Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Amy L. Mahan Date

Transcriptional regulation of Homer1a during Pavlovian Fear Conditioning

By

Amy L. Mahan

Doctorate of Philosophy

Graduate Division of Biological and Biomedical Sciences

Neuroscience

_____ [Advisor's signature]

Kerry J. Ressler

Advisor

_____ [Member's signature]

Gary Bassell

Committee Member

_____ [Member's signature]

Randy Hall

Committee Member

_____ [Member's signature]

Robert Liu

Committee Member

_____ [Member's signature]

Pete Wenner

Committee Member

Accepted:

Transcriptional Regulation of Homer1a During Pavlovian Fear Conditioning

By

Amy L. Mahan

B.S., Trinity University

Advisor: Kerry J. Ressler, M.D., Ph.D.

An abstract of

A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of

Emory University

in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate

Division of Biological and Biomedical Sciences

Neuroscience

2011

Abstract

Transcriptional Regulation of Homer1a during Pavlovian Fear Conditioning

By Amy L. Mahan

The consolidation of fear conditioning involves upregulation of genes necessary for long-term memory formation. We examined whether *homer1a*, which is required for memory formation, is downstream of BDNF - TrkB activation. In chapter 3 we demonstrate that *homer1a* mRNA 1) increases after fear conditioning *in vivo* within both amygdala and hippocampus, 2) increases after BDNF application to primary hippocampal and amygdala cultures *in vitro,* 3) these increases are dependent on transcription and MAPK signaling, and 4) we demonstrate that a trkB agonist, 7,8-DHF enhances long-term memory for Pavlovian fear conditioning *in vivo* as well as increasing expression level of *homer1a in vitro*, and that inhibiting trkB signaling impairs *homer1a* expression during Pavlovian conditioning in the amygdala and hippocampus. In chapter 4 we demonstrate that 1) Pavlovian fear conditioning induces distinct modifications of histones bound to the Homer1 promoter; 2) BDNF induced plasticity produced similar modifications of histones bound to the Homer1a promoter in primary amygdala and hippocampal cell culture; and 3) Sodium Butyrate, a histone deacetylase inhibitor, enhanced contextual fear memories, hippocampal mRNA expression and hippocampal H3 acetylation around the Homer1 promoter. In Chapter 5 we demonstrate that *homer1a* gene transcription and changes in histone modifications during fear conditioning are mediated through CREB binding around the Homer1 promoter by: 1) demonstrating that CREB and CBP are increasingly bound to the Homer1 promoter after fear conditioning and 2) that inhibiting CREB inhibits *homer1a* expression, CBP binding and histone modifications after fear conditioning. Together these data suggest that CREB and CBP are critical for regulation of *homer1a* expression, in part via modulation of histone acetylation regulating chromatin containing the *homer1* gene. This body of work provides evidence for dynamic regulation of *homer1a* following BDNF-induced plasticity or during a BDNF-dependent learning process.

Transcriptional Regulation of Homer1a During Pavlovian Fear Conditioning

By

Amy L. Mahan

B.S., Trinity University

Advisor: Kerry J. Ressler, M.D., Ph.D.

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate

Division of Biological and Biomedical Sciences

Neuroscience

2011

Table of Contents

Table of Figures

Chapter 1: Introduction

Fear Conditioning, Synaptic Plasticity, and the Amygdala:

Implications for Posttraumatic Stress Disorder

1.1 Abstract

Posttraumatic stress disorder (PTSD) is an anxiety disorder that can develop after a traumatic experience such as domestic violence, natural disasters or combat-related trauma. The cost of such disorders on society and the individual can be tremendous. In this chapter we will review how the neural circuitry implicated in PTSD in humans is related to the neural circuitry of fear. We then discuss how fear conditioning is a suitable model for studying the molecular mechanisms of the fear components which underlie PTSD, and the biology of fear conditioning with a particular focus on the brain derived neurotropic factor (BDNF)-TrkB, GABAergic and glutamatergic ligand-receptor systems. We then summarize how such approaches may help to inform our understanding of PTSD and other stress-related disorders and provide insight to new pharmacological avenues of treatment of PTSD.

1.2 Introduction

Irrational fear is a major impediment to success and productivity. When Franklin D. Roosevelt acknowledged, in 1933 "*the only thing we have to fear is fear itself*", he was commenting on the economic future of the United States, but unreasonable, over-generalized fear can have dramatic effects on all aspects of one's life. Over-generalized fear is one of the biggest symptoms of anxiety disorders, in particular disorders of fear regulation, including phobia, panic disorder, and posttraumatic stress disorder (PTSD). PTSD is an example of how excessive fear can impair quality of life. While fear learning is an evolutionarily advantageous response mechanism, when fear becomes too generalized, this mechanism may not only be unproductive, but harmful. PTSD is a disorder where learned fear due to a traumatic event becomes generalized to situations

that would normally be considered safe and results in autonomic hyperarousal in inappropriate situations.

Three types of symptoms are prevalent in PTSD: reexperiencing, avoidance and hyperarousal. Reexperiencing symptoms involve flashbacks, nightmares and frightening thoughts about the trauma, which can result in physical symptoms, including headaches, pains, and other symptoms of somatization. Avoidance symptoms include avoiding reminders of the experience, feeling emotionally numb, losing interest in previously enjoyable activities, and deficits in learning and memory. These symptoms may cause a person to change his or her personal routine. Finally, hyperarousal symptoms include being easily startled, feeling tense, having difficulty sleeping, and/or having angry outbursts. Reminders of the traumatic event usually trigger reexperiencing and avoidance symptoms whereas hyperarousal symptoms may be present more continuously (1994; Davidson et al., 2004; Hoge et al., 2007; Milliken et al., 2007; Gillespie et al., 2009; Wilcox et al., 2009).

There is a variability in the prevalence and severity of PTSD (Milliken et al., 2007). Trauma is necessary but not sufficient for the precipitation of PTSD. In fact one of the most critical current questions is why some trauma victims develop PTSD (between 5-30%)(Davidson et al., 2004; Milliken et al., 2007; Gillespie et al., 2009) while others experiencing the same trauma appear to be resilient. In addition, those who meet the criteria for PTSD vary widely in their symptom severity and in the type of symptoms they experience (Davidson et al., 2004; Lanius et al., 2006; Hoge et al., 2007; Milliken et al., 2007; Dickie et al., 2008; Gillespie et al., 2009; Wilcox et al., 2009). A variety of factors contribute to the magnitude of PTSD symptoms, including an individual's genetic

makeup, predisposition, social support network, and early-life experiences (Binder et al., 2008; Bradley et al., 2008; Green et al., 2010; Jovanovic and Ressler, 2010) (Box 1). In other words, these factors may determine an individual's resilience to trauma. Studying what accounts for this resilience in certain individuals could help target treatments and the prevention of PTSD in trauma victims predisposed to develop PTSD. Understanding the neurobiological mechanisms of PTSD as well as developing more rapid and cost effective treatments is of vital importance. The current review addresses recent molecular approaches to understanding PTSD using animal models of fear, limitations of these models, and speculation about how these models may lead to better treatment and understanding of PTSD and other fear-related disorders.

1.3 Pavlovian fear conditioning as a model for understanding the underlying mechanisms of pathological fear responses

The neural structures important to PTSD belong to the limbic system, a region important for emotional processing in both humans and animals (Heimer and Van Hoesen, 2006). The three regions within the limbic system most clearly altered in PTSD include the amygdala, the hippocampus, and the prefrontal cortex (PFC). The amygdala regulates learned fear in animal and human studies of Pavlovian fear conditioning (see Glossary) and receives projections from the hippocampus and PFC (Etkin and Wager, 2007; Francati et al., 2007; Quirk and Mueller, 2008; van Marle et al., 2009; de Carvalho et al., 2010). Subjects with PTSD show reduced activation of the PFC and hippocampus, which may coincide with reduced top-down control of the amygdala, possibly resulting in a hyper-responsive amygdala signal to fearful stimuli (Etkin and Wager, 2007). This may

result in the disordered fear regulation in PTSD and other fear-related disorders. Other regions involved with PTSD include the parahippocampal gyrus, orbitofrontal cortex, the sensorimotor cortex, the thalamus (Lanius et al., 2006), and the anterior cingulate cortex (Figure 1.1) (Abe et al., 2006; Rogers et al., 2009; Thomaes et al., 2010).

Patients with PTSD show markedly different responses to fear conditioning paradigms relative to trauma victims without PTSD (Blechert et al., 2007; Wessa and Flor, 2007; Griffin, 2008; Milad et al., 2008; Jovanovic et al., 2009; Pole et al., 2009; Shin, 2009; Ehlers et al., 2010; McLaughlin et al., 2010; Suendermann et al., 2010). They demonstrate behavioral sensitization to stress (Griffin, 2008; Ehlers et al., 2010; McLaughlin et al., 2010) and over-generalization of the conditioned stimulus (CS)-unconditioned stimulus (US) response (Pole et al., 2009; Suendermann et al., 2010). Such patients show impaired extinction of CS-US pairings (Blechert et al., 2007; Wessa and Flor, 2007; Milad et al., 2008) and show impaired fear inhibitory learning (Jovanovic et al., 2009). It is thought that this altered fear response may result in the intrusive memories and flashbacks, enhanced avoidance of reminder cues, and autonomic hyperarousal seen in PTSD (Yehuda and LeDoux, 2007; Jovanovic et al., 2009). The neural circuitry of fear conditioning is conserved across most vertebrate species, and its behavioral readout is both quick and robust (Lang et al., 2000; Belzung and Philippot, 2007). Therefore, fear conditioning is a tractable method of studying the fear response underlying PTSD. Many of the molecular tools that have been developed to study behavior in rodents can be applied to study mechanisms of fear dysregulation, and hence, to develop new therapeutics that may prove valuable for the treatment of PTSD.

