditioned mice ($M= 157.33, SD= 25.15$). Furthermore, stimulated barrels of trace-paired conditioned mice were greater than barrels in naïve mice ($M= 163.38, SD= 24.57$). Note, there were no statistical differences between the two non-stimulated hemispheres for the naïve mice, thus both hemispheres were combined into a single non-stimulated group. Together, the data demonstrates that stimulated barrels of trace-paired conditioned mice exhibited greater metabolic activity than non-stimulated barrels of trace-paired conditioned mice, both stimulated and non-stimulated barrels of unpaired conditioned mice and barrels of naïve mice (Figure 9C). These findings are consistent with our previous findings demonstrating metabolic expansion of stimulated barrels in trace-paired conditioned mice following WTEB acquisition (Galvez et al., 2006; Galvez et al., 2007). No significant differences were seen between stimulated and non-stimulated barrels of unpaired conditioned mice, or between non-stimulated barrels of trace-paired conditioned mice and stimulated and non-stimulated barrels of unpaired conditioned mice.

**Synapsin I Expression**

Analyses of synapsin I expression demonstrated a significant difference between training...
groups (trace-paired vs. unpaired) \( (F_{(1,17)}= 5.02, p < 0.05) \), and between whiskers (stimulated vs. non-stimulated) \( (F_{(1,17)}= 18.98, p < 0.05) \). Post hoc analyses using the Tukey criterion for significance indicated that stimulated barrels in trace-paired conditioned mice (\( M= 16496.00, SD= 1327.02 \)) exhibited more synapsin I positive puncta than non-stimulated barrels in trace-paired conditioned mice (\( M= 12997.80, SD= 1391.65 \)), stimulated barrels in unpaired conditioned mice (\( M= 14250.20, SD= 1212.50 \)) and non-stimulated barrels in unpaired conditioned mice (\( M= 13093.50, SD= 532.45 \)). Furthermore, stimulated barrels in trace-paired conditioned mice exhibited more synapsin I positive puncta than barrels in naïve mice (\( M= 13203.80; SD= 2149.32 \)), demonstrating that stimulated barrels in trace-paired conditioned mice have greater synapsin I expression than non-stimulated barrels in trace-paired conditioned mice, barrels in unpaired conditioned mice and barrels in naïve mice (Figure 10B). Note, there were again no statistical differences between the two non-stimulated hemispheres for the naïve mice, thus both hemispheres were combined into a single non-stimulated group.

**Discussion**

Studies have demonstrated that learning induces neocortical synaptic plasticity following acquisition of various learning and memory tasks (Globus et al., 1973; Diamond et al., 1975; Turner and Greenough, 1985; Kolb et al., 2003). However, to our knowledge, learning-induced neocortical synaptic plasticity following forebrain-dependent trace associative learning has not been closely investigated. It has been previously demonstrated that WTEB conditioning induces expansion of the cytochrome oxidase staining in layer IV of primary somatosensory cortex (Galvez et al., 2006; Galvez et al., 2011). The findings from this study replicated these previous findings (Figure 9C). Although unlikely, an alternative explanation for these previous findings could be that learning does not modify neocortical whisker representation, but rather, that
whisker stimulation in the absence of learning (unpaired conditioning) decreases the layer IV neocortical whisker cytochrome oxidase representation. With the inclusion of naïve mice in the current study as an additional control group, this study further demonstrates that whisker stimulation in the absence of learning (unpaired conditioning) does not significantly alter layer IV whisker barrel cytochrome oxidase staining representation. These findings further emphasize that trace associative learning, not neuronal stimulation, results in neocortical metabolic expansion.

Additional analyses revealed that acquisition for the WTEB association increased synapsin I expression in stimulated barrels of trace-paired conditioned mice (Figure 10B). These findings suggest that the observed increase in cytochrome oxidase staining representation in layer IV of primary somatosensory barrel cortex following trace associative learning is due to synaptic plasticity. More specifically, given the correlation between synapsin I expression and synapse quantity (Lohmann et al., 1978; Moore and Bernstein, 1989; Chin et al., 1995; Perlini et al., 2011), these results suggest that the learning-induced augmentation of neocortical cytochrome oxidase activity in the corresponding whisker barrel representation following trace associative learning is likely due to synaptic proliferation.

Our findings employing a trace associative learning paradigm are consistent with prior analyses of neocortical synaptic properties following acquisition of other associative learning tasks. For example, contextual fear conditioning, a hippocampal-dependent task, has been shown to increase the number of dendritic spines in the anterior cingulate cortex (Restivo et al., 2009), a region believed to play a role in attention mechanisms (Weible et al., 2003) and suggested to be a site for consolidation of contextual fear associations (Frankland et al., 2004; Frankland and Bontempi, 2005; Vetere et al., 2011). Furthermore, Arc/Arg3.1 mRNA, which
has been associated with neuronal plasticity (Steward et al., 1998; Peebles et al., 2010), is modified in the cerebral cortex twenty-four-hours following water maze acquisition (Gusev and Gubin, 2010). Finally, Maviel and colleagues (2004) demonstrated increased neocortical GAP-43, a presynaptic protein that can be used as a marker for synaptogenesis, thirty-days after performing a spatial memory task. These analyses, along with findings from the present study, strongly suggest that trace associative learning induces neocortical synaptic proliferation.

Although prior analyses using other learning and memory tasks, together with the findings using WTEB conditioning mentioned above, would suggest that synaptic proliferation is a very likely explanation for the data obtained, it is not the only explanation. An alternative interpretation for the observed findings could be enhanced synaptic efficiency. Synapsin I is a presynaptic protein involved in binding synaptic vesicles (Cesca et al., 2010); alterations in synapsin I expression alternatively could suggest changes in the size of the post-synaptic density, resulting in modification of the number of bound synaptic vesicles in already existing synapses. However, studies have previously demonstrated that long-term potentiation, a proposed mechanism for learning and memory, increases synapse size (Buchs & Muller, 1996) but does not significantly modulate synapsin I expression (Nayak et al., 1996). These findings further suggest that the observed learning-induced increase in synapsin I expression is due to the generation of new synapses and not the modification of existing synapses.

To date, most studies have assumed from findings utilizing other memory tasks that acquisition of trace associations result in neocortical synaptic proliferation. Findings from this study demonstrate that forebrain-dependent trace associative learning increases synapsin I expression in layer IV of primary somatosensory cortex, suggesting that trace associative learning induces neocortical synaptic proliferation.
Figure 6. Schematic of conditioning paradigms. Conditioned mice were trained with either an unpaired conditioning or trace-paired conditioning paradigm. (A) Unpaired conditioned mice randomly received either 250 ms of whisker stimulation or mild periorbital shock each trial. (B) Trace-paired conditioned mice received 250 ms of whisker stimulation (CS), followed by 250 ms of stimulus-free (trace) interval and 100 ms of periorbital shock (US) every trial. (C) Depiction of a typical CR with the upward deflection representing closure of eyelid. The first grey region (left) corresponds to the CS and the second grey region (right) corresponds to the US. Note the cyclic pattern during the CS is electrical noise generated by the whisker stimulator.
Figure 7. Trace-paired conditioned mice learned the whisker-stimulation-shock-association compared to unpaired conditioned mice. (A) Mean percent conditioned response (CR) (±SEM) across all training sessions. Trace-paired conditioned mice were trained until they exhibited four-CRs out of five consecutive trials. The arrow indicates the average training session it took trace-paired conditioned mice to meet criterion. (B) Mean percent CR (±SEM) for sessions leading up to criterion (C = day of criterion; C-1 = day before criterion; C-2 = two-days before criterion; C-3 = three-days before criterion). Trace-paired conditioned mice exhibited a significant increase in CR performance compared to unpaired conditioned mice, and in comparison to baseline performance. * p < 0.05.
Figure 8. Trace-paired conditioned mice exhibited learning-induced conditioned response adaptive properties. With training, trace-paired conditioned mice demonstrated (A) a decrease in CR onset ($F_{(3,18)}= 5.62, p < 0.05$), (B) a decrease in CR peak time ($F_{(3,18)}= 10.06, p < 0.05$), (C) an increase in CR duration ($F_{(3,18)}= 5.39, p < 0.05$) and (D) an increase in CR area ($F_{(3,18)}= 6.69, p < 0.05$). Note that all of the CR parameters are graphed over sessions to criterion to control for individual animal learning differences (C = day of criterion; C-1 = day before criterion; C-2 = two-days before criterion; C-3 = three-days before criterion).
Figure 9. Stimulated barrels of trace-paired conditioned mice exhibited significantly more metabolic activity compared to non-stimulated barrels of trace-paired conditioned mice and barrels from unpaired conditioned and naïve mice. (A) Representative photomicrograph of cytochrome oxidase stained barrels in layer IV of primary somatosensory cortex. Scale bar = 100 µm. (B) Mean gray value for the rectangular area depicted in A acquired from Image J. Note the elevated gray values delineating the barrel hollows. Barrel length was measured as the distance for each barrel hollow. (C) Mean barrel length (±SEM) of trace-paired conditioned, unpaired conditioned and naïve mice. Stimulated barrels in trace-paired conditioned mice were significantly larger compared to non-stimulated barrels from trace-paired mice and barrels from unpaired conditioned mice and naïve mice. * p < 0.05.
Figure 10. Trace-paired conditioning increases synapsin I expression in stimulated barrels of trace conditioned mice compared to non-stimulated barrels of trace conditioned mice and barrels of unpaired conditioned and naïve mice. (A) Representative photomicrographs depicting cytochrome oxidase (CO), NeuN and synapsin I stain. Representative photomicrographs at 2.5x magnification (top left) of CO stained barrels in layer IV of primary somatosensory cortex (gray) and NeuN stain from the adjacent section (green). Scale bar = 100 µm. Representative photomicrographs at 63x magnification (top right) of magnified NeuN stain (green) and synapsin I stain (red). Scale bar = 10 µm. (B) Mean number of synapsin I positive puncta (±SEM) following conditioning. Stimulated barrels in trace-paired conditioned mice exhibited significantly more synapsin I positive puncta compared to non-stimulated barrels from trace-paired mice and barrels from unpaired conditioned mice and naïve mice. These findings suggest that trace-paired conditioning induces learning- and region-specific synaptic proliferation. * p < 0.05.
Chapter 3: Golgi-Cox Analysis of S1 During & Following WTEB Conditioning*

Introduction

It is widely accepted that memory consolidation involves structural plasticity (for review, see Fu and Zuo, 2011). More specifically, dendritic spine modifications have been suggested to play a critical role in learning and memory consolidation. For example, classic studies utilizing general learning and memory tasks, such as environmental enrichment paradigms, have demonstrated robust increased dendritic spine density in the visual (Globus et al., 1973; Greenough and Volkmar, 1973; Diamond et al., 1975; Green et al., 1983; Turner and Greenough, 1985; Kolb et al., 2003), temporal (Greenough et al., 1973) and somatosensory cortex (Knott et al., 2002) following extended periods of sensory learning. Additionally, other general learning tasks, such as acrobatic training paradigms, have shown increased synaptic density in the motor cortex following various types of motor learning (Kleim et al., 1996). Together, findings from these general learning studies suggest that structural neuronal plasticity underlies memory consolidation.

Findings from these general learning and memory studies have been pivotal for establishing a now prominent theory that task acquisition and memory consolidation are mediated by the formation of new synaptic connections. Furthermore, based upon these and other learning analyses (Eichenbaum et al., 1992; Sutherland and McNaughton, 2000), most agree that synaptic modification in the neocortex underlies memory consolidation. However, this assertion is based upon general learning paradigms, where a subject undergoes multiple different learning events over several days of training, making it difficult to determine the synaptic time course mediating each learning event. Although it is generally accepted that neocortical synaptic modification mediates learning, the time course for neocortical learning induced synaptic changes in response to a single learning event has never been closely examined.
To examine neocortical structural plasticity at different time points during learning, the present study utilized the trace-eyeblink conditioning paradigm. During eyeblink conditioning, subjects are presented with a neutral, conditioned stimulus (CS) (i.e., tone, light, or whisker deflection) paired with a salient, unconditioned stimulus (US) (i.e., air-puff to the eye or a mild periorbital eyeshock) that elicits an unconditioned response (UR) (i.e., eyelink). With multiple CS-US pairings, subjects learn the CS-US association and exhibit a conditioned response (CR) (i.e., eyelink) when presented with the CS. In trace conditioning paradigms, there is a stimulus free interval between the CS and the US. Acquisition for this form of conditioning is forebrain-dependent because it requires an intact hippocampus (Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995; Weiss et al., 1999a; Takehara et al., 2002), medial prefrontal cortex (Weible et al., 2000; McLaughlin et al., 2002) and neocortex (Galvez et al., 2006; Galvez et al., 2007).

To investigate learning-induced neocortical plasticity, the present study took advantage of the whisker barrel system and utilized the trace-eyeblink conditioning paradigm with whisker stimulation as the CS (whisker-trace-eyeblink (WTEB) conditioning). In the rodent whisker system, sensory information from individual whiskers are sent contralaterally to a specific region in layer IV of primary somatosensory cortex (barrel cortex) in a 1:1 configuration (Woolsey and Van der Loos, 1970). Prior findings have reported that pre- and post-training lesions of the primary somatosensory cortex impairs WTEB acquisition and retention (Galvez et al., 2007), demonstrating that primary somatosensory cortex is required for both learning and expression of the learned CS-US association. Additionally, previous studies utilizing the WTEB conditioning paradigm have demonstrated that conditioning increases the size of the cytochrome oxidase stained whisker representation for the conditioned whisker barrels in layer IV of primary somatosensory cortex (Galvez et al., 2006; Galvez et al., 2011; Chau et al., 2013a). Furthermore,
recent findings from our laboratory demonstrated that WTEB conditioning increases synapsin I expression in conditioned barrels compared to control whisker barrels (Chau et al., 2013), suggesting that WTEB conditioning induces neocortical synaptic modification. Synapsin I is a phosphoprotein involved with regulating the release of neurotransmitters at the synapse (Cesca et al., 2010), and has been reported to be correlated with synapse number (Lohmann et al., 1978; Moore and Bernstein, 1989; Chin et al., 1995; Perlini et al., 2011). Collectively, these studies demonstrate that WTEB conditioning is a neocortical-dependent task that also induces neocortical synaptic modifications making it a suitable paradigm for investigating the timing of learning-induced structural plasticity. The present study used Golgi-Cox staining to examine neocortical spine modifications during and following acquisition for WTEB conditioning.

Methods

Subjects

Thirty-five three-month old male C57BL/6J mice were individually housed under a 12 h light/dark cycle with lights on at 7:00AM, and had access to food and water ad libitum.

Surgery

Mice were surgically implanted with a headpiece necessary for WTEB conditioning, as previously described (Galvez et al., 2009). Briefly, mice were anesthetized with a ketamine (1 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.) cocktail. Once anesthetized, a plastic strip connector containing two Teflon-coated stainless steel wires and one ground wire was fitted to the head. The Teflon-coated wires were surgically implanted underneath the skin and emerged around the right periorbital region. The headpiece was then secured to the skull with dental acrylic. Mice had at least five days to recover before behavioral training. All procedures were performed in accordance with guidelines approved by the University of Illinois at Urbana-Champaign’s
Institutional Animal Care and Use Committee.

**Behavioral Task**

Training chambers were standard laboratory cages placed inside a sound-attenuated chamber. Mice were connected to a tether via their headpiece and allowed to move freely in the training chamber for 20 min during habituation. Following habituation, mice were randomly assigned to either trace-paired conditioning (n = 15) or unpaired conditioning (n = 15). A computer running routines written on LabView software delivered all stimuli (whisker stimulation and mild periorbital eyeshock) and collected all behavioral data (eyeblinks). Trace-paired conditioned mice received 250 ms of whisker stimulation delivered via a custom-made whisker stimulator (see Galvez et al., 2009), 250 ms of stimulus-free (trace) interval followed by 100 ms of periorbital shock (0.1 to 1 mA periorbital square wave shock, 60 Hz, 0.5 ms pulses) (Figure 11A). Trace-paired conditioned mice were given 30 trials per session with a 45 s mean intertrial interval (ITI) ranging from 30 to 60 s. An optic sensor placed in front of the right eye was used to monitor eyelid closure. Using information from the optic sensor, a CR was defined as a 4 standard deviation change in voltage from baseline occurring within 35 ms of CS onset (Moyer et al., 1990; Tseng et al., 2004; Weiss and Disterhoft, 2011). Unpaired-conditioned mice randomly received either a whisker stimulation or periorbital shock each session with a 22 s mean ITI (varied randomly between 15 to 30 s) (Figure 11B). Note that unpaired-conditioned mice (stimulation-controls) are termed pseudo-conditioned mice in some studies. All trace-paired conditioned and unpaired-conditioned mice received one conditioning session consisting of 30 trials per day. Mice in the trace-paired conditioning group were further randomly assigned to either the acquisition (ACQ), learned (LRD) or over-trained (OT) group. ACQ mice were trained until three-CRs were exhibited out of five consecutive trials, LRD mice were trained until
four-CRs were exhibited out of five consecutive trails and OT mice were trained until two sessions of four-CRs exhibited out of five consecutive trials. Unpaired-conditioned (unpaired) mice were randomly yoked to trace-paired conditioned mice (unpaired-ACQ; unpaired-LRD; unpaired-OT), and collected at the same time. Naïve mice (n= 5) did not undergo any surgery or eyeblink conditioning, and were collected at the same time as all of the other mice.