Evidence from animal models and human neuroimaging studies suggest that one of the underlying mechanisms of PTSD may be aberrant synaptic plasticity (Quirk et al., 1995; McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Blair et al., 2001; Tsvetkov et al., 2002; Maren, 2005; Shumyatsky et al., 2005; Lanius et al., 2006; Francati et al., 2007; Sigurdsson et al., 2007; Sah et al., 2008; Pape and Pare, 2010). Synaptic plasticity describes the changes that occur at the synapse with prolonged synaptic activity. Such changes are physiological, morphological and molecular in nature. Synaptic plasticity is hypothesized to be the underlying basis of learning and memory (Quirk et al., 1995; McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Blair et al., 2001; Tsvetkov et al., 2002; Maren, 2005; Shumyatsky et al., 2005; Sigurdsson et al., 2007; Howland and Wang, 2008; Sah et al., 2008; Pape and Pare, 2010). Behaviorally, subjects with PTSD show increased sensitization to stress, overgeneralization of fear associations and failure to extinguish learned fear (Figure 1.2) (Blechert et al., 2007; Wessa and Flor, 2007; Griffin, 2008; Milad et al., 2008; Jovanovic et al., 2009; Pole et al., 2009; Shin, 2009; Ehlers et al., 2010; McLaughlin et al., 2010; Suendermann et al., 2010). Animal models that mimic these behavioral abnormalities, such as animals trained in the fear conditioning or extinction learning paradigms, require synaptic plasticity (Quirk et al., 1995; McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Blair et al., 2001; Tsvetkov et al., 2002; Maren, 2005; Shumyatsky et al., 2005; Sigurdsson et al., 2007; Sah et al., 2008; Pape and Pare, 2010). Therefore, impairment of fear or extinction processes in PTSD may be indicative of impaired synaptic plasticity. Much is known about the molecular mechanisms of synaptic plasticity, and understanding how PTSD

might be a disorder of synaptic plasticity within emotional circuits will provide new avenues for translational research.

There are two practical clinical benefits to understanding the biological mechanisms of PTSD: prevention and treatment. A better understanding the genetics and underlying molecular mechanisms of PTSD will hopefully lead to better predictions about which individuals might be more susceptible to developing PTSD after trauma through genetic, biomarker, and psychological screening. In addition, knowledge of the molecular underpinnings of PTSD will point towards novel molecular targets for drug development. By generating drugs that activate these molecular mediators of plasticity, one may be able to enhance extinction of inappropriate fear associations, or even prevent development of fear associations in at-risk individuals. This area of research shows great promise for potential new approaches to treat PTSD symptoms.

1.4 Neurotrophic mechanisms of synaptic plasticity in fear conditioning

The brain derived neurotropic factor (BDNF)-TrkB pathway provides one example of a ligand-receptor system which underlies synaptic plasticity and which has also been implicated in both PTSD in humans and in animal models of fear conditioning, extinction and inhibitory learning. Peripheral plasma and serum studies (Dell'osso et al., 2009; Berger et al., 2010; Hauck et al., 2010) as well as genetic studies have directly linked BDNF to PTSD (Zhang et al., 2006). In addition, transgenic, molecular and behavioral studies in rodents have provided insights into the underlying mechanisms of BDNF signaling in PTSD.

There is burgeoning evidence for an association between a single nucleotide polymorphism (SNP) in the BDNF gene (Val66Met) and various psychiatric disorders, including depression and schizophrenia (Zhang et al., 2006; Gonul et al., 2011). This mutation is thought to alter BDNF stability and activity-dependent secretion, hence leading to dysfunctional BDNF signaling (Egan et al., 2003). While there is limited evidence for a role of the Val66Met polymorphism in PTSD, the Val66Met polymorphism may also result in altered memory function (Egan et al., 2003; Hajcak et al., 2009; Dennis et al., 2010; Lonsdorf et al., 2010; van Wingen et al., 2010; Gonul et al., 2011). BDNF (met/met) carriers showed increased medial temporal lobe activation during episodic and encoding retrieval tasks (Dennis et al., 2010). Another study described greater recruitment of amygdala and PFC activity in Met/Met carriers during memory formation and retrieval of biologically relevant stimuli (van Wingen et al., 2010). Finally, BDNF(met/met) carriers exhibited impaired extinction learning, which was correlated with altered activation of the amygdala, PFC and the hippocampus (Hajcak et al., 2009; Lonsdorf et al., 2010; Soliman et al., 2010). Together these data suggest that this polymorphism may play a role in activation of the limbic system during memory formation and emotionally relevant learning.

Humanized BDNF (Val66Met) knock-in mice with the Met/Met phenotype show increased anxiety-related behaviors compared to Val carrier mice when placed in stressful settings (Chen et al., 2006; Li et al., 2010). BDNF (met/met) mice and humans carrying the Met allele show impaired extinction learning after fear conditioning (Yu et al., 2009; Soliman et al., 2010). Together these studies suggest that the transgenic mice share a similar phenotype to individuals at risk for PTSD, in that they appear to be more

sensitive to stress/anxiety and have impaired extinction of conditioned fear. In addition, BDNF (met/met) mice showed impaired NMDA receptor-dependent synaptic plasticity in the hippocampus (Ninan et al., 2010). It has not been reported whether these mice show impaired plasticity in the amygdala and PFC, though the extant data support the idea that PTSD is a disorder of aberrant plasticity mechanisms, and that these mechanisms are regulated by BDNF signaling.

BDNF-TrkB signaling has been shown to be necessary for various aspects of fear conditioning and extinction in all three of the regions implicated in PTSD: the amygdala, the hippocampus, and the PFC (Liu et al., 2004; Rattiner et al., 2004b; Rattiner et al., 2004a; Chhatwal et al., 2006; Heldt et al., 2007; Yee et al., 2007; Musumeci et al., 2009; Choi et al., 2010; Jang et al., 2010; Ou et al., 2010; Peters et al., 2010; Andero et al., 2011; Takei et al., 2011). In the amygdala, BDNF transcription is increased during the consolidation period 2 hours after fear conditioning (Takei et al., 2011)[60-62]. Inhibiting BDNF signaling in the amygdala impairs both the acquisition and consolidation of fear conditioning (Rattiner et al., 2004b) and the consolidation of extinction (Chhatwal et al., 2006). In addition, an increase in BDNF was observed after the normal window of consolidation at around 12 hours after fear conditioning and this peak in BDNF expression was shown to be crucial for persistence of the fear memory (Ou et al., 2010). Thus, BDNF signaling in the amygdala appears to play a significant role in synaptic plasticity events underlying the consolidation and the persistence of fear memories.

Mice heterozygous for the BDNF deletion (BDNF+/-) showed impaired contextual fear conditioning, which could be partially rescued with expression of BDNF in the hippocampus (Liu et al., 2004). Mice in which BDNF was selectively deleted from

the hippocampus did not show impaired acquisition of fear conditioning; however there was a marked decrease in extinction of conditioned fear (Heldt et al., 2007). This result suggests that normal hippocampal plasticity is required for normal context-dependent extinction of conditioned fear. Taken together with the findings of smaller hippocampal volumes in subjects with PTSD (Liu et al., 2004; Heldt et al., 2007), these convergent data suggest that impaired hippocampal function in PTSD may be causally related to these subjects' impairment in extinction of fear memories.

BDNF has also been implicated in differential roles in distinct subregions of the PFC in the retention and in the extinction of learned fear. Genetic deletion of BDNF selectively in the prelimbic area (PL) of the PFC causes impairment in consolidation of learned fear, but not extinction (Choi et al., 2010). In contrast, infusing BDNF into the infralimbic area (IL) of the PFC resulted in reduced fear expression for up to 48 hours after fear conditioning even in the absence of extinction training, but did not erase the original fear memory (Peters et al., 2010). Rats with impaired extinction showed less BDNF expression in the IL PFC compared to control rats, and infusing BDNF into the IL prevented extinction failure (Choi et al., 2010). These data suggest that BDNF may be a crucial mediator of neural plasticity in both regions. Due to the differential connectivity and functioning of IL and PL, BDNF in these areas also results in opposite effects. BDNF in the PL is necessary for fear memory formation and expression, whereas BDNF in the IL is apparently necessary for the inhibition, or extinction, of that fear. Thus, BDNF signaling in the PFC plays a critical role in the regulation of fear and emotion, and may serve as a target for enhancing extinction in subjects with PTSD.

The tyrosine kinase B (TrkB) receptor is composed of an extracellular domain that binds BDNF and an intracellular domain that activates signaling pathways through phosphorylation of two tyrosine residues, Y515 or Y816, which activate divergent signaling pathways (Figure 1.3). Phosphorylation of the Y515 residue allows recruitment of Src homology *2* domain containing)/fibroblast growth factor receptor substrate *2* (Shc/FRS-2) activating the RAS/mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase PI3K pathways. In contrast, phosphorylation of the Y816 residue allows recruitment of phospholipase C (PLC) which activates the Ca^{2+}/calmodulin-dependent protein kinase (CAMK)/ *cAMP* responsive element binding protein (CREB) signaling pathway (Ou and Gean, 2006). Genetic mouse models carrying single point mutations at each of these two sites (Y515F or Y816F) have been developed (Musumeci et al., 2009). TrkB(Y515F) knock-in heterozygous mice exhibited deficits in consolidation but not acquisition of fear conditioning, while TrkB(Y816F) mice, on the other hand, exhibited deficits in acquisition (Musumeci et al., 2009). How acquisition and consolidation lead to differential activation of the TrkB receptor at the Y515 site versus the Y816 site is currently unclear. Furthermore, it will be of interest to study the differentiation role of these phosphorylation sites in the extinction of learned fear.

Despite significant evident suggesting a role for the BDNF-TrkB system in fear-related and other affective disorders, a lack of high affinity ligands for the TrkB receptor has limited progress towards BDNF-related treatments for psychiatric and neurological disorders. However, 7,8-dihydroxyflavone (7,8-DHF) has recently been identified as a relatively specific TrkB agonist, which crosses the blood-brain barrier after oral or i.p. systemic administration in mice (Jang et al., 2010). It was subsequently demonstrated

that amygdala TrkB receptors are activated by systemic 7,8-DHF (5mg/kg, i.p.) (Andero et al., 2011). Additionally, systemic 7,8-DHF rescued the fear consolidation deficit observed in prelimbic BDNF knockout mice (Choi et al., 2010), and enhanced both the acquisition of fear and its extinction in wild-type mice (Andero et al., 2011). Furthermore, this agonist appears to rescue an extinction deficit in mice with a history of immobilization stress, which may serve as a face-valid animal model of PTSD. These data suggest that 7,8-DHF and other potential TrkB activating ligands may not only be valuable as pharmacological tools for achieving a better understanding of the role of BDNF-TrkB signaling pathways in learning and memory, but also as potential therapeutics for reversing learning and extinction deficits associated with psychopathology.