**Golgi Processing**

Subjects were given an overdose of sodium pentobarbital 1 h following the last conditioning session and transcardially perfused with 0.1 M phosphate buffered saline (PBS) before their brains were processed for Golgi-Cox staining (Glaser and Van der Loos, 1981; Galvez et al., 2003). Briefly, the neocortex was dissected, flattened and placed into a standard Golgi-Cox solution for 55 days. Once impregnated, the flattened neocortices were embedded in 10% celloidin and sectioned at 80 µm. The flattened neocortices were then stained with methylene blue for neocortical barrel visualization, mounted onto slides and coverslipped.

**Data Analysis**

Neocortical barrels were localized using the methylene blue staining at 2.5x magnification with a Zeiss AxioImage A1 light microscope (Figure 13A). Once a neocortical barrel was localized, spiny stellate neurons located in the inner one-third of the neocortical barrel wall were digitally traced at 100x magnification using Neurolucida Software (MicroBrightField, Williston, VT, USA; Figure 13B-C). Note, only sections with visible neocortical barrels were analyzed. Scholl sphere analyses were also conducted using the same software, with each ring 10 microns apart. For the neuronal bifurcation ratio (NR), the total number of bifurcating branches (all branch orders) was divided by the sum of all bifurcating and normal ending branches (all branch orders). Overall spine density and spine densities for the different spine
morphologies were examined on secondary and tertiary branches exhibiting more than ten spines. Only secondary and tertiary branches were examined due to the limited observations of dendritic spines on primary dendrites. Spines were characterized into four types of morphologies: filopodia-like, thin with bulbous head (bulbous), stubby and branched (similar to previously described neocortical dendritic spine morphologies (Jones et al., 1969; see Bourne and Harris, 2007 for review). More specifically, filopodia-like spines were thin spines that had a neck and head that were the same size, bulbous spines had a head that was wider than the neck, stubby spines resembled a box and branched spines had more than one head. These spine types were further categorized into immature (filopodia-like), intermediate (bulbous) and mature (stubby and branched) (Figure 13D).

**Statistics**

Behavioral analysis was conducted with a two-way ANOVA. Analyses of overall spine density and spine densities of the different spine morphologies (i.e., immature, intermediate and mature) were conducted with a one-way ANOVA. When appropriate, follow-up post hoc analyses used Fisher's LSD criterion for significance and all comparisons were considered statistically significant if \( p < 0.05 \) as calculated by SigmaPlot (Version 11.0, Systat Software, Chicago, IL, USA) and SPSS (Version 14.0, IBM Software, Armonk, NY, USA).

**Results**

**Behavioral Analysis**

A two-way ANOVA demonstrated a significant difference between groups (\( F_{(5,52)} = 13.26, p < 0.05 \)), days to criterion (\( F_{(2,52)} = 14.89, p < 0.05 \)) and interaction between groups and days to criterion (\( F_{(10,52)} = 2.17, p < 0.05 \); Figure 12). Post hoc analyses using Fisher's LSD criterion for significance indicated that OT mice (\( M = 61.83; SD = 11.70 \)) performed
significantly better than OT-yoked-unpaired mice (M = 6.67; SD = 8.17), LRD mice (M = 39.12; SD = 18.13) and ACQ mice (M = 35.45; SD = 8.15). Furthermore, LRD mice (M = 39.12; SD = 18.13) performed significantly better than LRD-yoked-unpaired mice (M = 13.33; SD = 4.71), and ACQ mice (M = 35.45; SD = 8.15) performed significantly better than ACQ-yoked mice (M = 6.67; SD = 0.00). On average, it took ACQ mice 2.25 days to reach the ACQ requirement, LRD mice 2.67 days to reach the LRD requirement and OT mice 3.80 days to reach the OT requirement (Supplementary Figure 1). Together, these results demonstrate that ACQ, LRD and OT mice, unlike their respectively yoked unpaired-conditioned mice, learned the WTEB conditioning task.

**Golgi Analyses**

There were no significant differences detected between unpaired-ACQ, unpaired-LRD and unpaired-OT mice for any of the subsequent Golgi analyses, so the data was combined into a single respective group (unpaired). Additionally, there were no significant differences in overall spine densities or spine densities of the different spine morphologies examined between secondary and tertiary branches within each treatment group, so these were collapsed into their respective groups as well. Also, note that spines were further classified into immature (filopodia-like), intermediate (bulbous) and mature (stubby and branched) spines.

**Overall Spine Density**

A one-way ANOVA demonstrated a significant difference between groups (F(4,45) = 4.89, p < 0.05; Figure 14A). Post hoc analyses using Fisher’s LSD criterion for significance indicated that ACQ mice exhibited significantly greater spine density (M = 0.18; SD = 0.08) compared to cage-control mice (M = 0.12; SD = 0.03), unpaired mice (M = 0.10; SD = 0.02) and OT mice (M = 0.11; SD = 0.05). Additionally, LRD mice (M = 0.18; SD = 0.10) exhibited significantly
greater spine density compared to cage-control mice (M = 0.12; SD = 0.03), unpaired mice (M = 0.10; SD = 0.02) and OT mice (M = 0.11; SD = 0.05). There were no significant differences in overall spine density between ACQ and LRD mice. Overall spine density of OT mice (M = 0.11; SD = 0.05) were significantly different from ACQ mice (M = 0.18; SD = 0.08) and LRD mice (M = 0.18; SD = 0.10), but were not significantly different from unpaired mice (M = 0.10; SD = 0.02) or cage-control mice (M = 0.12; SD = 0.03), suggesting that over-training returns overall spine density to control levels. Further analyses demonstrated that performance on the last WTEB conditioning session for ACQ and LRD mice were significantly correlated to their overall spine density, $R^2 = 0.74$, $p < 0.05$; Figure 14B).

Densities of Immature, Intermediate and Mature Spine Morphologies

**Immature Spines (Filopodia-Like).** A one-way ANOVA demonstrated a significant difference between groups ($F_{(4,45)} = 4.65, p < 0.05$; Figure 15A). Post hoc analyses using Fisher’s LSD criterion for significance indicated that ACQ mice exhibited greater density of filopodia-like spines (M = 0.04; SD = 0.03) compared to cage-control (M = 0.02; SD = 0.006), unpaired (M = 0.02; SD = 0.009) and OT mice (M = 0.017; SD = 0.01). Additionally, LRD mice exhibited greater density of filopodia-like spines (M = 0.03; SD = 0.02) compared to unpaired mice (M = 0.02; SD = 0.009). There were no differences detected between ACQ and LRD mice.

**Intermediate Spines (Bulbous).** A one-way ANOVA demonstrated a significant difference between groups ($F_{(4,43)} = 3.00, p < 0.05$; Figure 15B). Post hoc analyses using Fisher’s LSD criterion for significance indicated that both ACQ (M = 0.11; SD = 0.06) and LRD mice (M = 0.10; SD = 0.06) exhibited significantly greater density of bulbous spines than unpaired mice (M = 0.05; SD = 0.02).
Mature Spines (Combination of Stubby and Branched Spines). A one-way ANOVA did not detect any significant differences in spine density of mature spines between any of the groups. However, a pre-planned comparison indicated a trend (p = 0.056) for LRD mice (M = 0.024; SD = 0.03) to exhibit greater spine density of mature spines than cage-control (M = 0.017; SD = 0.01), unpaired (M = 0.015; SD = 0.02) and OT mice (M = .012; SD = 0.02) (Figure 15C).

Dendritic Branching

No significant differences in dendritic material or dendritic branching were detected between any of the groups from the scholl sphere and bifurcation ratio analyses, respectively (see Supplementary Figure 2).

Discussion

Classic findings utilizing general learning and memory paradigms demonstrating increased dendritic material and dendritic spine density in the neocortex have strongly suggested that memory consolidation involves neocortical structural plasticity (Globus et al., 1973; Greenough and Volkmar, 1973; Diamond et al., 1975; Green et al., 1983; Turner and Greenough, 1985; Kolb et al., 2003). However, the time course for these neocortical anatomical modifications during a specific learning task has not been closely examined. The present study utilized the forebrain-dependent trace associative learning paradigm, WTEB conditioning, to examine neocortical structural plasticity at different time points during learning.

Analyses from the current study demonstrated that layer IV spiny stellate neurons in ACQ and LRD mice exhibited a greater spine density compared to control, unpaired and OT mice (Figure 14A). These findings suggest that neocortical spine proliferation facilitates acquisition of associative learning tasks, consistent with previous reports from general learning and memory paradigms (Greenough and Volkmar, 1973; Greenough et al., 1973; Green et al.,
Additional analyses demonstrated a significant correlation between overall spine density and WTEB conditioning performance of ACQ and LRD mice (Figure 14B), offering further support that spine proliferation plays a role during task acquisition and memory formation. These findings are also consistent with previously proposed mechanisms of learning and memory (Bourne and Harris, 2007) and more recent reports of increased dendritic spine formation in the motor cortex during early training of various motor-learning tasks (Fu et al., 2012). Together, these findings paralleling previous analyses demonstrate that neocortical spine proliferation occurs during acquisition and further suggest that remodeling of neocortical networks play an essential role during memory formation.

Further analyses found that with over-training, the overall spine density in layer IV of primary somatosensory cortex returned to control levels (Figure 14A), suggesting a transient increase in overall neocortical spine density during learning. Though these findings appear inconsistent with the previously discussed analyses from general learning and memory tasks (Globus et al., 1973; Greenough and Volkmar, 1973; Diamond et al., 1975; Green et al., 1983; Turner and Greenough, 1985; Kolb et al., 2003), the time course of structural plasticity during learning in many of these general learning and memory paradigms precludes analyses of underlying time-specific mechanisms. Furthermore, this transient increase in spine density is consistent with hippocampal analyses of spine density following spatial learning tasks. For example, studies have found that hippocampal spine density increases and returns to baseline levels after learning of hippocampal-dependent tasks such as the morris water maze (O'Malley et al., 2000; Eyre et al., 2003) and avoidance learning (O'Malley et al., 1998). Similarly, other findings have reported a hippocampal time-dependent increase in spine density following long-term potentiation (Wosiski-Kuhn and Stranahan, 2012), one of the most common molecular
models of learning and memory. However, to our knowledge, the current study is the first to demonstrate a time-dependent transient increase in spine density during associative learning in the neocortex. Together, these findings suggest that learning, at least in some brain regions, results in a transient increase in spine density facilitating synaptic reorganization.

This time-dependent transient increase in neocortical spine density could have resulted from a number of different anatomical mechanisms. Experience-dependent plasticity studies have reported pruning of newly formed spines in the neocortex following sensory learning (Holtmaat et al., 2008). In contrast, motor learning tasks, such as forelimb reaching tasks, have demonstrated pruning of more mature spines following learning (Fu et al., 2012). The drop in overall spine density in layer IV spiny stellate neurons in primary somatosensory cortex following WTEB conditioning could be due to either mechanism. However, irrespective of which spine population is being selectively removed, these findings suggest that learning in layer IV results in reorganization of primary thalamic synaptic input.

Findings from this study also demonstrated significantly increased spine density of filopodia-like spines in ACQ mice compared to controls, unpaired and OT mice (Figure 15A), paralleling the overall spine density analysis previously discussed. These findings suggest that filopodia-like spines are contributing to the increase in overall spine density and that proliferation of these immature spines facilitates associative learning task acquisition. These analyses are consistent with previous reports proposing that thin, filopodia-like spines play a critical role in learning (for review, see Bourne and Harris, 2007). More specifically, previous studies have demonstrated increased density of thin spines in the cerebellum following complex motor learning (Lee et al., 2007). Additionally, studies have reported that the density of thin spines in the prefrontal cortex correlates with learning performance in aging subjects (Dumitriu
et al., 2010), further suggesting that thin spines are fundamental for learning. These analyses, in conjunction with our findings, suggest that the proliferation of filopodia-like spines is important for memory formation and plays a key role in initial neocortical rewiring during learning.

Further spine morphology analyses demonstrated that bulbous spines were significantly increased in ACQ and LRD mice compared to unpaired mice (Figure 15B). Previous studies have demonstrated that immature spines (i.e., filopodia-like spines) transition into intermediate spines (i.e., bulbous spines) following general sensory learning. In particular, experience-dependent plasticity studies utilizing whisker deprivation have reported the maturation of newly formed thin spines to bulbous spines following general sensory learning (Holtmaat et al. 2006). Furthermore, previous studies have reported synapse formation of newly formed spines four days after proliferation (Holtmaat et al., 2008), similar to the time frame of when LRD mice were collected (see Supplementary Figure 1), suggesting that these intermediate spines are able to communicate with other neurons and thus become further integrated into the neocortical network. Collectively, findings from these studies suggest that acquisition-induced filopodia-like spines are transitioning into bulbous spines during memory formation, and that these bulbous spines are important for rewiring of the neocortical network during learning.

There were no significant differences detected in mature spine densities between any of the groups, but there was a trend for increased spine density of mature spines in LRD mice compared to OT mice (p = 0.056) (Figure 15C) suggesting that the intermediate spines are transitioning into mature spines, as previously reported following sensory learning (Holtmaat et al., 2006). This trend is consistent with previous studies reporting increased branched spines in the hippocampus (Geinisman et al., 2001; Medvedev et al., 2012) and striatum (Comery et al., 1996) following associative learning and environmental enrichment, respectively. However, the
lack of a significant overall effect in mature spine morphologies in our analyses could be due to the amount of training the animals underwent. In the current study, animals were only trained one day beyond reaching learning criterion. In many prior analyses, animals were trained for several days beyond criterion (Ramirez-Amaya et al., 1999). Although this could account for the observed differences, more recent findings in the prefrontal cortex have also found no significant correlation between the number of mature spines and learning ability (Dumitriu et al., 2010), suggesting that the number of mature spines in the neocortex, unlike the hippocampus and striatum, do not correlate with learning.

To our knowledge, there have been few examinations of neocortical plasticity at different time points during learning for a more specific learning paradigm, such as trace associative learning. Classic learning and memory studies have suggested the importance of structural plasticity, especially dendritic spine proliferation, for memory consolidation, but few have closely examined neocortical dendritic plasticity at different time points during that learning process. Findings from this study demonstrate that forebrain-dependent trace associative learning induces time-dependent neocortical spine proliferation. Furthermore, our analyses of the different spine morphologies suggest that in the neocortex, filopodia-like spines proliferate during memory formation. Based upon previously discussed findings, these immature spines then transition into intermediate and mature spines, resulting in rewired neocortical input. Together with previous findings, these analyses suggest that the neuronal mechanisms underlying learning are a time-dependent process resulting in the reorganization of synaptic contacts beginning at the site of primary thalamic input to the neocortex, layer IV. Furthermore, these findings suggest that this reorganization of synaptic contacts would set the foundation for learning-induced neocortical modifications through the different neocortical layers. Subsequent
analyses are needed to determine the implications of these synaptic reorganizations on neuronal connections throughout all six neocortical layers.
Figures

A.

![Diagram of trace-paired conditioning](image)

- **whisker stimulation**: 250 ms
- **trace interval**: 250 ms
- **periorbital shock**: 100 ms

B.