An additional molecule that has been implicated in synaptic plasticity and BDNF regulation is pituitary adenylate cyclase-activating polypeptide (PACAP). PACAP is known to broadly regulate the cellular stress response, however, it was only recently demonstrated to also have a role in human psychological stress responses, such as PTSD. Specifically, a sex-specific (female) association of PACAP blood levels with fear physiology, PTSD diagnosis and symptoms was observed in a population of heavily traumatized subjects (Ressler et al., 2011). Additionally, a single SNP in a putative estrogen response element within the PACAP receptor (PAC1) was associated with PTSD symptoms in females only. This SNP also associated with enhanced levels of fear discrimination and with levels of PAC1 mRNA expression in human cortex. Methylation of the PAC1 gene in peripheral blood was also found to be significantly associated with PTSD (Ressler et al., 2011). Complementing these human findings, PAC1 mRNA

expression was induced with either fear conditioning or estrogen replacement in rodent models (Ressler et al., 2011). These data suggest that perturbations in the PACAP-PAC1 pathway are involved in abnormal stress responses underlying PTSD, and that some of the sex-specific differences in PTSD risk/resilience (Tolin and Foa, 2006) may be in part due to estrogen modulation of this pathway.

1.5 GABAergic Inhibitory Regulation of Neuronal Circuits in Fear Conditioning

GABAergic inhibitory control is crucial for the precise regulation of consolidation, expression and extinction of fear conditioning (Zhang and Cranney, 2008; Bolshakov, 2009; Makkar et al., 2010). Fear conditioning results in a reduction in GABAergic signaling in the basolateral nucleus of the amygdala (BLA) relative to non-fear conditioned controls (Rea et al., 2009) and genetic deletion of the α1 subunit of the $GABA_A$ receptor enhances auditory fear learning (Wiltgen et al., 2009). Many of the early papers used GABA agonists as a method of inactivating specific brain regions to determine their role in behavior. GABAergic inactivation of the amygdala, hippocampus, PFC and regions of the striatum resulted in impairments in various aspects of conditioned fear (Corbit and Janak, 2010; Raybuck and Lattal, 2011; Sierra-Mercado et al., 2011). In addition, GABAergic inactivation of the infralimbic cortex, BLA or ventral hippocampus also impaired fear extinction (Hart et al., 2010; Laurent and Westbrook, 2010; Sierra-Mercado et al., 2011). However, GABAergic signaling is more than a methodological tool for inactivating regions of the brain but appears to maintain tight regulatory control over microcircuits in a region- and cell-type specific manner.

Two recent papers have outlined how GABAergic inhibitory microcircuits may regulate acquisition and expression of fear memories in the central nucleus of the amgydala (CEA). It was originally thought that associative learning primarily occurs in the BLA, whereas the CEA mainly controlled the expression of fear (Ciocchi et al., 2010). Such regulation of fear expression occurs via projections from central amygdala output neurons, which are mainly located in the medial subdivision (CEm), to the brainstem and hypothalamus (Ciocchi et al., 2010). However, a role for the CeA in fear acquisition has now been demonstrated (Ciocchi et al., 2010). Activation of the CEm in mice by pharmacological and physiological techniques was found to result in strong and reversible freezing responses (Ciocchi et al., 2010). Inactivating the lateral division of the CEA (CEl), but not the CEm, was found to induce unconditioned freezing as well as to impair fear conditioning. From these results it was concluded that neuronal activity in the CEm is necessary and sufficient for driving the freezing response, but that the CEl is required for the acquisition of fear and produces tonic inhibitory control of the CEm, which is reduced during presentation of the conditioned stimulus (CS+) (Ciocchi et al., 2010).

Moreover, the above study also identified two distinct subpopulations of inhibitory GABAergic neurons in the CEl (Ciocchi et al., 2010). These neuronal subpopulations were termed CEl "on" and "off" neurons based on their response to fear conditioning. CEl "on" neurons acquired an excitatory response to the CS+ during and after fear acquisition, whereas CEI "off" neurons showed decreased responses to the CS+ during and after fear acquisition. CS evoked excitation of CEl "on" neurons began before the CEl "off" neurons, and both "on" and "off" neurons sent inhibitory projections to the

CEm (Ciocchi et al., 2010). CS evoked inhibition of "off" neurons started immediately prior to excitation of CEM neurons, indicating that increases in CEm firing may be due to a reduction of inhibition from CEl "off" neurons. It is also likely based on the short onset latency of the CS-evoked excitation of CEl "on" neurons that they receive direct input from the sensory thalamus. The CEm also receives thalamic input (Ciocchi et al., 2010), which may be inhibited by feed forward inhibition through the CE "on" pathway. Based on this physiological data, it is hypothesized that fear conditioning leads to a shift in the balance of activity between distinct classes of CEl neurons, which ultimately regulate the activity of CEm firing (Ciocchi et al., 2010).

A second recent study has added to the understanding of CEA inhibitory microcircuits by molecularly defining two subtypes of inhibitory neurons in the CEl by the presence or absence of the δ isoform of protein kinase C (PKC- δ) (Haubensak et al., 2010). Using molecular and genetic approaches, this study was able to map the functional connectivity of PKC- δ+ and PKC- δ- neurons. Specifically, optogenetic targeting was employed to examine the effect of reversibly silencing PKC- δ+ neurons on the activity of CEl-"on", CEl "off" and CEm neurons. PKC- δ+ neurons were found to be predominantly late firing neurons, which reciprocally inhibit PKC- δ- neurons. Inactivation of PKC- δ+ neurons evoked action potentials in the CEm output neurons. In addition, tonic activity of CEl "off" units was strongly suppressed by the inactivation of PKC- δ+ neurons. Taken together, these findings suggest that the PKC- δ+ neurons are likely to be the CEl "off" neurons (Haubensak et al., 2010) (Figure 1.4).

Together, these recent papers provide new insight into the role of GABAergic inhibitory microcircuits in the acquisition and expression of fear conditioning. One

outstanding question from this research is:if both CEl “off” and CEl “on” units send inhibitory projections to the CEm, why is CEm activity increased rather than decreased after fear conditioning? This may be due simply to a balance between on and off neuron firing, i.e. the effect of decreased CEl “off” firing is greater than the effect of increased CEl “on” firing. Another reason could be that the CEl “on” neurons project to a different subpopulation of CEm neurons. Such recent findings add another level of control to the acquisition of fear. Not only is the BLA complex crucial for fear conditioning, but the CEl appears to be crucial as well. The CEl is downstream of the BLA, but may also work in parallel to form fear memories, as it also receives connections from auditory thalamic nuclei and cortical areas. Because the CEA is downstream of these structures, the CEA might be able to override stimulus discrimination established in upstream structures such as sensory and association cortex and thalamic regions.

Furthermore, feed forward inhibition from intercalated (ITC) neurons may implicate the CEl as the primary target for fear extinction. ITC cells are a very small subpopulation of neurons located just medial to the BLA complex, and appear to be necessary for extinction. Selectively lesioning ITC neurons results in a marked impairment in extinction learning (Likhtik et al., 2008). ITC neurons receive glutamateric input from the PFC (Berretta et al., 2005; Amano et al., 2010) and directly project to both the CEl and CEm (Haubensak et al., 2010). Activating the infralimbic region of the PFC resulted in activation of the immediate early gene, c-fos, in ITC neurons (Berretta et al., 2005), and extinction produced an excitation in ITC neurons, which resulted in inhibition of the CEA output neurons (Berretta et al., 2005). The BLA also synapses onto ITC neurons (Izumi et al., 2011), providing another level of regulation of fear learning and

extinction (Figure 1.4). Clearly, fear conditioning and extinction are under tight regulatory control by GABAergic signaling, and as will be discussed in the next section, glutamatergic signaling also plays a key regulatory role.

1.6 Glutamatergic Signaling in Fear conditioning

Glutamate is the main excitatory neurotransmitter in the brain. Thus, it is not surprising that glutamatergic signaling is essential for the consolidation and extinction of fear. Glutamatergic cells in the BLA are activated after fear conditioning in rodents (Lin et al., 2010). The BLA receives glutamatergic input from the sensory thalamic and cortical structures as well as the hippocampus and PFC (Pape and Pare, 2010). In addition, the BLA sends glutamatergic signals to the CEA, which regulates the inhibitory microcircuits reviewed in the previous section. Glutamate acts on a variety of ionotropic (NMDA, AMPA) and metabotropic receptors (mGluR 1-8), which have been widely demonstrated to play a role in fear conditioning. Ionotropic glutamate receptors are the key mediators of synaptic plasticity required for long term fear memories, whereas mGluRs modulate synaptic plasticity through G-protein coupled signal transduction.

Fear conditioning appears to result in an activation of NMDA receptors (Nedelescu et al., 2010) and downstream signaling mechanisms result in a subsequent insertion of additional AMPA receptors at synaptic sites (Rumpel et al., 2005; Mokin et al., 2007; Brigman et al., 2010; Liu et al., 2010; Nedelescu et al., 2010). This increase in surface AMPA receptors results in LTP and an increased responsiveness of the synapse to future CS+ presentations. Antagonizing NMDA receptors in either the hippocampus or BLA impairs consolidation of fear conditioning (Dalton et al., 2008; Liu et al., 2009; Zimmerman and Maren, 2010). Blocking AMPA receptor insertion in the synaptic

membrane in the lateral amygdala blocks fear memory formation (Rumpel et al., 2005; Mokin et al., 2007). Extinction of fear conditioning also appears to be regulated by NMDA and AMPA receptor signaling. Antagonizing NMDA receptors can impair extinction in rodents (Falls et al., 1992; Liu et al., 2009). In addition, there appears to be a reduction in surface AMPA receptors after extinction, relative to fear-conditioned animals that were not extinguished (Clem and Huganir, 2010).