![Diagram of unpaired conditioning](image)

- **whisker stimulation**: 250 ms
- **periorbital shock**: 100 ms

*Figure 11.* Schematic of conditioning paradigms. Conditioned mice were trained with either a trace-paired conditioning or unpaired conditioning paradigm. (A) Trace-paired conditioned mice received 250 ms of whisker stimulation (CS), followed by 250 ms of stimulus-free (trace) interval and 100 ms of periorbital shock (US) every trial. (B) Unpaired conditioned mice randomly received either 250 ms of whisker stimulation or 100 ms of a mild periorbital shock each trial.
Figure 12. All trace-paired conditioned mice learned the WTEB conditioning task, in contrast to their respectively yoked unpaired-conditioned mice. (A) Mean percent conditioned response (CR) (±SEM) for ACQ mice each session until ACQ criterion (C-1 = day of ACQ criterion; C-2 = day before ACQ criterion. (B) Mean percent conditioned response (CR) (±SEM) for LRD mice each session until LRD criterion (C = day of LRD criterion; C-1 = day before LRD criterion; C-2 = two-days before LRD criterion). (C) Mean percent conditioned response (CR) (±SEM) for OT mice each session until OT criterion (C+1 = day of OT criterion; C = day before OT criterion; C-1 = two-days before OT criterion; C-2 = three-days before OT criterion). All trace-paired conditioned mice (ACQ, LRD and OT) exhibited a significant increase in WTEB conditioning performance compared to unpaired-conditioned mice, and in comparison to their baseline performance. *p < 0.05.
Figure 13. Representative photomicrographs of Golgi-Cox stained stellate neurons and spine morphology types. (A) Representative photomicrographs depicting Golgi-Cox and methylene blue co-staining at 2.5x magnification (left) and 20x magnification (right). Scale bar = 100 µm. Note that only spiny stellate neurons in the inner one-third of the barrel wall were examined. (B) Representative photomicrograph depicting Golgi-Cox staining at 60x magnification, with sample primary, secondary and tertiary branches labeled. Scale bar = 10 µm. (C) Representative Neurolucida tracing of a spiny stellate neuron. (D) Representative photomicrographs depicting bulbous, filopodia-like, stubby and branched spine types (top, white triangles from left to right). Scale bar = 50 µm. Depiction of immature, intermediate and mature spine types (bottom).
Figure 14. Increased spine proliferation during memory formation for WTEB conditioning. (A) ACQ and LRD mice exhibited greater overall spine density compared to cage-control, unpaired and OT mice. (B) Overall spine density of ACQ and LRD mice are positively correlated to WTEB conditioning performance on last session. * p < 0.05.
Figure 15. Spine density of immature, intermediate and mature spines at different time points of WTEB conditioning. (A) ACQ mice exhibited significantly more filopodia-like spines than cage-control, unpaired and OT mice. LRD mice exhibited significantly more filopodia-like spines than unpaired mice. (B) ACQ and LRD mice exhibited significantly more bulbous spines than unpaired mice. (C) LRD mice exhibited a trend for greater spine density of mature spines compared to OT mice. * p < 0.05.
Supplementary Figure 1. Mean training sessions for trace-paired conditioned mice to reach ACQ, LRD or OT. (A) Mean percent conditioned response (CR) (±SEM) for ACQ mice each session. The arrow indicates the mean training session it took ACQ mice to exhibit three-CRs out of five consecutive trials. (B) Mean percent conditioned response (CR) (±SEM) for LRD mice each session. The arrow indicates the mean training session it took LRD mice to exhibit four-CRs out of five consecutive trials. (C) Mean percent conditioned response (CR) (±SEM) for OT mice each session. The arrow indicates the mean training session it took OT mice to exhibit four-CRs out of five consecutive trials for two sessions.
Supplementary Figure 2. No significant difference in dendritic material or dendritic branching between groups. (A) Scholl sphere analysis did not detect any differences between groups. (B) Bifurcation ratio analysis did not detect any differences between groups.
Section Three: Examining Learning-Induced Molecular Correlates of Forebrain-Dependent Trace-Associative Learning
Chapter 4a: Arc/Arg3.1 Analysis of S1 Following WTEB Conditioning*

*My first-year project for the Behavioral Neuroscience Division (January 2011)
Introduction

The ability for our brain to learn new information, and the underlying mechanisms involved with this process, have captivated neuroscientists for decades. Although this process is still not well understood, most would agree that learning induces synaptic modifications. For example, enriched rearing, a classic learning and memory paradigm used to induce various types of learning, including visual learning, increases not only the amount of dendritic material (Greenough et al., 1973; Juraska et al., 1980; Juraska, 1984), but also the number of dendritic spines in the visual cortex (Globus et al., 1973; Diamond et al., 1975; Turner and Greenough, 1985; Kolb et al., 2003). Furthermore, acrobat training, a paradigm where rodents learn to traverse a series of obstacles thus increasing motor skill and coordination, increases the number of synapses per neuron in the motor cortex compared to activity controls (Kleim et al., 1996). Together, these studies, along with anatomical findings from other paradigms, suggest that learning results in synaptic modification.

Studies have further demonstrated that memory consolidation, and most likely the learning-induced synaptic modifications mentioned previously, require the production of various proteins. For example, protein synthesis inhibitors in the dorsal hippocampus disrupt long-term memory for spatial water maze, but not acquisition (Guzowski and McGaugh, 1997). This and other studies demonstrate that memory consolidation and most likely the learning-induced synaptic changes formerly mentioned require the production of new proteins. One family of proteins that are rapidly transcribed with neuronal activation are immediate early genes (IEGs).

Immediate early genes (IEGs) are transcriptional factors that are rapidly transcribed by a variety of stimuli. For example, whisker stimulation due to novel exploration (Bisler et al., 2002) or wheel running (Clark et al., 2010) has been shown to produce an increase in protein
expression for various IEGs. One IEG that has obtained considerable attention for potentially playing a critical role in learning and memory is the activity-regulated-cytoskeleton-associated protein (Arc). Blocking Arc in the hippocampus via antisense infusion prior to spatial water maze training impairs retention forty-eight hours after acquiring the task, but not acquisition (Guzowski et al., 2000). Guzowski et al (2000) also demonstrated that blocking Arc protein expression does not block induction of long-term potentiation (LTP: a neuronal property believed to be important for memory consolidation) but disrupted late phase LTP. These findings suggest that Arc is not critical for acquisition but is essential for memory consolidation. Additional studies utilizing fear conditioning, an associative learning task, found similar results. Global Arc knockouts or selectively inhibiting Arc translation in the lateral amygdala prior to conditioning, does not inhibit acquisition of the fear association; however, animals were unable to recall the association twenty-four hours following training (Plath et al., 2006; Ploski et al., 2008). These findings further support the hypothesis that while Arc is not essential for acquisition, Arc is critically involved in memory consolidation.

Further support for Arc involvement in synaptic modification and memory consolidation has come from various in vitro analyses. For example, analysis of dendritic Arc expression following high frequency stimulation of the perforant pathway demonstrated up-regulation of Arc mRNA at the site of synaptic activation. (Steward et al., 1998), suggesting that Arc was actively transported to the synaptic site of neuronal activation. Additionally, Arc mRNA has been demonstrated to be increased during LTP and decreased during the initiation of long-term depression (LTD; a synaptic mechanism believed to be responsible for weakening synaptic connections; Yilmaz-Rastoder et al., 2010) further suggesting that Arc plays a role in synaptic strength and thus modifying synapses. Together, these and the findings from the behavioral
experiments examining Arc involvement during memory consolidation and retrieval suggest that Arc plays a critical role in long-term memory consolidation.

Although these studies have strongly suggested a role for Arc in memory consolidation very few of them have actually examined Arc in the most likely site for long-term memory storage, the neocortex (Eichenbaum et al., 1992; Squire, 2004; Smith and Squire, 2009). To examine Arc expression in the neocortex, we utilized the associative learning paradigm, trace-eyeblink conditioning with whisker stimulation as the conditioned stimulus (CS). In rodents, whisker tactile information from individual whisker deflections is sent to primary somatosensory neocortex, in a 1:1 configuration. This projection results in a somatotopic map of the whisker pad, referred to as the barrel cortex, in layer IV of somatosensory neocortex (Woolsey and Van der Loos, 1970). This whisker to somatotopic neocortical projection in rodents is homologous to the finger to somatosensory (Recanzone et al., 1992) or eye to visual neocortical projection (Silver and Kastner, 2009) in non-human primates and humans.

In eyeblink conditioning, a neutral conditioned stimulus (CS), such as a light, tone, or deflection of a whisker, is paired with a salient unconditioned stimulus (US), such as an air-puff to the eye or a periorbital shock, eliciting an unconditioned response (UR), such as an eyelink. After multiple pairings, the subject learns to associate the CS with the US and exhibits a conditioned response (CR) when presented with the CS. When there is a separation in time between the CS and the US, this form of conditioning is referred to as trace conditioning.

Trace conditioning is an associative paradigm that is forebrain dependent. Hippocampal lesions prior to training inhibit acquisition for the trace-association (Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995; McGlinchey-Berroth et al., 1997; Clark and Zola, 1998; Weiss et al., 1999b; Takehara et al., 2002; Tseng et al., 2004); however, hippocampal lesions thirty-days
following learning have no effect on performance (Kim et al., 1995; Takehara et al., 2002). These studies suggest that though the hippocampus is essential for acquisition of this associative task, the hippocampus is not the site of long-term storage of trace-associations. As mentioned above, the most likely site for long-term storage of trace-associations is the neocortex (Eichenbaum et al., 1992). Neocortical lesions prior to and following trace conditioning prevent both acquisition and retrieval of the trace-association, respectively (Galvez et al., 2007).

Furthermore, studies using cytochrome oxidase staining to visualize the metabolic representation of neocortical barrels have demonstrated that acquisition for whisker-trace-eyeblink (WTEB) conditioning increases the functional representation of the conditioned barrels (Galvez et al., 2006), suggesting that the barrel cortex is not only necessary but is also modified with conditioning. These studies suggest that the neocortex is necessary for acquisition of associative learning and is a site of storage for long-term associative memory. Collectively, these studies demonstrate that acquisition for trace-eyeblink conditioning requires both hippocampal and neocortical involvement, making it a suitable paradigm for examining Arc expression following learning.

Various in vivo and in vitro analyses of Arc in the hippocampus and other brain regions have suggested that Arc plays a role in memory consolidation and synaptic modification. However, Arc expression following learning has never been examined in the neocortex, the most likely site for long-term memory storage. If Arc expression is necessary for learning induced synaptic plasticity, one would expect it to exhibit learning induced expression in the neocortex. The following analyses utilized WTEB conditioning to examine this hypothesis. Our findings demonstrated that Arc is increased in the somatosensory neocortex, in a time dependent fashion following both trace-paired (trace) and unpaired conditioning. These findings suggest that
neuronal activation, rather than learning, is responsible for modulating neocortical Arc expression.

Methods

Subjects

Fifteen male 3-month old C57BL/6J mice from Jackson Laboratories were individually housed with access to food and water ad libitum.

Surgery

After one week to acclimate to our animal facility, mice were surgically implanted with an electrical connector (Galvez et al., 2009). Briefly, mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (6 mg/kg). Once anesthetized, a plastic strip connector, containing two teflon coated stainless steel wires surgically implanted into the right periorbital region and one ground wire, was affixed to the rodent’s skull. All procedures were performed in accordance with guidelines approved by the University of Illinois at Urbana-Champaign’s Institutional Animal Care and Use Committee.

Behavioral Task

After a week of recovery, the mice were habituated to the training chambers. Following habituation, the mice were trained for five days on either a trace-paired (trace) or unpaired conditioning paradigm. Tethered mice in both conditioning groups were able to move freely in the training chambers. For trace conditioning, mice were conditioned with 250 ms whisker stimulation via a custom-made whisker stimulator (Galvez et al., 2009), followed by a 250 ms stimulus free (trace) interval and a 100 ms periorbital shock (60Hz). Mice were given 30 trials per session with a 45 s mean inter-trial interval (ITI) ranging from 30 s to 60 s and one training session per day. Unpaired conditioned mice randomly received 30 whisker stimulations and 30
periorbital shocks with a 22 s (randomly varied between 15 to 30 s) mean ITI per day. Closure of the eyelid, the CR, was defined as a 4 standard deviation change in voltage within 35 ms of whisker stimulation onset. Following the fifth training session, mice were randomly assigned to one of three post-training sacrificing time points (0 min, 45 min, and 90 min). Depending upon the post-training time point assigned, each mouse was then decapitated accordingly. Note that these time points are following approximately 25 minutes of training. The somatosensory cortex was then immediately dissected and placed into -80°C until homogenization.

**Tissue Preparation and Western Blotting**

Mice somatosensory cortex were homogenized in Radio Immuno Precipitation Assay (RIPA) buffer (50mM Tris (pH 8.0), 150mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and a protease inhibitor cocktail (Sigma-Aldrich) using a glass homogenizer. Protein measurements were made via the Bradford method using bovine albumin as an internal standard. Proteins were diluted in 6X loading dye (1M Tris (pH 6.8), SDS, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), glycerol, and bromophenol blue), boiled for 5 minutes, separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were then blocked in 5% dry milk powder for 30 minutes at room temperature and subsequently stained for polyclonal anti-Arc (1:1000, Synaptic Systems) overnight at 4°C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:700, Santa Cruz) was also stained for an additional loading control. The membranes were then incubated with goat-anti-rabbit antibody for 1.5 h at room temperature. For band visualization, the membranes were rinsed once with Tris-buffered saline with 0.1% Tween-20 (TBST) followed by three TBST washes for 10 minutes each before being immersed in
chemiluminescence (ECL) detection reagent (Bio-Rad) for 5 minutes, and then read by phosphorimager.

Data Analysis

Image J (http://rsbweb.nih.gov/ij/) was used to determine the optical density of Arc and GAPDH bands. The relative amount of Arc bands in each sample was quantified by dividing the optical density of Arc by the optical density of GAPDH for each animal.

Results

Behavioral Analysis

All subjects received one session of habituation and five sessions of either trace or unpaired conditioning. There were a total of thirty trials per session, and the percentage of CR was calculated by taking the total number of CRs exhibited by the subject and dividing it by thirty. Consistent with previous reports (Galvez et al., 2006), trace conditioned mice demonstrated an increase in the mean percent CR across training sessions, demonstrating that they learned the association, while unpaired conditioned mice did not exhibit a change in their mean percent CR across training sessions, demonstrating that they did not learn the association (Figure 16).

Arc Expression

Primary somatosensory cortices were collected at 0 min, 45 min, and 90 min following the fifth training session. Analysis of Arc expression demonstrated that trace conditioned mice exhibited a time dependent increase in Arc protein levels following the last training session. Analysis of Arc expression in unpaired conditioned mice demonstrated that protein levels also exhibited a time dependent increase following the last training session. Our analyses did not
detect any differences in Arc expression at any of the different sacrificing time points in trace compared to unpaired conditioned mice (Figure 17).

**Discussion**

Numerous studies have suggested that the IEG Arc is critically involved in learning and memory. Blocking Arc protein expression in the hippocampus via Arc antisense disrupts retention of behavioral tasks while having no affect on acquisition (Guzowski et al., 2000), suggesting a link between Arc expression and long-term memory. Various behavioral paradigms and learning theories have suggested that long-term memory is stored in the neocortex (Eichenbaum et al., 1992; Squire, 2004; Smith and Squire, 2009). However, little is known regarding Arc expression in the neocortex, the most likely site for long-term memory storage. The current study demonstrated that trace and unpaired conditioned mice exhibited similar neocortical Arc expression following conditioning. These data suggest that Arc is up-regulated by neuronal activation, not learning.

Our findings demonstrated that neocortical Arc expression is increased in primary somatosensory neocortex following trace conditioning. These results are consistent with Arc analyses from other brain regions using different learning paradigms. However, our findings also demonstrated that Arc expression is increased in primary somatosensory neocortex following unpaired conditioning, our stimulation control. Furthermore, our findings demonstrated that the pattern of Arc activation following conditioning is similar in both trace and unpaired conditioned mice. These observations suggest that Arc up-regulation in the neocortex is not directly dependent upon learning but rather due to neuronal activity.

The fact that Arc may not be modulated by learning but rather neuronal activation is consistent with prior analysis of hippocampal Arc expression following auditory fear
conditioning (Lonergan et al., 2010). Lonergan and colleagues (2010) found that auditory fear conditioned animals exhibited similar Arc expression in the dorsal hippocampus when compared to controls exposed to the tone but not the footshock. These findings are consistent with our observations and suggest that Arc, at least in the hippocampus and neocortex, is not exclusively activated during learning, but is activated following neuronal activation.

Although our findings strongly suggest that Arc expression in the neocortex is not selectively regulated by learning, this interpretation is dependent upon the fact that unpaired conditioning does not induce any form of neocortical learning. Studies have demonstrated that spatial learning as a result of spatial exploration also increases Arc expression in the hippocampus. Ramirez-Amaya and colleagues (2005) found that rats exploring a novel environment for two sessions exhibited elevated Arc protein expression compared to controls, demonstrating that spatial exploration is sufficient for hippocampal Arc induction. Based upon these findings, unpaired conditioned mice may have exhibited increased Arc expression in the neocortex simply due to exploration of their training chamber. However, to our knowledge, no one has demonstrated that spatial exploration modulates Arc in the neocortex. Moreover, our analysis of Arc expression was conducted after the fifth training session, thus decreasing the likelihood that the training chamber would appear novel and instigate exploration. Furthermore, we have personally witnessed that the mice do not typically explore their environment but rather try to sleep during conditioning. Based upon these observations, we believe that although both groups of mice were able to move within their training chamber, it is unlikely that exploration of the training chamber was responsible for the observed elevated Arc expression in the unpaired conditioned group.
Although we believe it is very unlikely that the observed neocortical Arc expression was due to exploration of their training chamber, an alternative hypothesis could be that the elevated neocortical Arc expression was due to a contextual association between the training chamber and the periorbital shock. However, the shock intensity used for conditioning is several magnitudes of order lower in intensity than that typically used for contextual fear conditioning. Furthermore, neither trace nor unpaired conditioned mice exhibited a fear response while in the training chamber. Finally, based upon the known neuronal pathway for contextual fear conditioning (Ciocchi et al., 2010), there is no reason to believe that primary somatosensory barrel cortex would be involved in forming this association. For these reasons, we believe that a contextual-association in the barrel cortex is a very unlikely explanation for the observed Arc expression following unpaired conditioning.

It is important to note that our observations are based upon Arc expression within an hour and a half of training. Studies using other behavioral paradigms have demonstrated that there are at least two-waves of Arc expression in the hippocampus, the first one between thirty minutes to two-hours and the second one between eight and twenty-four hours following learning (Ramírez-Amaya et al., 2005). These observations, along with our results and the fact that removing Arc in the hippocampus does not hinder acquisition, but prevents consolidation, suggests that the first wave of Arc expression is dependent upon neuronal activation and thus may be involved in a basic neuronal property such as synaptic repair due to neuronal stimulation. However, the second wave of Arc expression, that would typically occur much later, could play a vital role in synaptic modification necessary for memory consolidation. Note that Arc antisense studies decrease or prevent Arc expression in the desired region for approximately fifty-four hours.
following treatment (Guzowski and McGaugh, 1997), thus both waves of Arc expression would have been affected in these studies.

Numerous studies have suggested the importance of Arc for long-term memory. As previously discussed, blocking Arc in the hippocampus inhibits the subject’s ability to retain behavioral tasks, but not their ability to acquire the task (Guzowski et al., 2000). However, little was known regarding Arc expression following learning in the neocortex, the most likely site for long-term memory storage. Our findings failed to find a difference in the initial neocortical Arc expression following associative learning in unpaired and trace conditioned animals. These observations suggest that the first wave of Arc expression in the neocortex is dependent upon neuronal activity and not learning.
Figure 16. Learning Curve for Trace and Unpaired Conditioned Mice. All subjects received one session of habituation and five sessions of either trace or unpaired conditioning. There were a total of thirty trials per session, and the mean percent CR was calculated by taking the total number of CRs exhibited by the subject and dividing it by thirty. Analysis of trace conditioned mice over five consecutive days of conditioning demonstrated an increase in the percent CR, demonstrating that the trace conditioned mice acquired the trace association. In contrast, unpaired conditioned mice did not exhibit a change in their mean percent CR over the five sessions of conditioning.
Figure 17. Neocortical Arc Expression following Trace and Unpaired Eyeblink Conditioning. Primary somatosensory cortices were collected at 0 minutes, 45 minutes and 90 minutes following the fifth session (T = trace conditioned; P = unpaired conditioned). The relative amount of Arc bands in each sample was quantified by dividing the optical density of Arc by the optical density of GAPDH for each mouse. The results suggest that both trace and unpaired conditioned animals exhibit a similar pattern of Arc expression, suggesting that neocortical Arc is not directly related to learning.
Chapter 4b: Arc/Arg3.1 Analysis Following One-Trial Trace-Fear Conditioning*

Introduction

Associative learning paradigms have been widely employed to examine the neuronal mechanisms of learning and memory. More commonly, these studies have used fear conditioning paradigms, where a neutral stimulus (conditioned stimulus; CS) is presented simultaneously and co-terminates with a fear provoking stimulus (unconditioned stimulus; US) (delay fear conditioning). Alternatively, the US can be paired with the environment (contextual fear conditioning). With one or more pairings of these stimuli, the subject quickly learns the CS-US or context-US association. Many studies using delay or contextual fear conditioning paradigms have suggested that the amygdala plays an important role in fear-related associative memory. For example, amygdala lesions impair both contextual and delay fear conditioning (Phillips and LeDoux, 1992; Muller et al., 1997; Maren, 1998; Flavell and Lee, 2012). Furthermore, both contextual and delay fear conditioning have been shown to activate various immediate early genes (IEGs) in the amygdala (Milanovic et al., 1998; Ploski et al., 2008), suggesting that these forms of fear conditioning result in amygdala activation and synaptic plasticity. Together, these and other contextual and delay fear conditioning analyses (Kim and Jung, 2006; Johansen et al., 2011) strongly emphasize the importance of the amygdala in both acquisition and consolidation of contextual and delay fear conditioning.

Unlike contextual and delay conditioning, trace paradigms, such as trace-eyeblink conditioning, require the subject’s awareness of the task during acquisition (Manns et al., 2000) and have thus been proposed to engage higher cognitive processing (Knuttinen et al., 2001; Weiss and Disterhoft, 2011). Trace conditioning is when there is a separation in time between the CS and US. This form of conditioning has been shown to involve the hippocampus (Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995; McEchron et al., 1998; Buchel et al.,
primary neocortex (Galvez et al., 2007) and medial prefrontal cortex (McLaughlin et al., 2002; Runyan et al., 2004; Gilmartin and McEchron, 2005; Quinn et al., 2008). Together, reports from these studies suggest that trace conditioning is forebrain-dependent and taps into higher cognitive processing.

Although the amygdala’s involvement in contextual and delay fear conditioning has been extensively examined, the amygdala’s involvement in trace fear conditioning is not as well understood. Furthermore, the few trace fear conditioning analyses examining the amygdala’s involvement have resulted in conflicting findings. For example, Raybuck and colleagues (2011) found that amygdala inactivation with the GABA_A agonist muscimol impairs contextual and delay fear conditioning, but not acquisition for trace fear conditioning, suggesting that the amygdala is not necessary for acquisition of trace fear associations. In contrast, other studies found that inactivating the amygdala with the same GABA_A agonist (Guimarais et al., 2011; Gilmartin et al., 2012) or anisomycin (Kwapis et al., 2011) impairs acquisition for trace fear conditioning, suggesting that the amygdala is necessary for and may be critically involved in trace fear conditioning.

The present study used IEG expression to determine the amygdala’s and specific amygdala nuclei’s involvement in trace fear conditioning. Studies have demonstrated that the IEG, activity-regulated cytoskeleton-associated protein (Arc/Arg3.1), is up regulated in the amygdala following delay fear conditioning acquisition (Ploski et al., 2008). Furthermore, various studies have suggested that Arc expression is important for synaptic plasticity (Steward et al., 1998; Peebles et al., 2010) and memory consolidation (Guzowski et al., 2000). Together, these findings suggest that Arc expression is a suitable marker of neuronal activation necessary
for synaptic plasticity and memory consolidation. The present study used amygdalar Arc expression to determine the amygdala’s role in trace fear conditioning.

**Methods**

**Subjects**

Three-month-old male C57BL/6J mice were individually housed under a 12-h light/dark cycle with lights on at 7:00AM and had access to food and water *ad libitum*. All procedures were performed in accordance with guidelines approved by the University of Illinois at Urbana-Champaign’s Institutional Animal Care and Use Committee.

**Behavioral Task**

**Training**

Mice were randomly assigned to one of the following conditioning groups: trace conditioning, backward-trace conditioning (backward conditioning), delay conditioning or naïve. Note that the backward conditioning group acted as stimulation-controls. Conditioned mice did not receive any handling prior to training, and mice in the naïve group did not receive any behavioral training or handling prior to decapitation or perfusion. On the day of conditioning, trace and backward conditioned mice were placed into a rectangular training chamber (32 cm x 28 cm x 30 cm) with metal bars across the floor (Med Associates, St. Albans, VT, USA) for 120 s before presentation of the first stimulus. Trace conditioned mice were then trained as described in Kohman et al (2012). Briefly, trace conditioned mice received a tone (30 s; 68 db) followed by a stimulus-free (trace) interval (45 s) and a mild foot-shock (2 s; 0.6 mA) (Figure 18A). Backward conditioned mice received a mild foot-shock (2 s; 0.6 mA) followed by a stimulus-free (trace) interval (45 s) and a tone (30 s; 68 db) (Figure 18B). Trace and backward conditioned mice were then returned to their home cages 20 s following the last stimulus. Delay
conditioned mice were placed into the same training chamber for 167 s, received a tone (30 s; 68db) that co-terminated with a mild foot-shock (2s; 0.6mA) and were returned to their home cages 20 s later (Figure 18C). All conditioned mice were in the training chamber for the same amount of time and received one training session consisting of a single presentation of each stimulus. All groups were trained and collected in a counterbalanced fashion.

Testing

Cued learning of the fear association was assessed 24 h following training for a subset of the trace conditioned (n = 9), backward conditioned (n = 6) and delay conditioned mice (n = 4). Mice were placed into a novel, octagon-shaped testing chamber (26 cm x 30 cm) with a smooth floor. After 180 s, mice received two-tone presentations (30 s; 68 db) with 60 s between each tone presentation. Mice were then returned to their home cages 60 s following the last tone presentation offset. Freezing behavior (defined as a lack of movement) was assessed every 5 s during baseline (the first 20 s) and every 5 s during the tone presentations. This method of recording freezing behavior is similar to that used by other laboratories (Moore et al., 2010). Freezing behavior during the two-tone presentations was averaged into one score. To eliminate experimenter bias, the training condition (trace vs. backward vs. delay) was kept blind to the experimenter observing and scoring the behavior.

Two hours following cued testing, all mice were then tested for contextual learning. Mice were placed into the training chamber with metal bars across the floor for 4 m. Freezing behavior was assessed during the first 100 s during training for baseline and for the entire 4 m during contextual testing as described above.

Analysis of Data

Behavioral analysis was conducted with a two-way repeated measures ANOVA.
Differences in Arc protein expression and Arc-positive puncta count were conducted with a one-way ANOVA. When appropriate, follow-up post hoc analyses used Fisher’s LSD criterion.

**Experiment 1: Amygdalar Arc Protein Analysis**

**Tissue Preparation**

One-hour following behavioral training, a subset of the trace conditioned (n = 8) and backward conditioned (n = 7) mice were decapitated for western immunoblotting analyses. Naïve mice (n = 8) were also collected at this time. Brains were kept at -80°C until coronally sectioned at 400 µm. The amygdala was localized using Franklin and Paxinos (2007) as guidance and micropunched (Figure 19A). As a control for the specificity of these findings, Arc expression was also examined in the caudate (Supplementary Figure 3), dorsal hippocampus (Supplementary Figure 4) and primary auditory cortex (Supplementary Figure 5). Samples were then sonicated in sonication buffer (50mM Tris (pH 7.4), 150mM NaCl, 1% SDS, 50mM NaF and 5mM Na₃P₂O₇). Following sonication, protein estimations were determined using a BCA reagent kit (Pierce).

**Western Immunoblotting**

Samples were prepared with equal amounts of protein, boiled for 5 m at 95°C, separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were then blocked in 5% dry milk powder in tris-buffered saline with 1% Tween-20 (TBST) for 1 h and incubated in a primary antibody cocktail containing anti-Arc (1:1500; Synaptic Systems) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:750; Santa Cruz) overnight at 4°C. The membranes were then rinsed several times with TBST, incubated in a secondary antibody cocktail containing anti-rabbit (1:3000; Cell Signaling) and anti-mouse (1:1500; Cell Signaling) for 2 h. The membranes were then rinsed several times with TBST, immersed in
chemiluminescence detection reagent (Bio-Rad) for 5 m, and visualized with a phosphorimager (Alpha Innotech).

Analysis

Once protein bands were captured by the phosphoimager and digitalized, ImageJ Software (NIH, Version 1.45s) was used to determine the optical density of the Arc and GAPDH bands. The relative intensity of the Arc bands in each sample was determined by dividing the optical density of Arc by the optical density of GAPDH.

**Experiment 2: Amygdala Arc Localization**

Tissue Preparation

One-hour following conditioning, a subset of the trace conditioned (n = 8), backward conditioned (n = 8) and delay conditioned (n = 8) mice were given an overdose of sodium pentobarbital and transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Naïve mice (n = 6) were also collected at this time. The brains were collected and placed into 4% paraformaldehyde overnight at 4°C and transferred into 30% sucrose until sectioned. The brains were coronally sectioned at 30 µm and stored in cryoprotectant. Sections containing the amygdala were located using cellular landmarks (Franklin and Paxinos, 2007) and every sixth section was stained for Arc. Puncta counts were taken from both hemispheres of three sections per mouse (six amygdala per mouse). For analysis, puncta counts were averaged into a single score for each hemisphere of each mouse, and data were analyzed using ANOVA.

Immunohistochemistry

Sections containing the amygdala were washed several times in 0.1M PBS and treated with 0.6% H₂O₂. Sections were then blocked in PBS-X (2% normal goat serum and 0.5%
Triton-X in PBS) for 1 h and incubated overnight at 4°C in a primary antibody against Arc (1:1000; Synaptic Systems) in PBS-X. The sections were then washed several times in PBS-X and incubated for 2 h in a secondary antibody against rabbit made in goat (1:500) (Vector Laboratories). Sections were then washed several times in PBS-X and treated with an avidin-biotin complex (ABC) solution (Vectastain) for 1 h. Following several 0.1M PBS washes, the tissue was stained using a diaminobenzidine (DAB) solution. To minimize immunohistochemical reactivity variability, sections from all groups were reacted together. Sections were then washed several times in 0.1M PBS, mounted onto slides and coverslipped.

**Analysis**

An Olympus BX50 microscope with a Zeiss AxioCam ICc 1 camera was used to visualize Arc protein expression in the amygdala. Amygdala nuclei were localized using Franklin and Paxinos (2007) for guidance. Once localized, a digital image 320 x 238 µm^2^ in the center of the BLA, CeA and LA using a 20x objective was acquired. Arc-positive puncta between 250-2000 µ^2^ were then counted using the image particle analysis tool on ImageJ (Figure 20B).

**Results**

**Behavioral Analysis**

Analysis of cued learning 24 h following training demonstrated a significant difference in mean freezing behavior between baseline and tone (F(1,16) = 18.01, p < 0.05). Within-subject analyses demonstrated that trace conditioned mice exhibited a significant increase in freezing behavior between baseline (M= 3.33, SD= 8.17) and tone (M= 31.82, SD= 13.48; p <0.001). These data, consistent with prior findings (Guimarais et al., 2011; Kohman et al., 2012), demonstrate that mice are able to acquire trace fear conditioning with a single CS-US
presentation. Further within-subject analyses demonstrated that delay conditioned mice also exhibited a significant increase in freezing behavior between baseline (M= 5.00; SD= 10.00) and tone (M= 38.64; SD= 10.82; p= 0.001). Backward conditioned mice did not exhibit a significant difference in the mean percent freezing behavior between baseline and tone. Together, these analyses demonstrate that trace and delay conditioned mice learned the CS-US association compared to the stimulation-controls (Figure 18D).

Analyses of contextual learning demonstrated a significant difference in mean freezing behavior between baseline and contextual testing ($F_{(1,13)} = 25.85, p < 0.05$). Within-subject analyses demonstrated that trace conditioned mice exhibited a significant increase in freezing behavior between baseline (M= 1.50, SD= 0.93) and contextual testing (M= 14.90, SD= 13.07; p= 0.007). Within-subject analyses also demonstrated that backward conditioned mice exhibited a significant increase in freezing behavior between baseline (M= 1.43, SD= 1.40) and contextual testing (M= 19.23, SD= 3.90; p= 0.002). These analyses suggest that both trace and backward conditioned mice learned the context-US association (Supplementary Figure 6).

**Experiment 1: Amygdalar Arc Protein Analysis**

Analysis of Arc protein expression in the amygdala demonstrated a significant difference between conditioned groups ($F_{(2,13)} = 7.48, p < 0.05$; Figure 19B). Further post-hoc analyses indicated that trace conditioned mice (M= 0.81, SD= 0.21) exhibited more Arc protein expression in the amygdala compared to backward conditioned (M= 0.38, SD= 0.19; p= 0.002) and naïve (M= 0.52, SD= 0.19; p = 0.040) mice (Figure 19C).

**Experiment 2: Amygdalar Arc Localization**

Analysis of mean Arc-positive puncta in the BLA ($F_{(3,50)} = 28.08, p < 0.05$; Figure 20C) and in the LA ($F_{(3,50)} = 9.22, p < 0.05$; Figure 20D), but not in the CeA ($F_{(3,50)} = 0.66, p > 0.05$;
Figure 20E) demonstrated a significant difference between conditioning groups. Note, there were no within-subject hemispheric differences in amygdalar Arc expression in any of the nuclei examined, thus puncta counts from each hemisphere were combined in the overall ANOVA and subsequent Post-hoc analyses. Post-hoc analyses indicated that trace conditioned mice (M=14.72; SD= 2.23) exhibited more Arc-positive puncta in the BLA compared to backward conditioned (M= 1.14; SD= 0.90; p < 0.001), delay conditioned (M= 2.23; SD= 2.21; p < 0.001) and naïve mice (M= 3.11; SD= 3.26; p < 0.001). Post hoc analyses further demonstrated that delay conditioned mice (M= 7.24; SD= 2.47) exhibited more Arc-positive puncta in the LA compared to trace conditioned mice (M= 3.40; SD = 1.71; p = 0.007), backward conditioned mice (M= 2.78, SD= 4.08; p < 0.001) and naïve mice (M= 1.94; SD= 2.02; p < 0.001).