Changes in NMDA/AMPA ratios appear to happen rapidly during consolidation of memory, but the question remains: How is glutamatergic signaling translated into a long-term memory and how is that memory biologically maintained? Protein kinase M zeta (PKMζ) is an atypical isoform of PKC that can stay chronically active despite molecular turnover. Over-expression of PKMζ enhances long-term memory (Hardt et al., 2010) and inhibiting PKMζ can disrupt memory, even after that memory has been formed (Serrano et al., 2008; Kwapis et al., 2009; Cohen et al., 2010; Hardt et al., 2010; Migues et al., 2010; Parsons and Davis, 2011). In addition, PKMζ inactivation-induced impairment of fear memory appears to correlate with a decrease in expression of the GluR2 subunit of the AMPA receptor (Migues et al., 2010). Furthermore, blocking GluR2-dependent removal of postsynaptic AMPA receptors abolished behavioral impairment of PKMζ inhibition (Migues et al., 2010), suggesting that PKMζ may be a mechanistic switch that maintains memory over time through the regulation of AMPA receptor trafficking. However, a pharmacological inhibitor of PKMζ only temporarily disrupts expression of fear conditioning when administered to rats immediately prior to testing and does not completely abolish the fear memory (Parsons and Davis, 2011).

Thus, at least based on these findings, it appears that PKMζ is an unlikely drug target for PTSD.

An alternative promising avenue for the modulation of glutamatergic signaling has been the development of D-cycloserine (DCS), a NMDA partial agonist. DCS has been shown to facilitate extinction learning in animals and humans (Ressler et al., 2004; Ledgerwood et al., 2005; Guastella et al., 2007a; Guastella et al., 2007b; Kushner et al., 2007; Langton and Richardson, 2008; Norberg et al., 2008; Wilhelm et al., 2008; Kalisch et al., 2009; Langton and Richardson, 2009; McCallum et al., 2010; Otto et al., 2010a; Otto et al., 2010b). More recently, DCS has been suggested to reverse the reduction in AMPA receptors that is normally observed at synaptic sites in the lateral amygdala after fear learning (Lin et al., 2010). Clinically, DCS has been shown to be a valuable augmentation to behavioral therapies for a variety of anxiety-related disorders, including obsessive-compulsive disorder (Ressler et al., 2004; Guastella et al., 2007b; Kushner et al., 2007; Guastella et al., 2008; Norberg et al., 2008; Wilhelm et al., 2008; Otto et al., 2010b), however definitive trials specifically for PTSD treatment using DCS have yet to be completed. DCS is an example of a drug that enhances the extinction of fear in animals and humans, as well as enhancing behavioral therapy in individuals with anxiety disorders involving fear dysregulation.

mGluRs modulate synaptic plasticity in the brain and are critical for the consolidation of fear conditioning and extinction. While there have been mixed reports about the effect of mGluR agonists on fear conditioning, in general, mGluR antagonists and genetic deletion of mGluRs in the limbic regions of the brain appear to impair both consolidation and extinction of fear conditioning (Kim et al., 2007; More et al., 2007;

Goddyn et al., 2008; Siegl et al., 2008; Fendt et al., 2010; Fontanez-Nuin et al., 2011). Activation of mGluR1-containing receptors in the BLA is known to enhance fear learning (Rudy and Matus-Amat, 2009).

Many other receptor-ligand systems play a modulatory role in Pavlovian fear conditioning and likely contribute to PTSD, mostly by modulating GABAergic and glutamatergic signaling (Table 1). Two retrograde signaling systems (involving nitric oxide and endocannibinoids as the retrograde messengers) have been shown to be important for presynaptically-regulated plasticity in consolidation and extinction, respectively (Ota et al., 2008; Chhatwal et al., 2009; Kelley et al., 2010; Lisboa et al., 2010; Ota et al., 2010; Paul et al., 2010). Noradrenergic signaling from the locus coeruleus (Fu et al., 2007; Mueller et al., 2008; Lazzaro et al., 2010; Mueller and Cahill, 2010), and dopaminergic projections to the amygdala from the ventral tegmental area (VTA) and nucleus accumbens (Mueller et al., 2010; Ortiz et al., 2010; Biojone et al., 2011; de Oliveira et al., 2011) also play important roles in modulating synaptic plasticity and fear conditioning. These transmitter systems may provide additional potential molecular targets for the pharmacological augmentation of behavioral therapy for PTSD.

1.7 Conclusions

The molecular pathways discussed in this chapter are crucial for fear conditioning and extinction. Recent research has advanced our understanding of many of the downstream molecular mechanisms of these forms of learning. By understanding the genetics of PTSD we may eventually be better able to predict which individuals might be more susceptible to developing PTSD after trauma. In addition, knowing the molecular underpinnings of PTSD will provide important new insights into molecular targets for

drug development. By generating drugs that modulate signaling pathways involved in fear conditioning and synaptic plasticity in the amgydala, we may be able to enhance extinction of inappropriate fear associations, or even prevent the development of fear associations in individuals more susceptible to PTSD. Research in this area shows great promise for potential new approaches to better understand the physiology of circuits mediating fear responses, as well as to potentially further the prevention and treatment of PTSD (Box 2). Given the rising numbers of traumatized civilians and veterans, in addition to our increasing understanding of the prevalence, comorbidity, and sequelae of PTSD, developing better preventions and treatments are vital.

1.8 Figures

Figure 1.1 A schematic of the human brain illustrating how the limbic system is involved in PTSD. The PFC and the hippocampus both have dense connections to the amygdala, which is important for conditioned fear and associative emotional learning. The PFC is thought to be responsible for reactivating past emotional associations and is decreased in both responsiveness and density (Lanius et al., 2006; Etkin and Wager, 2007; Francati et al., 2007; Dickie et al., 2008). The hippocampus is thought to play a role in explicit memories of the traumatic events and in mediating learned responses to contextual cues, and in PTSD the hippocampus is decreased in volume (Gilbertson et al., 2002) and responsiveness to traumatic stimuli (Gilbertson et al., 2002; Thomaes et al., 2010). The top down control of the amygdala by the hippocampus and PFC may result in the increased activation of the amygdala, as is observed in subjects with PTSD. The end result of these neuroanatomical alterations is increased stress sensitivity, generalized fear responses and impaired extinction. Other regions including the anterior cingulate cortex, the orbitofrontal cortex, the parahippocampal gyrus, the thalamus and the sensorimotor cortex also play a secondary role in the regulation of fear and PTSD (Shin and Liberzon, 2010).

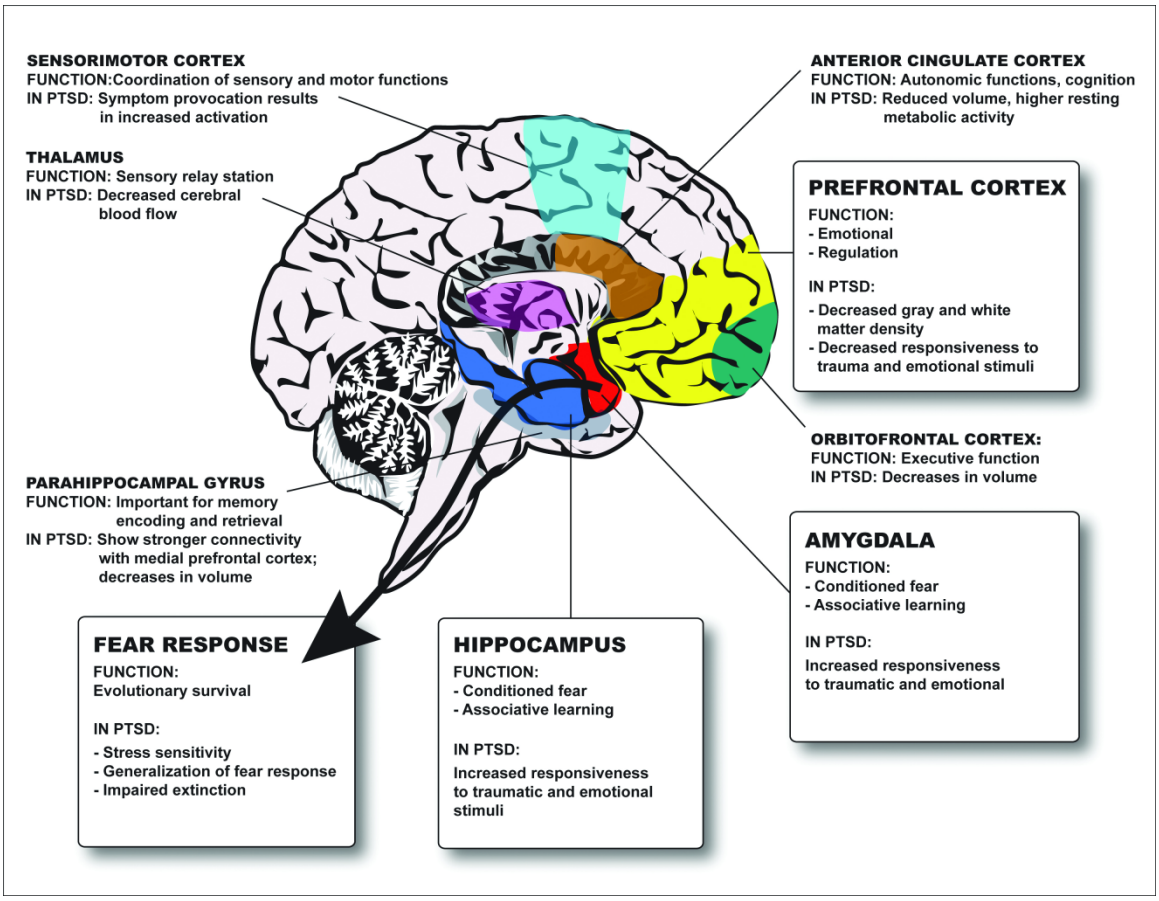

SENSORIMOTOR CORTEX
FUNCTION:Coordination of sensory and motor functions
IN PTSD: Symptom provocation results in increased activation
THALAMUS
FUNCTION: Sensory relay station
IN PTSD: Decreased cerebral blood flow
PARAHIPPOCAMPAL GYRUS
FUNCTION: Important for memory encoding and retrieval
IN PTSD: Show stronger connectivity with medial prefrontal cortex; decreases in volume
ANTERIOR CINGULATE CORTEX
FUNCTION: Autonomic functions, cognition
IN PTSD: Reduced volume, higher resting metabolic activity
PREFRONTAL CORTEX
FUNCTION:
- Emotional
- Regulation
IN PTSD:
- Decreased gray and white matter density
- Decreased responsiveness to trauma and emotional stimuli
ORBITOFRONTAL CORTEX:
FUNCTION: Executive function
IN PTSD: Decreases in volume
AMYGDALA
FUNCTION:
- Conditioned fear
- Associative learning
IN PTSD:
Increased responsiveness to traumatic and emotional
FEAR RESPONSE
FUNCTION:
Evolutionary survival
IN PTSD:
- Stress sensitivity
- Generalization of fear response
- Impaired extinction
HIPPOCAMPUS
FUNCTION:
- Conditioned fear
- Associative learning
IN PTSD:
Increased responsiveness to traumatic and emotional stimuli

Figure 1.2 Disordered fear regulation in PTSD. Individuals with PTSD typically show increased sensitization to stress, overgeneralization of fear to irrelevant stimuli, and impaired extinction of fear memories. Individuals who demonstrate resilience to PTSD, and/or who recover from traumatic/ stressful experiences, are able to discriminate between fearful and non-fearful stimuli, as well as displaying normal extinction of fear memories.

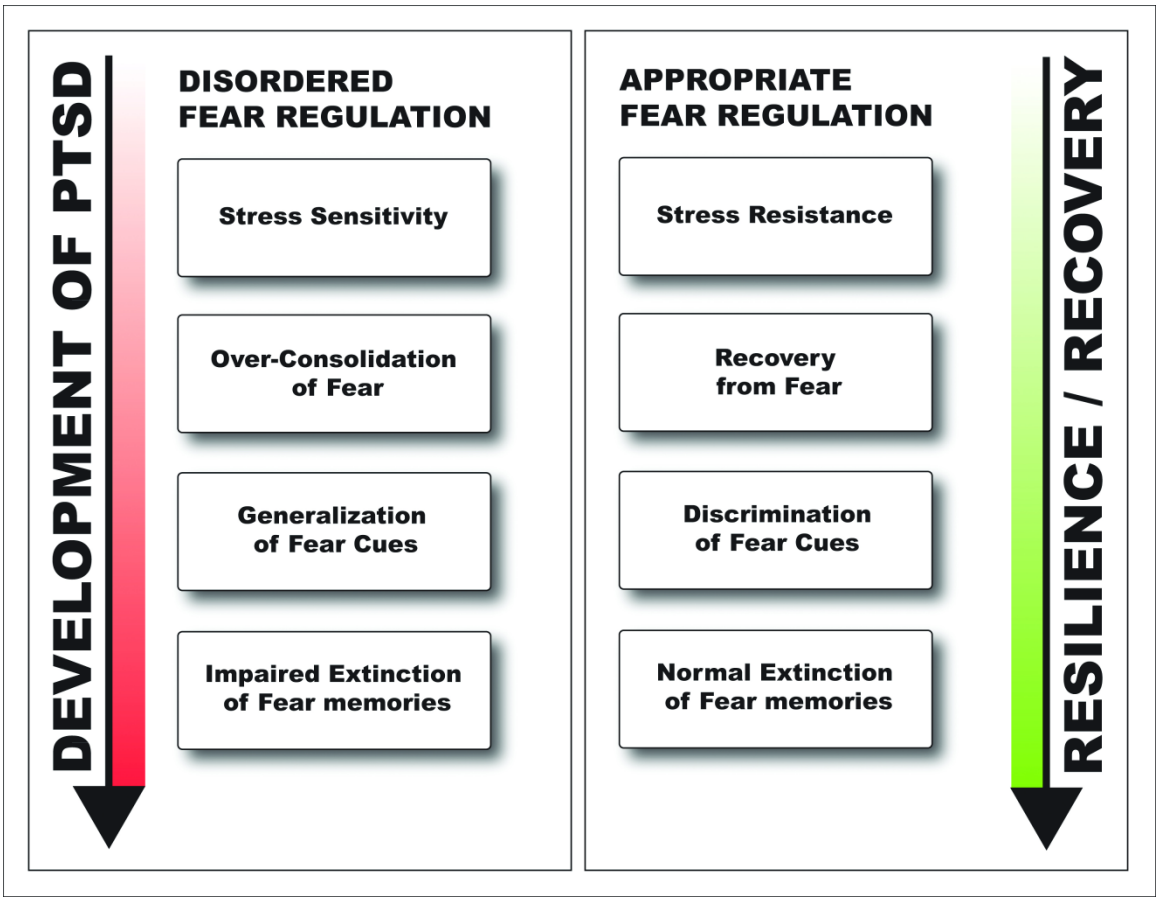
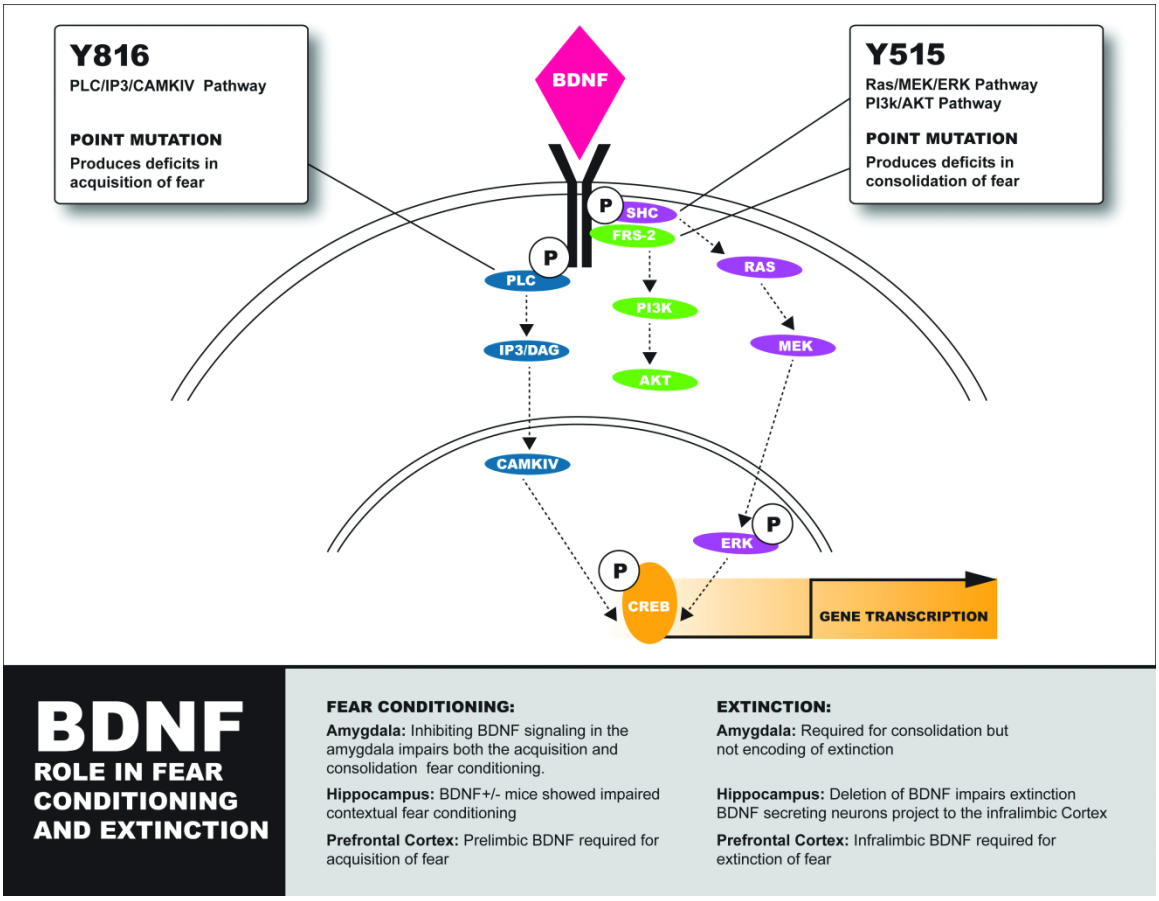

DEVELOPMENT OF PTSD
DISORDERED FEAR REGULATION
Stress Sensitivity
Over-Consolidation of Fear
Generalization of Fear Cues
Impaired Extinction of Fear memories
APPROPRIATE FEAR REGULATION
Stress Resistance
Recovery from Fear
Discrimination of Fear Cues
Normal Extinction of Fear memories
RESILIENCE / RECOVERY

Figure 1.3 BDNF – TrkB induced signaling pathway. BDNF binds to the TrkB receptor, resulting in the phosphorylation of two tyrosine sites (Y515 and Y816) on the intracellular domain of the TrkB receptor. Phosphorylation of the Y515 residue allows recruitment of Shc/FRS-2, which subsequently activates the Ras/MAPK and PI3K pathways. In contrast, phosphorylation of the Y816 residue allows recruitment of PLC, which activates the CAMK/CREB signaling pathway. Point mutations of the Y515 residue produce deficits in consolidation but not acquisition of fear conditioning (Musumeci et al., 2009). Point mutations of the Y816 residue, on the other hand, produce deficits in acquisition (Musumeci et al., 2009). Evidence exists for a role of BDNF signaling in the amygdala, hippocampus and PFC with respect to both the consolidation and extinction of fear conditioning.



BDNF
ROLE IN FEAR CONDITIONING AND EXTINCTION

FEAR CONDITIONING:

Amygdala: Inhibiting BDNF signaling in the amygdala impairs both the acquisition and consolidation fear conditioning.