Discussion

Numerous studies have demonstrated that the amygdala plays an essential role in fear-related learning and memory (Blanchard and Blanchard, 1972; Gallagher and Kapp, 1978; Kapp et al., 1979; Gallagher et al., 1981; Applegate et al., 1982; Pascoe and Kapp, 1985; Iwata et al., 1986; Phillips and LeDoux, 1992). However, most of these studies utilized either contextual or delay fear conditioning paradigms. To our knowledge, the amygdala’s involvement in forebrain-dependent trace fear associative learning has not been closely examined. The present study used Arc expression as an activity marker to determine the amygdala’s involvement in trace fear associative learning, and to further examine which amygdala nuclei is involved with trace fear conditioning.

Findings from our first experiment examining amygdalar Arc expression demonstrated elevated expression one-hour following trace fear conditioning (Figure 19C), suggesting amygdalar involvement in acquisition of the trace association. These findings are consistent with
previous studies demonstrating trace fear conditioning impairments following amygdala inactivation (Guimarais et al., 2011; Gilmartin et al., 2012). Together, these studies suggest that the amygdala is involved in acquisition and subsequent consolidation of trace fear associations.

Closer examination from our second experiment demonstrated that the BLA, but not the CeA or the LA, was significantly activated one-hour following trace fear conditioning compared to backward conditioned, delay conditioned and naïve mice (Figure 20C). To our knowledge, this is the first experiment to look at the different amygdala nuclei involved with trace fear associative learning. Consistent with this amygdala nuclei specific involvement in trace fear conditioning, many have postulated that contextual and delay fear conditioning utilize different amygdala nuclei pathways. For example, delay fear conditioning using an auditory stimulus as the CS has been shown to involve projections from the auditory cortex to the LA (LeDoux, 2000). Additionally, previous findings, along with our analyses (Figure 20D), have demonstrated increased amygdalar LA expression following delay fear conditioning (Ploski et al., 2008). Unlike delay fear conditioning, contextual fear conditioning using the same auditory CS has been shown to recruit projections from the ventral hippocampus to the BLA (LeDoux, 2000). Reports from various studies have further demonstrated hippocampal involvement in contextual, but not delay fear conditioning (for review, see Anagnostaras et al., 2001; Fanselow, 2000). Additionally, relatively recent studies have demonstrated that trace fear conditioning, similar to contextual fear conditioning, also requires the hippocampus (Czerniawski et al., 2011; Guimarais et al., 2011; Gilmartin et al., 2012) suggesting that acquisition of trace and contextual fear conditioning requires hippocampal involvement. Furthermore, performance in both trace and contextual fear conditioning are enhanced in hippocampal GABA$_A$ receptor $\alpha_4$ subunit knockout mice (Moore et al., 2010), further suggesting similar underlying mechanisms of these two fear
conditioning paradigms compared to delay fear conditioning. Together with previous findings, analyses from the present study demonstrating augmented BLA activation following trace fear associative learning, suggests that contextual and trace fear conditioning paradigms tap into a similar hippocampal-BLA pathway and possible neuronal mechanisms.

Although we believe the current analyses strongly suggest BLA activation following trace fear conditioning, an alternative hypothesis could be that the BLA activation is due to acquisition of a contextual association. However, we believe this interpretation to be unlikely due to our contextual learning analyses demonstrating that both trace and backward conditioned mice learned the context-shock association (see Supplementary Figure 6). However, only the mice that learned the cued association (trace conditioned mice) exhibited elevated BLA Arc expression one-hour following training (Figure 20C). These data suggest that the elevated BLA Arc expression is due to acquisition of the cued-shock rather than the context-shock association. The lack of a significant increase in BLA Arc expression in the backwards-conditioned mice is surprising based upon prior analyses of BLA activation following contextual fear conditioning (Barot et al., 2009; Figge et al., 2012). However, this discrepancy may be due to differences in the timing of Arc protein expression patterns in trace compared to contextual learning. To our knowledge, no one has looked at amygdala nuclei Arc protein expression profiles following different fear conditioning paradigms. Future analyses will be needed to determine the specific timing for amygdala nuclei activation (Arc expression) following fear conditioning.

**Conclusion**

To date, there have been limited studies investigating the amygdala’s involvement in trace fear conditioning. Furthermore, the amygdala nuclei involved with trace fear conditioning has not been examined. To our knowledge, this is the first study examining amygdala activation
in closer detail following trace fear associative learning. The present experiments demonstrate that the amygdala, specifically the BLA, is activated following trace fear conditioning. Activation was not found in the CeA or the LA following trace fear conditioning compared to backward conditioned, delay conditioned and naïve mice, suggesting that the BLA plays a more critical role in trace fear associative learning than delay fear associative learning. Additionally, findings from this study demonstrate that delay conditioned mice exhibited more Arc expression in the LA compared to any of the other conditioned groups and controls, suggesting that delay fear conditioning taps into an underlying pathway different from trace fear conditioning. Together, these findings suggest that the underlying pathway and thus possible neuronal mechanisms for acquisition and subsequent consolidation for trace fear associations are more similar to contextual fear associations than delay fear associations.
Figure 18. Schematic of cued trace, backward and delay conditioning paradigms along with the behavioral analyses. Trace and delay conditioned mice learned the tone-shock-association. Conditioned mice received a single presentation of each stimulus during training. (A) Trace conditioned mice received a tone (30 s; 68 db) followed by a trace interval (45 s) and a mild foot-shock (2 s; 0.6 mA). (B) Backward conditioned mice received a mild foot-shock (2 s; 0.6 mA) followed by a trace interval (45 s) and a tone (30 s; 68 db). (C) Delay conditioned mice received a tone (30 s; 68 db) that co-terminated with a mild foot-shock (2 s; 0.6 mA). (D) When tested 24 h later, trace conditioned mice (Moscovitch et al.) exhibited a significantly higher mean percent freezing (±SEM) during the tone presentation compared to baseline, suggesting that trace conditioned mice learned the tone-shock-association. Delay conditioned mice (dark grey) exhibited a significantly higher mean percent freezing (±SEM) during the tone presentation compared to baseline, suggesting that delay conditioned mice learned the tone-shock-association. No significant difference in mean percent freezing (±SEM) in the backward conditioned mice (light grey) during testing was detected. * p < 0.05.
Figure 19. Trace conditioned mice exhibited significantly greater Arc expression in the amygdala one-hour following fear conditioning compared to backward conditioned and naïve mice. (A) Schematic of a coronal section of the mouse amygdala sampled (Franklin and Paxinos, 2007). (B) Representative photomicrographs of the western immunoblots for Arc expression and the loading control, GAPDH, expression in naïve (left), backward conditioned (middle) and trace conditioned (right). (C) Mean relative Arc expression (±SEM) in trace conditioned mice following training was significantly elevated compared to backward conditioned and naïve mice. * p < 0.05.
Figure 20. Trace conditioned mice exhibited significantly greater Arc-positive puncta in the basolateral amygdala (BLA) compared to backward conditioned, delay conditioned and naïve mice. (A) Representative areas of the amygdala sampled (Franklin and Paxinos, 2007). Scale bar= 100 µm (B) Representative photomicrographs of Arc staining. Scale bar= 50 µm (C) Trace conditioned mice exhibited significantly more Arc-positive puncta in the BLA compared to backward conditioned, delay conditioned and naïve mice. (D) Delay conditioned mice exhibited significantly more Arc-positive puncta in the LA compared to trace conditioned, backward conditioned and naïve mice. (E) There were no significant Arc-positive puncta differences in the CeA between all groups. * p < 0.05.
Supplementary Figure 3. Trace and backward conditioned mice exhibited significantly greater Arc expression in the caudate compared to naïve mice, but there were no differences in Arc expression between trace and backward conditioned mice. (A) Arc expression in trace conditioned and backward conditioned mice following training was significantly elevated compared to naïve mice. However, there were no differences in Arc expression between trace conditioned and backward conditioned mice suggesting that stimulation or contextual learning may be resulting in caudate activation. (B) Schematic of a coronal section of the mouse caudate sampled (Franklin and Paxinos, 2007). * p < 0.05.
Supplementary Figure 4. Trace and backward conditioned mice demonstrated significantly greater Arc expression in the dorsal hippocampus compared to naïve mice, but there were no Arc expression differences between trace and backward conditioned mice. (A) Arc expression in trace and backward conditioned mice was significantly greater following training compared to naïve mice. There were, however, no differences in Arc expression between trace conditioned and backward conditioned mice, suggesting that stimulation or contextual learning may be producing the dorsal hippocampus activation observed. (B) Schematic of a coronal section of the dorsal hippocampus sampled (Franklin and Paxinos, 2007). * p < 0.05.
Supplementary Figure 5. Trace conditioned mice demonstrated significantly reduced Arc expression in the primary auditory cortex following conditioning compared to naïve mice, but there were no Arc expression differences between trace and backward conditioned mice. (A) Arc expression in trace conditioned mice was significantly reduced following training compared to naïve mice, consistent with findings from prior studies demonstrating a negative correlation between Arc expression and neuronal activity in the primary auditory cortex (Carpenter-Hyland et al., 2010). However, there were no differences in Arc expression between trace conditioned and backward conditioned mice. (B) Schematic of a coronal section of primary auditory cortex sampled (Franklin and Paxinos, 2007). * p < 0.05.
Supplementary Figure 6. Contextual testing demonstrated that both trace and backward conditioned mice exhibited significantly more freezing behavior compared to baseline ($F_{(1,13)} = 25.85$, $p < 0.05$). Within-subject analyses demonstrated that trace conditioned mice exhibited a significant increase in freezing behavior between baseline ($M= 1.50$, $SD= 0.93$) and contextual testing ($M= 14.90$, $SD= 13.07$; $p= 0.007$). Further within-subject analyses demonstrated that backward conditioned mice also exhibited a significant increase in freezing behavior between baseline ($M= 1.43$, $SD= 1.40$) and contextual testing ($M= 19.23$, $SD= 3.90$; $p= 0.002$). Together, our contextual learning analyses suggest that both trace and backward conditioned mice learned the context-shock association. * $p < 0.05$.
Chapter 5: Genome-Wide Analysis of S1 During & Following WTEB Conditioning*

*In Prep
Introduction

The underpinnings of memory formation remain an important area of research for neuroscientists. In these analyses, many studies utilizing behavioral learning and memory paradigms have suggested that the neocortex plays a critical role in long-term memory consolidation (Squire et al., 1984; Eichenbaum et al., 1992; Sutherland and McNaughton, 2000). Moreover, it is widely accepted that molecular modifications within the neocortex underlie learning and memory. For example, mutant mice with autophosphorylation impairments of alpha-CaMKII exhibited impaired plasticity in the primary somtaosensory cortex during whisker deprivation compared to controls (Glazewski, Giese, Silva, Fox 2000). Additionally, acquisition for the morris water maze has been shown to increase Arc mRNA in layers II/III and IV in parietal and visual cortices one month following training compared to lower layers of (Gusev and Gubin, 2010). Arc is an immediate early gene that has been suggested to be important for synaptic plasticity (Steward et al., 1998; Peebles et al., 2010) and memory consolidation (Guzowski et al., 2000). Together, these neocortical molecular analyses focusing on specific genes have offered much insight into their specific roles in learning and memory. However, to date, limited studies have employed a genome-wide analysis to examine neocortical molecular modifications during different time points of forebrain-dependent associative learning.

To examine molecular modifications during forebrain-dependent learning, the present study utilized the trace-eyeblink conditioning paradigm. During eyeblink conditioning, subjects are presented with a neutral, conditioned stimulus (CS) (i.e., tone, light, or whisker deflection) paired with a salient, unconditioned stimulus (US) (i.e., air-puff to the eye or a mild periorbital eyeshock) that elicits an unconditioned response (UR) (i.e., eyeblink). With multiple CS-US pairings, subjects learn the CS-US association and exhibit a conditioned response (CR) (i.e.,
eyeblink) when presented with the CS. In trace conditioning paradigms, there is a stimulus free interval between the CS and the US. Acquisition for this form of conditioning is forebrain-dependent because it requires both the hippocampus (Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995; Takehara et al., 2002) and the neocortex (Galvez et al., 2006; Galvez et al., 2007).

To investigate learning-induced neocortical plasticity, the present study took advantage of the whisker barrel system and utilized the trace-eyeblink conditioning paradigm with whisker stimulation as the CS (whisker-trace-eyeblink (WTEB) conditioning). In the rodent whisker system, sensory information from individual whiskers is sent to a specific region in layer IV of primary somatosensory cortex (whisker barrel cortex) in a 1:1 configuration (Woolsey and Van der Loos, 1970). Research findings have demonstrated that pre- and post-training lesions of primary somatosensory cortex impairs WTEB acquisition and expression of the already learned association, respectively (Galvez et al., 2007), suggesting that primary somatosensory cortex is required for acquisition and retention of WTEB conditioning. Additionally, studies have demonstrated that WTEB conditioning increases the size of the cytochrome oxidase stained whisker representation for the conditioned whisker barrels in layer IV of primary somatosensory cortex (Galvez et al., 2006; Galvez et al., 2011; Chau et al., 2013a). Furthermore, recent analyses demonstrated that WTEB conditioning increases synapsin I expression, a phosphoprotein correlated to synapse number (Lohmann et al., 1978; Moore and Bernstein, 1989; Chin et al., 1995; Perlini et al., 2011) and involved with regulation of neurotransmitter release at the synapse (Cesca et al., 2010), suggesting that WTEB conditioning induces neocortical synaptic modifications (Chau et al., 2013). Together, these studies demonstrate that
WTEB conditioning induces neocortical plasticity and that it is a suitable paradigm for examining neocortical molecular modifications during associative learning.

The present study utilized WTEB conditioning in conjunction with a microarray analysis, quantitative PCR (qPCR) and immunohistochemistry to examine neocortical mRNA and protein expression modifications during task acquisition and memory consolidation.

**Methods**

**Subjects**

Three-month old male C57BL/6J mice were individually housed under a 12 h light/dark cycle with lights on at 7:00AM and had access to food and water *ad libitum*.

**Surgery**

Mice were surgically implanted with a headpiece necessary for WTEB conditioning (Galvez et al., 2009). Briefly, mice were anesthetized with a ketamine (1 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.) cocktail. Once anesthetized, a plastic strip connector containing two Teflon-coated stainless steel wires and one ground wire was fitted to the head. The Teflon-coated wires were surgically implanted underneath the skin and emerged in the right periorbital region. The headpiece was secured to the skull with dental acrylic. Mice had at least five days to recover before any behavioral training. All procedures were performed in accordance with guidelines approved by the University of Illinois at Urbana-Champaign’s Institutional Animal Care and Use Committee.

**Behavioral Task**

Training chambers were standard laboratory cages placed inside a sound-attenuated chamber. For habituation, mice were connected to a tether via their headpiece and allowed to move freely in the training chamber for 20 min. Following habituation, mice were randomly
assigned to either trace-paired-conditioning or unpaired-conditioning. A computer running routines written on LabView software delivered all stimuli (whisker stimulation and mild periorbital eyeshock) and acquired all behavioral data (eyelid closure). Trace-paired conditioned mice received 250 ms of whisker stimulation delivered via a custom-made whisker stimulator (see Galvez et al., 2009), 250 ms of stimulus-free (trace) interval followed by 100 ms of periorbital shock (0.1 to 1 mA periorbital square wave shock, 60 Hz, 0.5 ms pulses) (Figure 21A). Trace-paired conditioned mice were given 30 trials per session with a 45 s mean intertrial interval (ITI) ranging from 30 to 60 s. An optic sensor placed in front of the right eye was used to monitor eyelid closure. Using information from the optic sensor, a CR was defined as a 4 standard deviation change in voltage from baseline occurring within 35 ms of CS onset (Moyer et al., 1990; Tseng et al., 2004; Weiss and Disterhoft, 2011). Unpaired conditioned mice randomly received either a whisker stimulation or periorbital shock each session with a 22 s mean ITI (varied randomly between 15 and 30 s) (Figure 21B). Note that the unpaired conditioned group (stimulation-control) is consistent with pseudo conditioning groups used in some studies. All trace-paired conditioned and unpaired conditioned mice received one conditioning session consisting of 30 trials per day. Mice in the trace-paired conditioning group were further randomly assigned to either the acquisition (ACQ) group or the learned (LRD) group. ACQ mice were trained until three-CRs were exhibited during five-consecutive trials and LRD mice were trained until four-CRs were exhibited during five-consecutive trials. Unpaired-conditioned mice were randomly yoked to trace-paired conditioned mice, and collected at the same time. Naïve mice did not undergo surgery or eyeblink conditioning, but were collected at the same time as all of the other mice.
**Microarray and Functional Analyses**

The following were conducted under RNase-free conditions. One-hour following reaching ACQ (n = 2) or LRD (n = 2) criterion, primary somatosensory cortex was located (Franklin & Paxinos, 2007), dissected and placed into cold TRIzol Reagent. Unpaired (n = 4) and naïve mice (n = 2) were also collected at this time.