Hippocampus: BDNF+/- mice showed impaired contextual fear conditioning

Prefrontal Cortex: Prelimbic BDNF required for acquisition of fear

EXTINCTION:

Amygdala: Required for consolidation but not encoding of extinction

Hippocampus: Deletion of BDNF impairs extinction BDNF secreting neurons project to the infralimbic Cortex

Prefrontal Cortex: Infralimbic BDNF required for extinction of fear

Figure 1.4 Schematic diagram illustrating the key amygdala nuclei involved in fear conditioning. Microcircuits within the amygdala demonstrate multiple levels of regulation with response to fear consolidation, extinction, and the expression of fear. Initially, it was thought that the BLA complex was solely responsible for fear acquisition and was the main recipient of thalamic and cortical inputs. The central amygdala was thought to be crucial only for the expression of conditioned fear responses via activation of downstream neural structures (see (Pape and Pare, 2010; Shin and Liberzon, 2010) for a review). Now significant evidence supports the idea that the lateral division of the central amgydala (CeL) is also critical for acquisition of fear and also receives cortical and thalamic inputs. In addition, intercalated neurons may regulate firing of central amgydala output neurons and the expression of extinction. The intercalated neurons receive projections from the infralimbic cortex (a region critical for extinction) and project GABAergic inhibitory neurons onto the medial division of the central amygdala (CeM).

Circuitry in the Amygdala

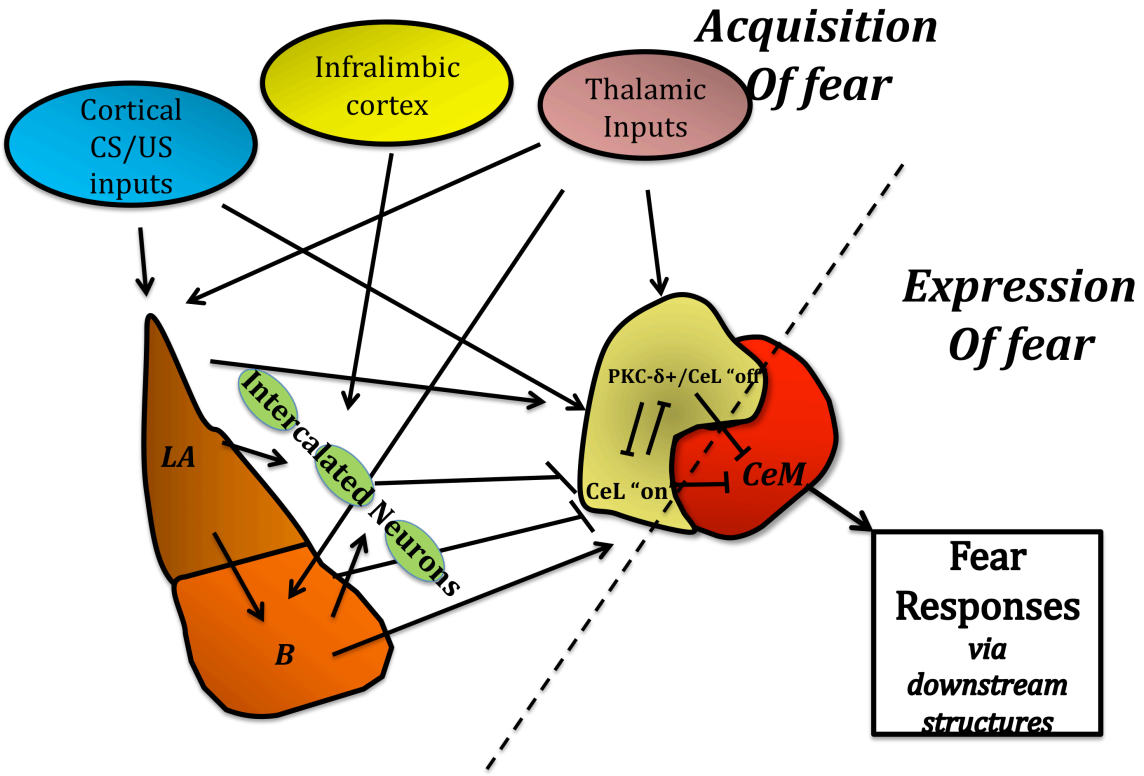


Table 1. Other ligand-receptor systems involved in the regulation of Pavlovian fear conditioning[1]

System	Function	Supporting Evidence
Norepinephrine (NE)	Consolidation	Enhanced with alpha1-adrenergic receptor antagonists (Lazzaro et al., 2010) Impaired by siRNA for beta(1) adrenergic receptors (Fu et al., 2007)
	Extinction	Impaired by antagonizing NE receptors in the infralimbic cortex (Mueller et al., 2008; Mueller and Cahill, 2010)
NOS-cGMP	Consolidation	Enhanced by PKG activation in the LA (Ota et al., 2010) Impaired contextual conditioning in nNOS KO mice (Kelley et al., 2010) Impaired in cGMP mutant mice (Paul et al., 2010) Impaired by NOS and PKG inhibition in the LA (Ota et al., 2008)
Endocannabinoid	Consolidation	CB1 mRNA increases 48 hrs after fear conditioning (Lisboa et al., 2010) Enhanced by inverse agonist of CB1 in the CeA or BLA (Lisboa et al., 2010) Impaired by CB1 receptor agonist or AEA transport inhibition into the vmPFC (Lisboa et al., 2010)
	Extinction	Impaired by pharmacological blockade or genetic deletion of CB1 receptors (Lin et al., 2008; Chhatwal et al., 2009)
Dopamine (DA)	Consolidation	Enhanced by D2 receptor agonists in the VTA(Biojone et al., 2011; de Oliveira et al., 2011) D2 receptor antagonists in the BLA impair fear potentiated startle (de Oliveira et al., 2011) Impaired by D1 receptor loss (genetic KO or siRNA in hippocampus)(Ortiz et al., 2010)
	Extinction	Impaired by systemic or intra-IL PFC infusion of D2 antagonist (Mueller et al., 2010)
Acetylcholine (Ach)	Consolidation	Enhanced by nicotinic Ach (nACh) agonists in the hippocampus (Davis and Gould, 2007; Kenney et al., 2010; Andre et al., 2011) Impaired by alpha7 nAch receptor antagonists(Chess et al., 2009).
	Extinction	Impaired by nAch agonists (Prado-Alcala et al., 1994)

[1] Abbreviations: CB1 (Cannabinoid Receptor type 1), PKG (cGMP-dependent protein kinase), NOS (Nitric Oxide Synthase), KO (Knock out), siRNA (Small Interfering RNA), IL (Infralimbic)

Glossary

Classical Conditioning: Classical conditioning is a learning paradigm that pairs a neutral/conditioned stimulus (CS) with an unconditioned stimulus (US) that evokes a reflex or unconditioned response (UR) until the neutral stimulus evokes the same conditioned response (CR) in the absence of the US

Contextual conditioning: a model of fear conditioning based solely on the context and not a discrete cue such as a light or a tone.

Extinction: The conditioning phenomenon in which a previously learned response to a cue is reduced when the cue is presented in the absence of a previously paired aversive or appetitive stimulus

Pavlovian Fear Conditioning: Pavlovian fear conditioning is a version of classical conditioning, where the CS (eg. tone, light, odor) is paired with an aversive US (eg. foot-shock, air-blast) that evokes a CR (eg. freezing, acoustic startle response, autonomic arousal).

Box 1. Genetic Association Studies in PTSD

How it works: These studies compare the DNA of two groups of participants: trauma victims with PTSD and trauma victims without PTSD. Each person gives a sample of cells from their cheek, saliva, or blood. DNA is extracted from these cells and gene chip analyses are performed. Rather than reading DNA sequence, these systems SNPs that are markers for regional DNA variation. If genetic variations are more frequent in the affected participants, then the variations are said to be associated with the disorder.

Some replicated genetic associations found in PTSD:

BDNF (Val66Met) SNP:

- Function: Neurotrophic Factor
- Result of Polymorphism:
  - Met allele has been shown to have altered trafficking and secretion in neurons compared to Val allele (Egan et al., 2003).
  - Met/met carriers showed increased medial temporal lobe activation (perhaps compensatory) during episodic and encoding retrieval tasks (Dennis et al., 2010).
  - Greater recruitment of amygdala and PFC activity in Met/Met carriers during memory formation and retrieval of biologically relevant stimuli (van Wingen et al., 2010).
  - Met/Met carriers exhibited impaired extinction learning ,which was correlated with altered activation of the amygdala, PFC and the hippocampus (Lonsdorf et al., 2010).

Serotonin transporter (SERT) - short vs. long Allele:

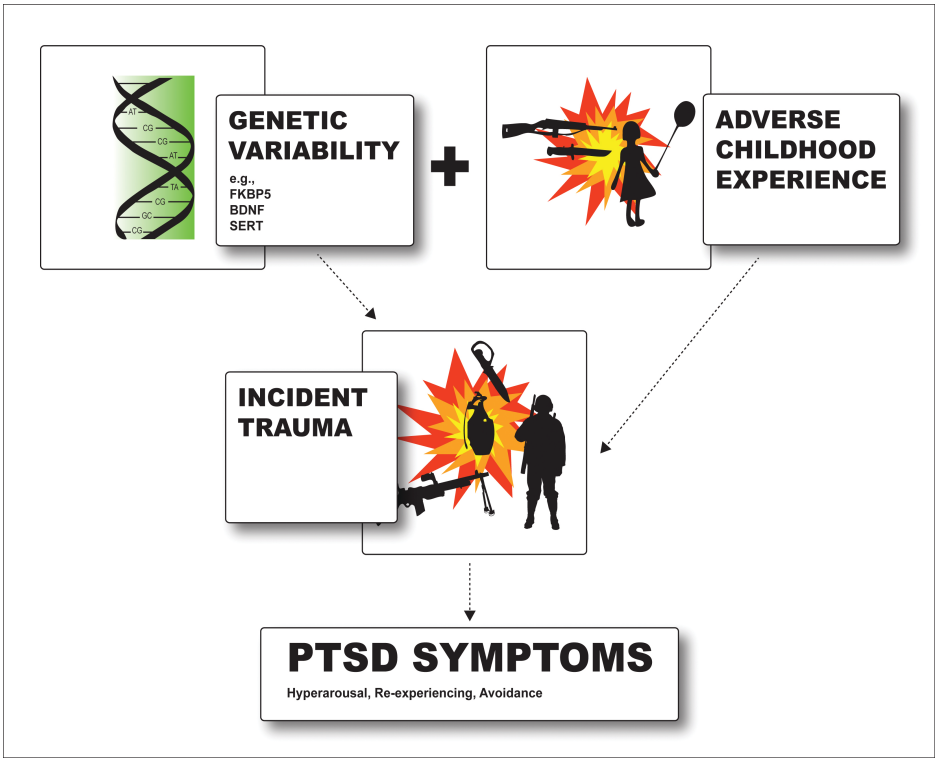
- Function: Serotonin transport/reuptake
- Result of Polymorphism
  - Different alleles have been associated with altered SERT gene expression/translation (Grabe et al., 2009; Xie et al., 2009; Bryant et al., 2010)
  - Findings have been reported in individuals for an increased risk of PTSD with both the long (Grabe et al., 2009; Xie et al., 2009) and short allele (Grabe et al., 2009; Bryant et al., 2010).
  - Recent data suggest that the short allele is associated with decreased risk of PTSD in low-risk environments (e.g., low crime/unemployment rates) but increased risk of PTSD in high-risk environments (Grabe et al., 2009). This suggests that environment modifies the effect of serotonin-transporter-linked polymorphic region (5-HTTLPR) genotype on PTSD risk