Samples were then processed for mRNA extraction. Once extracted, 5 µg of mRNA was plated onto an Affymetrix mouse Gene ST1.0 chip and sent to the University of Illinois Biotechnology Center for chip hybridization and analysis. RNA prepared from cells harvested from different experimental groups and negative control samples were hybridized together on human HT 12 Illumina bead station arrays after labeling according to the manufacturers’ instructions. Data were analyzed using the Bioconductor “lumi” package (http://bioconductor.org/packages/release/bioc/html/lumi.html). The samples from each group were compared as a pool to duplicate untreated groups controls using the bioconductor multi-test package (http://www.bioconductor.org/packages/2.3/bioc/html/multtest.html). Genes identified as differentially expressed with p < 0.05 in comparison to negative controls, and which also changed in expression in the same positive or negative direction in comparison to the controls, were considered for further analysis. Up-regulated and down-regulated genes were analyzed for function separately using the DAVID (Huang et al., 2009) functional clustering algorithm with default settings.

**Quantitative PCR**

The same primary somatosensory cortex samples from the microarray analysis were processed for quantitative PCR (qPCR) analyses for the following genes: *Arc, Cacng4, Capns2, Car8, Egr3, Fat3, Glra3, Homer2, Megf6, Mir153, Mir154, Mir199a1, Mir690, Mycbp2, Necab2,*
Nexn, Oprk1, Pbx3, Prima1, Prkce, Rasgrp2, Rcn, Rfx5, Sgk1, Syt9 and Zfp738. The internal control GAPDH was used to determine relative expressions.

Total RNA was isolated from tissues using TRIzol (Invitrogen, Carlsbad, CA), and cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen, CA) according to manufacturer’s instructions. We designed custom primer sets for use in quantitative reverse-transcript PCR (qRT-PCR), using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). RNA levels for each gene were calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Figure 23).

**Immunohistochemistry**

One-hour following reaching LRD criterion, mice (n = 8) were given an overdose of sodium pentobarbital and transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Yoked-unpaired (n = 12) and naïve mice (n = 12) were also collected at this time. The brains were collected and placed into 4% paraformaldehyde overnight at 4°C and transferred into 30% sucrose until sectioned. The brains were coronally sectioned at 30 µm and stored in cryoprotectant (30% sucrose and 30% ethylene glycol in 0.1M PBS).

Sections containing primary somatosensory cortex was located (Franklin & Paxinos, 2007) and stained for the following proteins: Necab2, Oprk1 and Sgk1. These proteins were selected based upon the robust mRNA changes during WTEB conditioning observed from our microarray analysis.

Sections were stained using a standard immunohistochemistry protocol. Briefly, sections were washed several times in PBS, treated with 0.6% H₂O₂, blocked in PBS-X (2% normal goat serum and 0.5% triton-x in PBS) for 1 h at room temperature and incubated overnight at 4°C in primary antibody [Necab2 (1:500 Sigma-Aldrich, St. Louis, MO, USA); Oprk1 (1:500 MBL]
International Corporation, Woburn, MA, USA); Sgk1 (1:500 Abcam, Cambridge, England)].
The sections were then washed several times in PBS-X, incubated for 2 h in secondary antibody at room temperature, washed several times in PBS-X and treated with an avidin-biotin complex (ABC) solution (Vectastain) for 1 h. Following several PBS washes, the tissue was stained using a diaminobenzidine (DAB) solution. To minimize the immunohistochemical reactivity variability, sections from all groups were reacted together. Sections were then washed several times in PBS, mounted onto slides and coverslipped (Figure 24).

An Olympus BX50 microscope with an attached Zeiss AxioCam ICc 1 camera was used for positive protein puncta visualization. Once the primary somatosensory cortex was localized (Franklin & Paxinos, 2007), a digital image was captured with the Zeiss AxioCam ICc 1 camera at 10x for Necab2 and Oprk1 images and at 20x for Sgk1 images. Then, Necab2 positive puncta between 60-1000 µm², Oprk1 positive puncta greater than 30 µm² and Sgk1 positive puncta greater than 120 µm² were counted using the Analyze Particle feature on ImageJ (Version 1.45s, NIH).

Results

Behavioral Performance

A two-way ANOVA demonstrated a significant difference between groups ($F_{(3,77)} = 5.23$, $p < 0.05$), days to criterion ($F_{(3,77)} = 14.87$, $p < 0.05$) and interaction between groups and days to criterion ($F_{(9,77)} = 4.54$, $p < 0.05$; Figure 22), suggesting that WTEB conditioning performance is dependent on days to criterion. For WTEB conditioning performance by conditioning sessions, see Supplementary Material. Post hoc analyses using the Tukey criterion for significance indicated that on criterion day, LRD mice ($M = 43.50$; $SD = 13.62$) performed significantly better than ACQ mice ($M = 23.30$; $SD = 8.32$) and LRD-yoked-unpaired mice ($M = 7.62$; $SD = \ldots$)
Additionally, ACQ mice (M = 23.30; SD = 8.32) performed significantly better than ACQ-yoked-unpaired mice (M = 6.71; SD = 6.20). Together, these results demonstrate that LRD and ACQ mice, unlike unpaired conditioned mice, learned the WTEB association. Furthermore, these results demonstrate that LRD mice performed significantly better than ACQ mice.

**Microarray and qPCR Analyses**

Findings from our microarray analysis demonstrated various gene modifications during different time points of WTEB conditioning. To test the accuracy of the microarray analysis, twenty-six genes were selected for subsequent qPCR analyses (see Figure 23). More specifically, we tested *Arc, Cacng4, Capns2, Car8, Egr3, Fat3, Glra3, Homer2, Megf6, Mir153, Mir154, Mir199a1, Mir690, Mycbp2, Necab2, Nexn, Oprk1, Pbx3, Prima1, Prkce, Rasgrp2, Rcn, Rfx5, Sgk1, Syt9* and *Zfp738*. We determined that they exhibited a similar distribution profile as observed in the microarray analysis.

**Immunohistochemistry of Selected Genes**

*Necab2*. A one-way ANOVA exhibited a significant difference between groups ($F_{(4,35)} = 19.92, p < 0.05$; Figure 24A). Post hoc analyses using Fisher's LSD criterion for significance indicated that naive barrels (M = 0.000179; SD = 0.0000321) had significantly more Necab2+ puncta compared to stimulated LRD barrels (M = 0.0000795; SD = 0.0000338), non-stimulated LRD barrels (M = 0.000109; SD = 0.0000551), stimulated unpaired barrels (M = 0.0000552; SD = 0.0000298) and non-stimulated unpaired barrels (M = 0.0000456; SD = 0.0000204). Non-stimulated LRD barrels (M = 0.000109; SD = 0.0000551) demonstrated significantly more Necab2+ puncta compared to both stimulated unpaired barrels (M = 0.0000552; SD = 0.0000298) and non-stimulated unpaired barrels (M = 0.0000456; SD = 0.0000204).
*Oprk1.* A one-way ANOVA demonstrated a significant difference between groups ($F_{(4,20)} = 4.06, p < 0.05$; Figure 24B). Post hoc analyses using Fisher's LSD criterion for significance indicated that stimulated LRD barrels ($M = 0.00224; SD = 0.000114$) exhibited significantly fewer Oprk1+ puncta compared to stimulated unpaired barrels ($M = 0.00274; SD = 0.000316$) and naive barrels ($M = 0.00302; SD = 0.000388$).

*Sgk1.* A one-way ANOVA revealed a significant difference between groups ($F_{(4,40)} = 8.82, p < 0.05$; Figure 24C). Post hoc analyses using Fisher's LSD criterion for significance indicated that stimulated LRD barrels ($M = 0.000524; SD = 0.000104$) exhibited significantly more Sgk1+ puncta compared to non-stimulated LRD barrels ($M = 0.000275; SD = 0.0000954$) and naive barrels ($M = 0.000375; SD = 0.0000733$). There were no differences detected between stimulated LRD barrels and stimulated unpaired barrels. Stimulated unpaired barrels ($M = 0.000527; SD = 0.000129$) also showed significantly more Sgk1+ puncta compared to non-stimulated LRD barrels ($M = 0.000275; SD = 0.0000954$) and naive barrels ($M = 0.000375; SD = 0.0000733$). Additionally, non-stimulated LRD barrels exhibited significantly fewer Sgk1+ puncta compared to non-stimulated unpaired barrels ($M = 0.000433; SD = 0.000116$) and naive barrels ($M = 0.000375; SD = 0.0000733$).

**Discussion**

Previous studies have examined the involvement of single genes in the neocortex, the most likely site for long-term memory consolidation (Eichenbaum et al., 1992; Squire, 2004; Smith and Squire, 2009), following various learning and memory paradigms. However, to date, few studies have employed a genome-wide analysis to determine essential neocortical molecular modifications at different time points of forebrain-dependent trace-associative learning.
Analyses from this study examining protein expression of select genes in primary somatosensory cortex demonstrated that the overall pattern between protein expression and mRNA expression is similar (Figures 23 - 24), though more studies are needed to closely examine how each gene contributes to learning and memory. Together, the similarity between mRNA and protein expression suggests that the array of genes from our microarray analysis could also be applied to its proteins.

Additional analyses from this study comparing neocortical mRNA changes between ACQ mice and stimulation controls demonstrated a down-regulation of various microRNAs (miRNAs) at the acquisition phase, but not at the learned phase. These findings are consistent with relatively recent studies reporting that knocking down hippocampal miRNAs in adult mice improved learning performance for spatial water maze, trace fear conditioning and contextual fear conditioning tasks (Konopka et al., 2010), suggesting that miRNA down-regulation is a necessary component for task acquisition. More specifically, analyses from the current study demonstrating a down-regulation of Mir379 miRNA with previous reports of Mir379 miRNA playing a role in dendritic spine size modification (Fiore et al., 2009) further suggest that Mir379 could be contributing to the structural plasticity observed in neurons during learning. Neuronal plasticity has been shown using various learning tasks and has been proposed to be important for learning and memory (Globus et al., 1973; Greenough and Volkmar, 1973; Diamond et al., 1975; Green et al., 1983; Turner and Greenough, 1985; Kolb et al., 2003). Together, these findings suggest that these miRNAs could be contributing to the structural plasticity observed during learning. Additional studies are needed to more closely examine the role of these miRNAs in learning and memory.
An overall functional analysis with the DAVID program indicated significant differences in calcium-related genes (Table 1), suggesting that both calcium-related mRNAs and proteins are modified in the neocortex during learning. More specifically, there is an up-regulation of calcium-related genes at the acquisition phase that returns to baseline levels by the learned phase (Figure 25), similar to the structural plasticity demonstrated in Chapter 3. Together, findings from these studies suggest that the downstream effects of these calcium-related genes could be underlying the synaptic modifications occurring during learning. Additionally, previous studies demonstrating the importance of AMPA receptor trafficking with synaptic modifications (for review, see Bassani et al., 2013) in conjunction with findings from this current study demonstrating an up-regulation of Cacng4 during the acquisition phase, a gene responsible for encoding a transmembrane AMPA receptor regulatory protein that assists in trafficking AMPA receptors (Cho et al., 2007), further supports that the downstream effects of these calcium-related genes contribute to the time-dependent synaptic modifications occurring during learning.

Moreover, findings from the current study demonstrating modifications of calcium-related genes during learning are consistent with previous studies using molecular and in vivo models of learning and memory. For example, studies utilizing long-term potentiation (LTP), one of the most common molecular models for learning and memory, established that calcium is essential for LTP induction (Dunwiddie and Lynch, 1979). Additionally, reports from in vivo learning and memory models indicating that calcium plays an essential role in the cerebellum for motor learning (for review, see Lamont, 2012) and alterations in calcium-dependent hippocampal biophysical properties following eyeblink conditioning (Disterhoft et al., 1986) parallel findings from this current study. Moreover, previous studies demonstrating age-related calcium-dependent hippocampal afterhyperpolarization modification contributing to eyeblink
conditioning acquisition impairments in aged animals (Disterhoft et al., 1996) and more recent studies pointing to calcium dysregulation as a factor for age-related neurological disorders, such as Alzheimer’s disease (for review, see Thibault, et al. 2007), further indicate that calcium regulation is critical for learning and memory. Findings from current analyses examining Necab2, a gene involved with neuronal calcium binding (Sugita et al., 2002), demonstrated that Necab2 protein and mRNA expression was significantly elevated in naive barrels compared to stimulated LRD barrels (Figure 24A), suggesting that there is a down-regulation of Necab2 following learning. Recent reports have pointed to the deletion of 16q23.3-q24.1 in the Necab2 gene underlying some forms of autism (Sakai et al., 2011), further indicating that modifications of Necab2 may play a role in certain forms of learning and memory impairments. Together with the previously discussed findings, our findings demonstrating neocortical changes in calcium-related gene modifications suggest the importance of calcium-related genes with learning and memory.

Findings from this current study also demonstrated neocortical synapse-related gene modifications during learning (Table 1). More specifically, there is an up-regulation of synapse-related genes at the acquisition phase that returns to baseline levels by the learned phase (Figure 26), suggesting that the downstream effects of these synapse-related genes, similar to the changes in the calcium-related genes previously discussed, could also be contributing to the synaptic modifications previously demonstrated during learning (see Chapter 3). Findings from the current study indicating synapse-related gene modifications are consistent with previous studies reporting learning-induced synaptic changes. For example, synaptic modifications have also been previously reported following WTEB conditioning (Chau et al., 2013a). Synaptic plasticity has been demonstrated following learning while synaptic abnormalities have been
reported in individuals with neurological disorders involving learning and memory impairments, such as autism (for review, Penzes, et al. 2011). In particular, findings from this current study demonstrated a down-regulation of GLRA3, a synapse gene that encodes glycine receptor subunits, in learned mice compared to stimulation-controls (Table 1). Previous studies have observed a deletion of GLRA3 in autistic children (Ramanathan et al., 2004). Together, these findings suggest that irregular synaptic modifications may contribute to learning and memory impairments. Collectively, findings from these studies emphasize the importance of synapse-related gene changes during learning and memory, and further suggest that synapse-related genes play an important role in the underlying mechanisms of learning and memory.

Additional analyses demonstrated that both stimulated LRD and stimulated unpaired barrels expressed more Sgk1 protein and mRNA expression, a gene involved with neuronal function regulation (Lang et al., 2010), than naive barrels (Figure 24C) suggesting an up-regulation of Sgk1 following either learning or stimulation. Studies demonstrating a faster rate of spatial water maze learning following increased hippocampal Sgk (Tsai et al., 2002), are consistent with our findings that Sgk1 is important for learning.

Findings from our analyses are consistent with a genome-wide neocortical analyses following whisker deprivation (Valles et al., 2011). In particular, our findings also demonstrated an up-regulation of Egr3 mRNA following WTEB conditioning compared to cage-controls (Figure 23). Previous reports have shown that Egr3 knockouts exhibit abnormalities in early- and late-LTP as well as learning impairments of hippocampal- and amygdala-dependent learning and memory tasks (Li et al., 2007), suggesting that Egr3 facilitates some underlying mechanisms of learning and memory. The similarity in Egr3 mRNA expression between these two studies suggests that sensory learning and forebrain-dependent trace associative learning have some
overlap in essential gene involvement, though previous reports utilizing whisker deprivation paradigms suggest that there are some underlying mechanistic differences (Chau et al., 2013b). Furthermore, many sensory learning-induced neocortical gene modifications previous observed, such as Btg2, Cyr61, Fos, Nptx2 and Nr4a2 (Valles et al., 2011), were not modified following WTEB conditioning, continuing to suggest underlying differences between associative learning and sensory adaptation.

In addition to these synaptic and calcium modulating genes, our analyses also revealed modulation of several interesting genes that will require further investigation. For example, our protein and mRNA analyses of Oprk1 expression demonstrated that it was significantly reduced in stimulated LRD barrels compared to stimulated unpaired barrels and naive barrels (Figure 24B), suggesting a down-regulation of neocortical Oprk1 expression following learning. Recent studies have reported that k-opioid receptor signaling plays a role in fear extinction (Bilkei-Gorzo et al., 2013), further suggesting that changes in Oprk1 could be underpinning learning. However, additional studies are needed to more closely examine the role Oprk1 plays in learning and memory.

In summary, findings from this study revealed modifications of a wide variety of genes in the neocortex during and following trace-associative learning. Moreover, analyses from this study demonstrating a time-dependent increase in calcium- and synapse-related genes during learning paralleling the transient structural plasticity seen in Chapter 3 suggest that these genes could be playing a role in learning-induced structural plasticity and thus be important for learning and memory. To our knowledge, this was the first genome-wide analysis to examine neocortical changes at different time points of forebrain-dependent trace-associative learning.
Together, these analyses provide a strong foundation for future studies to more closely examine the neuronal underpinnings of learning and memory.
Figure 21. Schematic of conditioning paradigms. Conditioned mice were trained with either a trace-paired conditioning or unpaired conditioning paradigm. (A) Trace-paired conditioned mice received 250 ms of whisker stimulation (CS), followed by 250 ms of stimulus-free (trace) interval and 100 ms of periorbital shock (US) every trial. (B) Unpaired conditioned mice randomly received either 250 ms of whisker stimulation or 100 ms of a mild periorbital shock each trial.
Figure 22. All trace-paired conditioned mice (ACQ and LRD) learned the WTEB conditioning task, in contrast to unpaired conditioned mice. (A) Mean percent conditioned response (CR) (±SEM) of ACQ mice per session until ACQ criterion (C-1 = day of ACQ criterion; C-2 = day before ACQ criterion; C-3 = two-days before ACQ criterion). (B) Mean percent conditioned response (CR) (±SEM) of LRD mice per session until LRD criterion (C = day of LRD criterion; C-1 = day before LRD criterion; C-2 = two-days before LRD criterion; C-3 = three-days before LRD criterion). All trace-paired conditioned mice exhibited a significant increase in WTEB conditioning performance compared to unpaired conditioned mice, and in comparison to baseline performance. Furthermore, LRD mice performed significantly better than ACQ mice on criterion day. * p < 0.05.
Figure 23. Relative mRNA expression (±SEM) of genes from qPCR analyses. (Top) Relative mRNA expression (±SEM) of Arc, CaenG4, Car8, Egr3, Fat3, Homer2, Mycbp2, Necab2, Prkce, Rasgrp2, Rcn, Sgk1 and Zfp738 across all groups. (Bottom) Relative mRNA expression (±SEM) of Capns2, Glra3, Megf6, Mir153, Mir154, Mir199a1, Mir690, Nexn, Oprk1, Pbx3, Prima1, Rfx5 and Syt9 across all groups.
Figure 24. Mean (±SEM) puncta count of selected genes (Necab2, Oprk1, and Sgk1) from immunohistochemical analyses. (A) Mean (±SEM) Necab2+ puncta count across all groups (left) and representative microphotograph of primary somatosensory cortex stained for Necab2 (right). (B) Mean (±SEM) Oprk1+ puncta count across all groups (left) and representative microphotograph of primary somatosensory cortex stained for Oprk1 (right). (C) Mean (±SEM) Sgk1+ puncta count across all groups (left) and representative microphotograph of primary somatosensory cortex stained for Sgk1 (right).
Figure 25. Relative mRNA expression (±SEM) of calcium-related genes (Cacng4, Fat3, Necab2, Rasgrp2 and Rcn) from qPCR analyses. * p < 0.05.
Figure 26. Relative mRNA expression (±SEM) of synapse-related genes (*Glra3, Nexn, Prima1 and Rcn*) from qPCR analyses. * p < 0.05.
### Table 1

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<tr>
<th>Calcium-Binding Genes</th>
<th>Synaptic Genes</th>
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<tr>
<td>Fat3</td>
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<td>Nacab3</td>
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<td>Rasgrp2</td>
<td>Rasgrp2</td>
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<td>Ano2</td>
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*Table 1.* List of calcium binding and synapse genes that were determined significantly different between LRD and yoked-LRD-unpaired mice from the overall functional analysis with the DAVID program.
Supplementary Figure 7. ACQ and LRD mice learned the WTEB conditioning task compared to unpaired-conditioned mice. (A) Mean percent conditioned response (CR) (±SEM) across all training sessions for ACQ mice. ACQ mice were trained until they exhibited three-CRs out of five-consecutive trials. ACQ mice took an average of 2.5 training sessions to meet ACQ criterion (arrow). (B) Mean percent conditioned response (CR) (±SEM) across all training sessions for LRD mice. LRD mice were trained until they exhibited four-CRs out of five-consecutive trials. LRD mice took an average of 2.6 training sessions to meet LRD criterion (arrow).
Section Four: Examining Learning-Induced Mechanisms at the Systems-Level
Chapter 6: Systems-Level Learning-Induced Mechanisms*

Chapter 6 will explore the underpinnings of memory consolidation at a systems-level focusing on the amygdala’s involvement in eyeblink conditioning.

Introduction

It is widely accepted that the more emotionally arousing an event is (whether positive or negative), the better the event will be remembered (Cahill and McGaugh, 1995; van Stegeren et al., 1998; Cruciani et al., 2011). Such emotionally arousing events have been shown to peripherally cause many physiological changes, such as increased cortisol levels and elevated dehydroepiandrosterone (Schwartz, 2002; Dickerson and Kemeny, 2004). Investigations of the neurobiology of emotion have similarly demonstrated that emotionally arousing events modulate glucocorticoid and epinephrine levels in the brain. Many of these investigations have further suggested that the amygdala plays a key role in regulating these biochemical changes by regulating our emotional response to an event. For example, brain imaging analyses in humans have demonstrated a positive correlation between the amount of amygdala activation and degree of emotional arousal (Cahill et al., 1996; Costafreda et al., 2008). Furthermore, patients with amygdala damage exhibit impairments in their ability to recognize and express emotion (Adolphs et al., 1994; Adolphs et al., 1995). These analyses, along with rodent and non-human primate studies of amygdala function, (Thompson et al., 1977; Lukaszewska et al., 1980; Swartzwelder, 1981; Rosen and Davis, 1988) have suggested that the amygdala plays a central role in mediating our emotional response to an event.

In addition to regulating the response to an emotional event, further analyses have also demonstrated that amygdala activation is directly tied to how well the emotional event is remembered. For example, memory tests in humans have found a positive correlation between the level of consolidation and the extent of amygdala activation (Cahill et al., 1996; LaBar et al., 1998). Furthermore, amygdala lesions in various species, including humans (Cahill et al., 1995), have been shown to dramatically impair a subject’s ability to remember an emotional event.
(Werka et al., 1978; Liang et al., 1982; Jellestad and Bakke, 1985; Peinado-Manzano, 1988).
Likewise, pharmacological activation of the amygdala produces a dose-dependent enhancement of memory for emotionally-motivated behavioral paradigms (Introini-Collison, Miyazaki, & McGaugh, 1991; Introini-Collison, Dalmaz, & McGaugh, 1996; Liang, 1986; Liang, McGaugh, & Yao, 1990). These, and other similar analyses, have strongly suggested that the amygdala plays a role in facilitating memory consolidation for emotionally arousing events.

Although most would agree with the amygdala’s importance in memory consolidation, there is still debate regarding the amygdala’s role as an actual site of memory storage versus simply modulating storage of memory in other brain regions. Many learning theories suggest that the most likely site for long-term memories is the neocortex (Eichenbaum et al., 1992; Squire et al., 2004). However, some findings suggest that an aspect of some memories is stored in the amygdala, especially with fear associative learning paradigms. The following review will discuss findings utilizing fear- and non-fear-motivated Pavlovian behavioral paradigms to illustrate our current understanding of how the amygdala facilitates memory acquisition and consolidation.

**Amygdala’s Role in Memory Storage**

**Fear Associative Learning**

Studies utilizing fear conditioning paradigms, a type of Pavlovian conditioning, have demonstrated that the amygdala plays a role in both acquisition and consolidation of cued-fear associative learning (Kim and Jung, 2006; Johansen et al., 2011). In this review, the term *subjects* will be used when similar findings have been reported with multiple species. In cued-fear associative learning, a subject learns to associate a cue, such as a light or tone (the conditioned stimulus; CS), with an unpleasant stimulus evoking fear, such as a footshock (the
unconditioned stimulus; US). To measure the strength of the tone-footshock-association, subjects are presented with the same cue in a novel environment and the fear response is recorded. Support for the amygdala playing a key role in fear associative memories stems from a myriad of studies varying in techniques, including lesioning (Blanchard and Blanchard, 1972; Kapp et al., 1979; Iwata et al., 1986; Phillips and LeDoux, 1992), electrophysiological recordings (Applegate et al., 1982; Pascoe and Kapp, 1985) and pharmaceutical manipulations (Gallagher and Kapp, 1978; Gallagher et al., 1981). The following section will focus on findings illustrating the role of the amygdala in consolidating cued-fear associations.

**Amygdala as a site of storage**

Analyses of amygdala function with cued-fear-conditioning have led many to suggest that the amygdala acts as a possible site of storage for these associations. In support of this theory, studies have demonstrated that the amygdala plays an essential role in retrieval of long-term fear associations (Lee et al., 1996; Maren et al., 1996; Schafe et al., 2001; Gale et al., 2004). For example, findings demonstrated that rats with lesions to the basolateral amygdala one-day, two-weeks, one-month (Lee et al., 1996; Maren et al., 1996) or sixteen-months (Gale et al., 2004) following cued-fear-conditioning exhibit significantly less freezing behavior compared to sham controls. Additionally, inactivation of the amygdala prior to retention testing results in significantly fewer conditioned responses, compared to controls (Muller et al., 1997). Furthermore, studies disrupting protein synthesis in the amygdala, a molecular mechanism believed to be important for long-term memory consolidation (Guzowski et al., 2000; Kandel, 2001), have demonstrated impairments in fear-related memory. For example, various studies have demonstrated that disruptions in protein synthesis in the amygdala following acquisition via infusion of a protein synthesis inhibitor impair fear memory retention (Schafe and LeDoux,
These studies, collectively, provide strong support for the amygdala either playing an essential role in retrieval of fear memories or that the amygdala is a site of storage for long-term fear associations.

To date, most investigations of amygdala’s involvement in fear-conditioning, summarized in the discussion above, utilize a delay-conditioning paradigm; not many studies have examined the amygdala’s role in a trace-fear-conditioning paradigm. In delay-conditioning, there is no separation in time between presentation of the CS and US. In contrast, there is a stimulus-free interval between the CS and US in trace-conditioning (Figure 27). Trace-fear-conditioning has been demonstrated to be dependent upon a number of distinct brain regions, including normal hippocampal (McEchron et al., 1998; Czerniawski et al., 2011) and medial prefrontal cortical activity (Runyan et al., 2004; Gilmartin and McEchron, 2005). However, the amygdala’s role in trace-fear-conditioning is not as well understood as the hippocampus and medial prefrontal cortex. Raybuck and Lattal (2011) found that global amygdala inactivation via GABA\(_A\) agonist muscimol infusion prior to trace-fear-conditioning resulted in no significant differences in freezing behavior, compared to sham and vehicle controls, suggesting that acquisition for the trace-fear-association is independent of the amygdala. In contrast, studies have found that global amygdala inactivation via infusion of the same GABA\(_A\) agonist muscimol or blocking protein synthesis in the amygdala hinders acquisition for trace-fear-conditioning compared to controls (Kwapis et al., 2011; Gilmartin et al., 2012), suggesting that acquisition for the trace-fear association is dependent upon amygdala involvement. Although further analyses are needed to decipher the discrepancy between these findings, one possible explanation could reside in the extent of the amygdala inactivation. Studies have shown that different amygdala nuclei play specific roles in delay-fear-conditioning (Nader et al., 2001). Such nuclei specific
analyses have not been as well examined with trace-fear-conditioning and could account for the conflicting findings. Although these analyses of amygdala function in trace-fear-conditioning conflict, analyses with delay-fear associations suggest that the amygdala is critically involved and could act as a possible site of storage for trace-fear associations.

Amygdala not as a site of storage

Although most analyses of cued-fear-conditioning suggest that the amygdala is a site of storage, most learning theories suggest that the neocortex is the most likely site of storage for long-term memories (Eichenbaum et al., 1992; Squire et al., 2004). In support of this theory, studies have demonstrated that training on an object orientation task, a paradigm where non-human primates learn to direct their attention towards a specific visual stimulus, alters both neuronal sensitivity and preferred orientation in primary visual neocortex (Schoups et al., 2001; Ghose and Maunsell, 2002). Likewise, rearing rodents in an enriched environment, a learning condition where subjects are reared in an environment facilitating enhanced motor, visual, and social stimulation, induces various forms of neocortical plasticity, such as increased dendritic material (Greenough and Volkmar, 1973; Juraska et al., 1980; Juraska, 1984) and increased number of dendritic spines in primary visual neocortex (Globus et al., 1973; Diamond et al., 1975; Turner and Greenough, 1985; Kolb et al., 2003). Furthermore, findings from frequency discrimination training, where a subject learns to preferentially favor a specific tone, have been shown to alter the preferred frequency receptive field in primary auditory neocortex (Disterhoft and Stuart, 1976; Kitzes et al., 1978; Kraus and Disterhoft, 1982; Diamond and Weinberger, 1986; Edeline et al., 1993; Recanzone et al., 1993; Rutkowski and Weinberger, 2005). Finally, studies utilizing tactile discrimination, where a subject learns to dissociate two tactile stimuli, have been shown to alter somatosensory neocortical map hand representation (Jenkins et al.,
and alter neuronal firing rate in primary somatosensory barrel neocortex (Krupa et al., 2004) for digit and whisker stimulation, respectively. These, and similar studies, along with various learning theories, have strongly suggested that the neocortex is modulated in response to learning and is a likely location for storage of most long-term memories.

In addition to these analyses suggesting that the neocortex is a likely site of long-term memory storage, some studies have also suggested that fear associations are not stored in the amygdala, but rather stored in other brain regions, such as the neocortex. These analyses have argued that the amygdala does not act as a site of consolidation for fear, but rather facilitates our ability to express fear. For example, studies have found that inactivation of the amygdala impairs freezing behavior in rodents when presented with cat fur, a non-learned stimulus that naturally induces fear in rodents (Vazdarjanova et al., 2001). These findings suggest that amygdala lesion-induced abnormalities in cued-fear-conditioning are due to an inability to express fear rather than removal of the site responsible for fear-related memory consolidation. Further support for this theory has come from analyses utilizing inhibitory avoidance conditioning. With inhibitory avoidance conditioning, a subject learns that a dark compartment (CS) is associated with an unpleasant stimulus, a footshock (US). However, rather than demonstrating this learned association with a fear response, the rodent demonstrates the learned association by avoiding entering the dark compartment. Note, there are many variations of this paradigm that can add other forms of learning such as an operant component; however, for the purpose of this review, we will focus on the associative aspects. Studies utilizing the inhibitory avoidance conditioning paradigm have found that post-training amygdala lesions do not impair expression of the learned fear-association (Liang et al., 1982; Parent et al., 1995). These findings
suggest that the amygdala is not a site of storage for inhibitory avoidance fear-associations. Furthermore, these findings suggest that the amygdala may not be a site of storage for cued-fear-conditioning. However, the molecular analyses demonstrating that post-training amygdala infusion of protein synthesis inhibitors following cued-fear-conditioning impair memory retention (Kwapis et al., 2011; Gilmartin et al., 2012) disagree with these findings, and suggest that an aspect of the cued-fear memory is stored in the amygdala. Irrespective of the specific site of storage for fear-associations, these, and other studies, have collectively demonstrated that the amygdala plays an essential role in either storing fear-related memories or facilitating consolidation of fear-related memories in other brain regions.

**Non-Fear Associative Learning: Eyeblink Conditioning**

The studies previously discussed, along with various others analyses examining amygdala function with fear-associative paradigms, have strongly suggested a role for the amygdala in fear-associations; however, amygdala involvement in classic non-fear associative paradigms, such as eyeblink conditioning, are not as well understood. In eyeblink conditioning, a subject learns that a neutral stimulus (CS), such as a tone or whisker stimulation, predicts delivery of a second stimulus (US) that elicits an eyeblink. After repeated CS-US pairings, the subject learns to blink when presented with the CS in anticipation of the US. In delay-eyeblink conditioning, the US co-terminates with the CS; thus there is no separation in time between the two stimuli (Figure 27). This form of learning is mediated by brainstem-cerebellar processing (Clark et al., 1984; Mauk and Thompson, 1987) and is not dependent upon neocortical processing (Norman et al., 1977; Oakley and Russell, 1977; Mauk and Thompson, 1987). Furthermore, various lesion and electrophysiological analyses have suggested that consolidation for delay-eyeblink associations occur in the cerebellum. For a detailed review of mechanisms
for memory consolidation with delay-eyeblink-conditioning see Thompson and Steinmetz (2009). Based upon current understanding of the neuronal pathways necessary for delay-eyeblink-conditioning, the amygdala is not believed to play a prominent role in acquisition of the association (Thompson and Steinmetz, 2009). Furthermore, unlike fear associative paradigms, this form of conditioning is not predominantly believed to be fear-motivated. Although analyses of heart rate and blood pressure, factors that increase with fear, have demonstrated increased levels within the first few CS-US pairings, these properties decrease, while the associative behavior increases with conditioning (Hein, 1969; Powell and Kazis, 1976). These studies suggest that acquisition for eyeblink conditioning is not dependent upon fear, thus further suggesting that the amygdala would not play a dominating role in task acquisition. However, studies have found that under certain conditions, the amygdala does play a role in modulating acquisition for eyeblink associations.