Box 1 Continued…

FK506-binding protein 5 (***FKBP5***):

- Function: Glucocorticoid Chaperone Protein
- Result of Polymorphism:
  - PTSD associated with differential FKBP5 mRNA and protein expression (Mehta et al., 2011)
  - No main effect of FKBP5 genotype on PTSD (Binder et al., 2008)
  - FKBP5 SNPs *interact* with child maltreatment history as a predictor of the severity of adult PTSD symptoms (Binder et al., 2008).
  - FKBP5 SNPs may contribute to increased sensitivity of the amygdala / HPA axis response to adult stress

Box 1 Figure 1. Genetic and environmental factors influence the risk for developing PTSD in certain individuals, as well as the severity of PTSD symptoms.



Box 2. Outstanding Questions:

Individual Differences: Why are some individuals at risk for developing PTSD, but despite similar trauma, others appear to be resistant? Furthermore, as with many common diseases, PTSD will likely represent a final common pathway of a 'broken brain' at the intersection of trauma and biology. How many different 'subtypes' of PTSD might there be? Will our current syndromal nomenclature be predictive of these subtypes, or will future biomarkers provide new ways of dissecting this syndrome?

Resilience: Is the resilience that we define as lack of PTSD, despite severe trauma, simply the absence of PTSD symptoms (along with comorbid depression and substance abuse) or is resilience an orthogonal construct that is uniquely protective?

Genetic Risk: Up to 30-35% of risk for PTSD appears to be heritable (True et al., 1993). Similar to a number of other disorders, will this be made up of many common gene variants, which each contribute only a small percentage of risk, or will there be a larger number of rare variants which each contribute higher levels of risk?

Gene x Environment interaction: With sufficient trauma loading, almost anyone is susceptible to PTSD. Genes appear to differentially modulate the level of susceptibility at a given trauma level or trauma 'dose'. How do the effects of childhood and adult trauma interact though neural circuitry with genes that contribute risk, and which may act in an additive fashion on this same circuitry?

Neural Circuitry of PTSD: The neural circuitry modulating fear, including the amygdala, PFC and hippocampal regions are conserved across mammals. This makes research on PTSD and other anxiety-related disorders more readily accessible to translation compared to many other mental disorders. Utilizing human dynamic and structural neuroimaging techniques combined with rodent and other laboratory model species, we can ask, how do these different regions, which organize and modulate the emotion of fear, work in concert?

References

(1994) American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, 4th ed Edition. Washington D.C.: American Psychiatric Association.

Abe O, Yamasue H, Kasai K, Yamada H, Aoki S, Iwanami A, Ohtani T, Masutani Y, Kato N, Ohtomo K (2006) Voxel-based diffusion tensor analysis reveals aberrant anterior cingulum integrity in posttraumatic stress disorder due to terrorism. Psychiatry Res 146:231-242.

Amano T, Unal CT, Pare D (2010) Synaptic correlates of fear extinction in the amygdala. Nat Neurosci 13:489-494.

Andero R, Heldt SA, Ye K, Liu X, Armario A, Ressler KJ (2011) Effect of 7,8-dihydroxyflavone, a small-molecule TrkB agonist, on emotional learning. Am J Psychiatry 168:163-172.

Andre JM, Leach PT, Gould TJ (2011) Nicotine ameliorates NMDA receptor antagonist-induced deficits in contextual fear conditioning through high-affinity nicotinic acetylcholine receptors in the hippocampus. Neuropharmacology 60:617-625.

Belzung C, Philippot P (2007) Anxiety from a phylogenetic perspective: is there a qualitative difference between human and animal anxiety? Neural Plast 2007:59676.

Berger W, Mehra A, Lenoci M, Metzler TJ, Otte C, Tarasovsky G, Mellon SH, Wolkowitz OM, Marmar CR, Neylan TC (2010) Serum brain-derived neurotrophic factor predicts responses to escitalopram in chronic posttraumatic stress disorder. Prog Neuropsychopharmacol Biol Psychiatry 34:1279-1284.

Berretta S, Pantazopoulos H, Caldera M, Pantazopoulos P, Pare D (2005) Infralimbic cortex activation increases c-Fos expression in intercalated neurons of the amygdala. Neuroscience 132:943-953.

Binder EB, Bradley RG, Liu W, Epstein MP, Deveau TC, Mercer KB, Tang Y, Gillespie CF, Heim CM, Nemeroff CB, Schwartz AC, Cubells JF, Ressler KJ (2008) Association of

FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. JAMA 299:1291-1305.

Biojone C, Casarotto PC, Resstel LB, Zangrossi H, Jr., Guimaraes FS, Moreira FA (2011) Anti-aversive effects of the atypical antipsychotic, aripiprazole, in animal models of anxiety. J Psychopharmacol 25:801-807.

Blair HT, Schafe GE, Bauer EP, Rodrigues SM, LeDoux JE (2001) Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning. Learn Mem 8:229-242.

Blechert J, Michael T, Vriends N, Margraf J, Wilhelm FH (2007) Fear conditioning in posttraumatic stress disorder: evidence for delayed extinction of autonomic, experiential, and behavioural responses. Behav Res Ther 45:2019-2033.

Bolshakov VY (2009) Nipping fear in the bud: inhibitory control in the amygdala. Neuron 61:817-819.

Bradley RG, Binder EB, Epstein MP, Tang Y, Nair HP, Liu W, Gillespie CF, Berg T, Evces M, Newport DJ, Stowe ZN, Heim CM, Nemeroff CB, Schwartz A, Cubells JF, Ressler KJ (2008) Influence of child abuse on adult depression: moderation by the corticotropin-releasing hormone receptor gene. Arch Gen Psychiatry 65:190-200.

Brigman JL, Wright T, Talani G, Prasad-Mulcare S, Jinde S, Seabold GK, Mathur P, Davis MI, Bock R, Gustin RM, Colbran RJ, Alvarez VA, Nakazawa K, Delpire E, Lovinger DM, Holmes A (2010) Loss of GluN2B-containing NMDA receptors in CA1 hippocampus and cortex impairs long-term depression, reduces dendritic spine density, and disrupts learning. J Neurosci 30:4590-4600.

Bryant RA, Felmingham KL, Falconer EM, Pe Benito L, Dobson-Stone C, Pierce KD, Schofield PR (2010) Preliminary evidence of the short allele of the serotonin transporter gene predicting poor response to cognitive behavior therapy in posttraumatic stress disorder. Biol Psychiatry 67:1217-1219.

Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, Lee FS (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science 314:140-143.

Chess AC, Landers AM, Bucci DJ (2009) L-kynurenine treatment alters contextual fear conditioning and context discrimination but not cue-specific fear conditioning. Behav Brain Res 201:325-331.

Chhatwal JP, Stanek-Rattiner L, Davis M, Ressler KJ (2006) Amygdala BDNF signaling is required for consolidation but not encoding of extinction. Nat Neurosci 9:870-872.

Chhatwal JP, Gutman AR, Maguschak KA, Bowser ME, Yang Y, Davis M, Ressler KJ (2009) Functional interactions between endocannabinoid and CCK neurotransmitter systems may be critical for extinction learning. Neuropsychopharmacology 34:509-521.

Choi DC, Maguschak KA, Ye K, Jang SW, Myers KM, Ressler KJ (2010) Prelimbic cortical BDNF is required for memory of learned fear but not extinction or innate fear. Proc Natl Acad Sci U S A 107:2675-2680.

Ciocchi S, Herry C, Grenier F, Wolff SB, Letzkus JJ, Vlachos I, Ehrlich I, Sprengel R, Deisseroth K, Stadler MB, Muller C, Luthi A (2010) Encoding of conditioned fear in central amygdala inhibitory circuits. Nature 468:277-282.

Clem RL, Huganir RL (2010) Calcium-permeable AMPA receptor dynamics mediate fear memory erasure. Science 330:1108-1112.

Cohen H, Kozlovsky N, Matar MA, Kaplan Z, Zohar J (2010) Mapping the brain pathways of traumatic memory: inactivation of protein kinase M zeta in different brain regions disrupts traumatic memory processes and attenuates traumatic stress responses in rats. Eur Neuropsychopharmacol 20:253-271.

Corbit LH, Janak PH (2010) Posterior dorsomedial striatum is critical for both selective instrumental and Pavlovian reward learning. Eur J Neurosci 31:1312-1321.

Dalton GL, Wang YT, Floresco SB, Phillips AG (2008) Disruption of AMPA receptor endocytosis impairs the extinction, but not acquisition of learned fear. Neuropsychopharmacology 33:2416-2426.

Davidson JR, Stein DJ, Shalev AY, Yehuda R (2004) Posttraumatic stress disorder: acquisition, recognition, course, and treatment. J Neuropsychiatry Clin Neurosci 16:135-147.

Davis JA, Gould TJ (2007) beta2 subunit-containing nicotinic receptors mediate the enhancing effect of nicotine on trace cued fear conditioning in C57BL/6 mice. Psychopharmacology (Berl) 190:343-352.

de Carvalho MR, Rozenthal M, Nardi AE (2010) The fear circuitry in panic disorder and its modulation by cognitive-behaviour therapy interventions. World J Biol Psychiatry 11:188-198.

de Oliveira AR, Reimer AE, de Macedo CE, de Carvalho MC, Silva MA, Brandao ML (2011) Conditioned fear is modulated by D2 receptor pathway connecting the ventral tegmental area and basolateral amygdala. Neurobiol Learn Mem 95:37-45.