*Delay-Eyeblink Conditioning*

In support of a role for the amygdala in facilitating acquisition of eyeblink associations, studies examining delay-eyeblink-conditioning have found that amygdala stimulation increases the rate of acquisition for the association (Whalen and Kapp, 1991; Canli and Brown, 1996; Neufeld and Mintz, 2001). These studies strongly suggest that the amygdala can play a role in modulating memory for eyeblink conditioning, similar to fear associative learning paradigms. In support of this role, lesion studies have further suggested a more direct role for the amygdala in acquisition of eyeblink associations. Studies have found that post-training amygdala lesions do not have an effect on performance; however, pre-training amygdala lesions impair acquisition for the delay-eyeblink association (Weisz et al., 1992; Choi et al., 2001; Lee and Simons, 2004; Lindquist and Brown, 2004; Sakamoto and Endo, 2010). Furthermore, amygdala lesions have
been found to reduce the rate of learning by dramatically impairing acquisition for the association during the initial days of training (Rescorla and Solomon, 1967; Choi et al., 2001; Mintz and Wang-Ninio, 2001; Lee and Simons, 2004). These findings suggest that the amygdala plays a critical role in enhancing the effectiveness of the CS early in training to assist with delivery of conditioned responses (CRs). These, and other analyses of amygdala involvement in acquisition of the delay-eyeblink association, have offered support towards a two process model for consolidation (Figure 28). In this model, the initial phase of learning activates the amygdala and other emotional responses, possibly increasing the saliency of the CS. In the second (later) phase of learning, amygdala involvement decreases while motor and sensory regions solidify the association and generate well-timed CRs (Rescorla and Solomon, 1967; Choi et al., 2001; Mintz and Wang-Ninio, 2001; Lee and Simons, 2004). In support of this hypothesis, many non-specific emotional responses (e.g., increased heart rate and respiration) have been found to dissipate as appropriately timed CRs emerge (Hein, 1969; Powell and Kazis, 1976).

This theory, that the amygdala plays an initial role in learning by increasing the saliency of the behavioral events, is believed to be a general property in acquisition for other non-fear-motivated paradigms. Such a theory would suggest that the amygdala focuses one’s attention on behaviorally relevant events or stimuli to facilitate acquisition and consolidation. In support of this argument, anatomical analyses of amygdala projections have found that the amygdala directly projects to the inhibitory thalamic reticular nucleus (TRN) (Zikopoulos and Barbas, 2012). The TRN receives projections from the neocortex and thalamus, but only sends inhibitory projections to the thalamus (Crick, 1984; Pinault, 2004), thus facilitating its ability to directly mediate or filter thalamocortical interactions (Figure 29). Further analyses have demonstrated that the TRN is activated when a subject is attending to a stimulus (Montero, 1997; McAlonan et
Furthermore, TRN lesions have been found to impair a rat’s ability to attend to a stimulus (Weese et al., 1999). These findings, along with its anatomical connections facilitating inhibition of thalamic activation of the neocortex, have strongly suggested a role for the TRN in regulating what our brains are attending to (Crick, 1984; Pinault, 2004). Amygdala to TRN projections would allow the amygdala to directly modulate what information is conveyed to the neocortex. Such regulation would empower the amygdala to determine what our brains should attend to and thus would have tremendous implications towards more rapid acquisition of behaviorally relevant stimuli for any learning task (Figure 29).

Although the rodent literature has offered much support for the amygdala involvement in initial acquisition and this two process model for memory consolidation, not all studies examining amygdala involvement have supported this theory. Some rodent studies have observed a general reduction in the rate of acquisition with amygdala lesions (Sakamoto and Endo, 2010). Furthermore, studies using rabbits have suggested that the amygdala’s involvement in delay-eyeblink-conditioning is not as prominent as suggested from rodent analyses. Analysis of delay-eyeblink-conditioning in rabbits have demonstrated only mildly impaired performance with amygdala lesions (Weisz et al., 1992). In their analysis, Weisz and colleagues (1992) further demonstrated that the impairing effects of amygdala lesions in rabbits can be diminished by increasing the intensity of the auditory stimulation used for the CS. These findings suggest that the saliency of the CS could have dramatic implications towards amygdala involvement and may account for possible discrepancies with amygdala lesions across species.

Another possible explanation for some of the discrepancies between these lesion studies could reside in the size of the lesion. Anatomically, it is known that the lateral amygdala receives converging input from both the auditory CS and somatosensory US pathways (Burton
and Craig, 1979; LeDoux et al., 1987; LeDoux et al., 1990; Whalen and Kapp, 1991; Weisz et al., 1992). The lateral amygdala then projects to the basolateral amygdala and finally to the central amygdala. From the central amygdala, information projects directly to the pontine nuclei that then feeds information to the cerebellum. Although these regions are interconnected, there is no reason to believe each of these nuclei, or even every cell within each nuclei, would have equal involvement in acquisition for the delay-eyeblink association. Analyses of training-induced neuronal activation in the amygdala found that about 60% of the neurons responded to the CS while about 70% responded to the US (Richardson and Thompson, 1984). Thus, partial lesions could disproportionately alter the amygdala’s involvement in delay-eyeblink associations. Furthermore, when neuronal activity from specific amygdala nuclei were examined, it was determined that unlike the central amygdala, which exhibited increased activity with conditioning, the basolateral amygdala did not exhibit a learning-specific pattern of activation (Rorick-Kehn and Steinmetz, 2005). Furthermore, additional analyses determined that although the central amygdala exhibited learning-specific activation, the extent of this activation could be modulated by simply varying the intensity of the US (Rorick-Kehn and Steinmetz, 2005). These findings strongly suggest that discrepancies in amygdala lesion studies could be due to differences in training conditions and the specificity of nuclei lesioned.

**Trace-Eyeblink Conditioning**

Although there are some inconsistencies in amygdala analyses, most studies suggest that the amygdala plays a critical role in acquisition of delay-eyeblink associations; however, analyses with trace-eyeblink-conditioning have not found that the amygdala plays as prominent of a role in acquisition of the association. In trace-eyeblink-conditioning, the CS and US are temporally separated by a stimulus-free interval (Figure 27). This form of learning is both
hippocampal- and neocortical-dependent in that pre-conditioning lesions of the hippocampus and specific regions of the neocortex impairs a subject’s ability to learn the trace-eyeblink association (Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995; McGlinchey-Berroth et al., 1997; Clark and Squire, 1998; Kronforst-Collins and Disterhoft, 1998; Weiss et al., 1999; Weible et al., 2000; McLaughlin et al., 2002; Takehara et al., 2002; Han et al., 2003; Takehara et al., 2003; Tseng et al., 2004; Galvez et al., 2007). Unlike delay-eyeblink-conditioning, where consolidation for the association is believed to reside in the cerebellum, trace-eyeblink associations are believed to also reside in the neocortex. For example, analyses of neocortical plasticity following trace-eyeblink-conditioning have demonstrated unilateral learning-specific metabolic expansion of the primary neocortical area receiving input from the CS, compared to pseudo-conditioned controls (Galvez et al., 2009, 2011). Further analyses have demonstrated that neocortical lesions prevent acquisition for the trace-eyeblink association (Galvez et al., 2007). These, and other similar studies, have strongly suggested that the neocortex is a site of storage for trace-eyeblink associations.

With the neocortex acting as a site of storage for trace-eyeblink associations, most would speculate that the amygdala, similar to delay-eyeblink-conditioning, would play a role in facilitating consolidation. However, in trace-eyeblink-conditioning the amygdala does not appear to play as prominent of a role as observed in delay-eyeblink-conditioning. Analysis of metabolic activity in the central amygdala following eyeblink conditioning acquisition demonstrated increased activation with delay-eyeblink-conditioning; however, only a trend towards increased activation following trace-eyeblink-conditioning was observed (Plakke et al., 2009). Although this is only a single analysis, it suggests decreased involvement of the amygdala with trace-eyeblink-conditioning. However, based upon the two process model for
consolidation (Figure 28) one would expect the amygdala to play a significant role during initial acquisition, but not once the association was learned. Furthermore, based upon the model, as the association is learned, the amygdala would decrease its involvement. This prediction of the model, along with the fact that trace-eyeblink associations require significantly more CS-US pairings, decreases the likelihood that the amygdala would still be activated following acquisition. Obviously, additional analyses of amygdala involvement in trace-eyeblink conditioning are necessary in order to make any definitive statements; however, analyses with delay-eyeblink-conditioning and the two process model for consolidation (Figure 28) suggest that the amygdala plays a role in facilitating initial acquisition for trace-eyeblink associations.

Conclusion

Over the last several decades, there has been overwhelming evidence that the amygdala plays an essential role in facilitating acquisition and consolidation of fear-associations. Although there is some question regarding the specific location of long-term memory storage (whether the amygdala or another region), these analyses strongly suggest that the amygdala plays a critical role in acquisition and consolidation of fear-related memories. However, the amygdala’s role is not as clearly defined when examining non-fear-related memories. Utilizing eyeblink-conditioning as a non-fear-motivated task, this review suggests that there is also substantial support for amygdala involvement in acquisition of non-fear-motivated tasks. Analyses of amygdala involvement in these non-fear-motivated tasks suggest that the amygdala acts to increase the saliency of the learned stimuli so that other brain regions can consolidate the learned response. These findings suggest a two process model for memory consolidation. In this proposed model, the amygdala facilitates determining what thalamic information is conveyed to the neocortex. In support of this model, studies have found anatomical projections from the
amygdala to the thalamic reticular nucleus, a brain region critically involved in directing attentional activation of the neocortex, the most likely site of storage for long-term memories. This model would suggest that amygdala lesions would decrease the rate of consolidation by not facilitating the initial phase of learning, but these lesions would not hinder a subject’s ability to eventually acquire the association. These predictions are entirely consistent with the amygdala analyses with eyeblink conditioning mentioned above. Although this model was proposed under the framework of the eyeblink paradigm, the implications of these findings would have a broader role in other non-fear-motivated tasks. Additionally, such a model would also have a role in fear-motivated tasks. However, due to the amygdala’s multifaceted role in different aspects of fear-motivated tasks, it is difficult to determine if the amygdala’s role in modulating thalamocortical communication decreases during task acquisition similar to that of non-fear-motivated tasks. Together, these findings suggest that the amygdala plays a promiscuous role in directing our attention towards behaviorally relevant stimuli, thus facilitating acquisition and memory consolidation for both fear- and non-fear related memories. Currently, many analyses of the amygdala’s role in humans have focused on individuals suffering from fear-related disorders such as post-traumatic-stress-disorder; however, the findings presented in this review demonstrate that the amygdala may also play a critical role in non-fear-related learning, suggesting that amygdala abnormalities could also plague many other neurological disorders of learning and memory.
Figures

A. Delay-Conditioning

B. Trace-Conditioning

Figure 27. Schematic of Pavlovian conditioning paradigms. (A) In delay-conditioning, the conditioned stimulus (CS) (e.g., tone, whisker stimulation) co-terminates with the unconditioned stimulus (US) (e.g., mild footshock, eye shock). (B) In trace-conditioning, there is a stimulus-free separation in time between the CS and US.
Figure 28. Schematic of amygdala involvement in a two process model for memory consolidation. In phase 1 of the model, the amygdala, receiving event information, increases the saliency of the event to motor and sensory regions, thus facilitating memory consolidation and behavioral response to the event. In phase 2 of the model, motor and sensory regions, primed with amygdala activation from phase 1, begin to solidify the memory and generate appropriate behavioral responses.
Figure 29. Schematic of amygdala and thalamic reticular nucleus involvement with eyeblink conditioning. Information from the conditioned stimuli (CS) first projects to the thalamus, where it will then project to the neocortex and thalamic reticular nucleus. The thalamic reticular nucleus can then compare information from the neocortex, amygdala and thalamus. Then, via selective inhibition of thalamic activity, the thalamic reticular nucleus can modulate what information the neocortex receives. Modulation of neocortical input would modulate neocortical nuclei that directly assists in generating the appropriate conditioned response “Blink”. Note in the above illustration, the amygdala can facilitate appropriate behavioral responses by not only modulating neocortical activation of the pontine nuclei via thalamic reticular nuclear stimulation, but also via direct projections to the pontine nuclei.
Figure 30. Schematic of brain regions involved with eyeblink conditioning.
Section Five: Conclusion
Together, studies in this dissertation examined neocortical anatomical and molecular correlates of learning-induced plasticity at different time points of WTEB conditioning. Learning-induced anatomical plasticity in the barrel cortex during different time points of WTEB conditioning was explored in Section Two. Findings from Chapter 2 demonstrated that expression of the presynaptic marker synapsin I is increased in conditioned barrels following WTEB conditioning acquisition; however, the exact synaptic modifications were indiscernible from these analyses. To more directly investigate synaptic anatomical modifications, Chapter 3 examined dendritic spine properties of Golgi-Cox stained neurons in layer IV of primary somatosensory cortex at different time points of WTEB conditioning. Findings from Chapter 3 demonstrated time-dependent anatomical plasticity in level IV of the primary somatosensory cortex during WTEB conditioning. This transient increase in spine density is consistent with some hippocampal analyses of spine density following spatial learning tasks. For example, studies have found that hippocampal spine density increases and returns to baseline levels after learning of hippocampal-dependent tasks such as the morris water maze (O'Malley et al., 2000; Eyre et al., 2003) and avoidance learning (O'Malley et al., 1998). Similarly, other findings have also reported a time-dependent increase in spine density of hippocampal slices following long-term potentiation (Wosiski-Kuhn and Stranahan, 2012), one of the most common molecular models of learning and memory. However, to our knowledge, findings from Chapter 3 are the first to demonstrate a time-dependent increase in spine density during trace-associative learning in the neocortex. These findings demonstrating neocortical plasticity during memory formation together with previous reports of hippocampal plasticity (Geinisman et al., 2001; Leuner et al., 2003) during trace eyeblink conditioning suggests that rewiring in multiple brain regions are
involved during task acquisition (see Figure 30 for schematic of brain regions involved during WTEB conditioning).

The learning-induced molecular plasticity at different time points of WTEB conditioning was investigated in Section Three. Chapter 4a examined Arc expression at various time points following WTEB conditioning in primary somatosensory cortex. Arc is an immediate early gene that has been suggested to be important for synaptic plasticity (Steward et al., 1998; Peebles et al., 2010) and memory consolidation (Guzowski et al., 2000). Findings from Chapter 4a did not detect any significant differences in neocortical Arc expression between trace conditioned and control subjects in primary somatosensory cortex following five-sessions of WTEB conditioning at any of the time points examined. Taking into consideration the time-dependent expression profile of Arc, Chapter 4b investigated Arc expression in multiple brain regions, including primary auditory cortex, following a one-trial cued fear conditioning session. Primary auditory cortex, similar to primary somatosensory cortex's role in WTEB conditioning, is important for cued fear conditioning (Song et al., 2010; Ide et al., 2012). Findings from Chapter 4b showed that there were only significant differences in Arc expression between trace conditioned and control subjects in the amygdala, but not in primary auditory cortex or any of the other brain regions examined (see Chapter 4b supplementary material). Though more studies are needed to determine when the primary auditory cortex is involved during one-trial cued fear conditioning, findings from this study suggest that plasticity at the systems-level is time-dependent, consistent with that proposed in Chapter 6. Findings from Chapter 4b suggest that learning-induced plasticity at the systems-level also occurs during the early phases of memory formation. To further explore learning-induced neocortical molecular modifications, a genome-wide analysis in primary somatosensory cortex during WTEB conditioning was conducted in Chapter 5.
Calcium-related and synapse-related genes were up-regulated at the acquisition phase and returned to baseline levels by the learned phase. The transient up-regulation of calcium-related and synapse-related genes, similar to the transient spine proliferation found in Chapter 3, further suggest that these calcium-related and synapse-related genes could play an important role in learning-induced structural plasticity.

Together, findings from this dissertation examining anatomical and molecular plasticity during WTEB conditioning demonstrating increased spine proliferation during task acquisition suggest that neocortical rewiring, as well as systems-level rewiring, occurs during learning. In particular, findings discussed in Chapter 6 showing that the amygdala is involved during the early phases of both fear and non-fear learning tasks and findings from Chapter 4b demonstrating increased amygdalar Arc expression one hour following a one trial cued trace-fear conditioning suggest that the amygdala is initially involved with learning and that amygdalar synaptic changes occur during the very early phases of memory formation. Moreover, findings from Chapter 3 demonstrating transient neocortical spine proliferation during learning and findings from Chapter 5 indicating a similar transient up-regulation in calcium-related and synapse-related genes suggest that modifications of calcium-related and synapse-related genes contribute to the neocortical rewiring observed during learning. Once learning has occurred, neocortical spine density in layer IV returns to control levels (see Chapter 3) suggesting that rewiring in these regions occurs during learning, and to a lesser degree, if at all, during over-training. Collectively, findings from these studies suggest time-dependent plasticity at both the systems-level and neocortical-level during learning.
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