Dell'osso L, Carmassi C, Del Debbio A, Dell'osso MC, Bianchi C, da Pozzo E, Origlia N, Domenici L, Massimetti G, Marazziti D, Piccinni A (2009) Brain-derived neurotrophic factor plasma levels in patients suffering from post-traumatic stress disorder. Prog Neuropsychopharmacol Biol Psychiatry 33:899-902.

Dennis NA, Cabeza R, Need AC, Waters-Metenier S, Goldstein DB, Labar KS (2010) Brain-derived neurotrophic factor val66met polymorphism and hippocampal activation during episodic encoding and retrieval tasks. Hippocampus.

Dickie EW, Brunet A, Akerib V, Armony JL (2008) An fMRI investigation of memory encoding in PTSD: influence of symptom severity. Neuropsychologia 46:1522-1531.

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger DR (2003) The BDNF val66met polymorphism

affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 112:257-269.

Ehlers A, Suendermann O, Boellinghaus I, Vossbeck-Elsebusch A, Gamer M, Briddon E, Martin MW, Glucksman E (2010) Heart rate responses to standardized trauma-related pictures in acute posttraumatic stress disorder. Int J Psychophysiol 78:27-34.

Etkin A, Wager TD (2007) Functional neuroimaging of anxiety: a meta-analysis of emotional processing in PTSD, social anxiety disorder, and specific phobia. Am J Psychiatry 164:1476-1488.

Falls WA, Miserendino MJ, Davis M (1992) Extinction of fear-potentiated startle: blockade by infusion of an NMDA antagonist into the amygdala. J Neurosci 12:854-863.

Fendt M, Burki H, Imobersteg S, van der Putten H, McAllister K, Leslie JC, Shaw D, Holscher C (2010) The effect of mGlu8 deficiency in animal models of psychiatric diseases. Genes Brain Behav 9:33-44.

Fontanez-Nuin DE, Santini E, Quirk GJ, Porter JT (2011) Memory for fear extinction requires mGluR5-mediated activation of infralimbic neurons. Cereb Cortex 21:727-735.

Francati V, Vermetten E, Bremner JD (2007) Functional neuroimaging studies in posttraumatic stress disorder: review of current methods and findings. Depress Anxiety 24:202-218.

Fu AL, Yan XB, Sui L (2007) Down-regulation of beta1-adrenoceptors gene expression by short interfering RNA impairs the memory retrieval in the basolateral amygdala of rats. Neurosci Lett 428:77-81.

Gilbertson MW, Shenton ME, Ciszewski A, Kasai K, Lasko NB, Orr SP, Pitman RK (2002) Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma. Nat Neurosci 5:1242-1247.

Gillespie CF, Bradley B, Mercer K, Smith AK, Conneely K, Gapen M, Weiss T, Schwartz AC, Cubells JF, Ressler KJ (2009) Trauma exposure and stress-related disorders in inner city primary care patients. Gen Hosp Psychiatry 31:505-514.

Goddyn H, Callaerts-Vegh Z, Stroobants S, Dirikx T, Vansteenwegen D, Hermans D, van der Putten H, D'Hooge R (2008) Deficits in acquisition and extinction of conditioned responses in mGluR7 knockout mice. Neurobiol Learn Mem 90:103-111.

Gonul AS, Kitis O, Eker MC, Eker OD, Ozan E, Coburn K (2011) Association of the brain-derived neurotrophic factor Val66Met polymorphism with hippocampus volumes in drug-free depressed patients. World J Biol Psychiatry 12:110-118.

Grabe HJ, Spitzer C, Schwahn C, Marcinek A, Frahnow A, Barnow S, Lucht M, Freyberger HJ, John U, Wallaschofski H, Volzke H, Rosskopf D (2009) Serotonin transporter gene (SLC6A4) promoter polymorphisms and the susceptibility to posttraumatic stress disorder in the general population. Am J Psychiatry 166:926-933.

Green KT, Calhoun PS, Dennis MF, Beckham JC (2010) Exploration of the resilience construct in posttraumatic stress disorder severity and functional correlates in military combat veterans who have served since September 11, 2001. J Clin Psychiatry 71:823-830.

Griffin MG (2008) A prospective assessment of auditory startle alterations in rape and physical assault survivors. J Trauma Stress 21:91-99.

Guastella AJ, Dadds MR, Lovibond PF, Mitchell P, Richardson R (2007a) A randomized controlled trial of the effect of D-cycloserine on exposure therapy for spider fear. J Psychiatr Res 41:466-471.

Guastella AJ, Lovibond PF, Dadds MR, Mitchell P, Richardson R (2007b) A randomized controlled trial of the effect of D-cycloserine on extinction and fear conditioning in humans. Behav Res Ther 45:663-672.

Guastella AJ, Richardson R, Lovibond PF, Rapee RM, Gaston JE, Mitchell P, Dadds MR (2008) A randomized controlled trial of D-cycloserine enhancement of exposure therapy for social anxiety disorder. Biol Psychiatry 63:544-549.

Hajcak G, Castille C, Olvet DM, Dunning JP, Roohi J, Hatchwell E (2009) Genetic variation in brain-derived neurotrophic factor and human fear conditioning. Genes Brain Behav 8:80-85.

Hardt O, Migues PV, Hastings M, Wong J, Nader K (2010) PKMzeta maintains 1-day- and 6-day-old long-term object location but not object identity memory in dorsal hippocampus. Hippocampus 20:691-695.

Hart G, Harris JA, Westbrook RF (2010) Systemic or intra-amygdala infusion of the benzodiazepine, midazolam, impairs learning, but facilitates re-learning to inhibit fear responses in extinction. Learn Mem 17:210-220.

Haubensak W, Kunwar PS, Cai H, Ciocchi S, Wall NR, Ponnusamy R, Biag J, Dong HW, Deisseroth K, Callaway EM, Fanselow MS, Luthi A, Anderson DJ (2010) Genetic dissection of an amygdala microcircuit that gates conditioned fear. Nature 468:270-276.

Hauck S, Kapczinski F, Roesler R, de Moura Silveira E, Jr., Magalhaes PV, Kruel LR, Schestatsky SS, Ceitlin LH (2010) Serum brain-derived neurotrophic factor in patients with trauma psychopathology. Prog Neuropsychopharmacol Biol Psychiatry 34:459-462.

Heimer L, Van Hoesen GW (2006) The limbic lobe and its output channels: implications for emotional functions and adaptive behavior. Neurosci Biobehav Rev 30:126-147.

Heldt SA, Stanek L, Chhatwal JP, Ressler KJ (2007) Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. Mol Psychiatry 12:656-670.

Hoge CW, Terhakopian A, Castro CA, Messer SC, Engel CC (2007) Association of posttraumatic stress disorder with somatic symptoms, health care visits, and absenteeism among Iraq war veterans. Am J Psychiatry 164:150-153.

Howland JG, Wang YT (2008) Synaptic plasticity in learning and memory: stress effects in the hippocampus. Prog Brain Res 169:145-158.

Izumi T, Boku S, Shinmin W, Inoue T, Konno K, Yamaguchi T, Yoshida T, Matsumoto M, Watanabe M, Koyama T, Yoshioka M (2011) Retrieval of conditioned fear activates the basolateral and intercalated nucleus of amygdala. J Neurosci Res 89:773-790.

Jang SW, Liu X, Yepes M, Shepherd KR, Miller GW, Liu Y, Wilson WD, Xiao G, Blanchi B, Sun YE, Ye K (2010) A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. Proc Natl Acad Sci U S A 107:2687-2692.

Jovanovic T, Ressler KJ (2010) How the neurocircuitry and genetics of fear inhibition may inform our understanding of PTSD. Am J Psychiatry 167:648-662.

Jovanovic T, Norrholm SD, Fennell JE, Keyes M, Fiallos AM, Myers KM, Davis M, Duncan EJ (2009) Posttraumatic stress disorder may be associated with impaired fear inhibition: relation to symptom severity. Psychiatry Res 167:151-160.

Kalisch R, Holt B, Petrovic P, De Martino B, Kloppel S, Buchel C, Dolan RJ (2009) The NMDA agonist D-cycloserine facilitates fear memory consolidation in humans. Cereb Cortex 19:187-196.

Kelley JB, Anderson KL, Itzhak Y (2010) Pharmacological modulators of nitric oxide signaling and contextual fear conditioning in mice. Psychopharmacology (Berl) 210:65-74.

Kenney JW, Wilkinson DS, Gould TJ (2010) The enhancement of contextual fear conditioning by ABT-418. Behav Pharmacol 21:246-249.

Kim J, Lee S, Park H, Song B, Hong I, Geum D, Shin K, Choi S (2007) Blockade of amygdala metabotropic glutamate receptor subtype 1 impairs fear extinction. Biochem Biophys Res Commun 355:188-193.

Kushner MG, Kim SW, Donahue C, Thuras P, Adson D, Kotlyar M, McCabe J, Peterson J, Foa EB (2007) D-cycloserine augmented exposure therapy for obsessive-compulsive disorder. Biol Psychiatry 62:835-838.

Kwapis JL, Jarome TJ, Lonergan ME, Helmstetter FJ (2009) Protein kinase Mzeta maintains fear memory in the amygdala but not in the hippocampus. Behav Neurosci 123:844-850.

Lang PJ, Davis M, Ohman A (2000) Fear and anxiety: animal models and human cognitive psychophysiology. J Affect Disord 61:137-159.

Langton JM, Richardson R (2008) D-cycloserine facilitates extinction the first time but not the second time: an examination of the role of NMDA across the course of repeated extinction sessions. Neuropsychopharmacology 33:3096-3102.

Langton JM, Richardson R (2009) The role of context in the re-extinction of learned fear. Neurobiol Learn Mem 92:496-503.

Lanius RA, Bluhm R, Lanius U, Pain C (2006) A review of neuroimaging studies in PTSD: heterogeneity of response to symptom provocation. J Psychiatr Res 40:709-729.

Laurent V, Westbrook RF (2010) Role of the basolateral amygdala in the reinstatement and extinction of fear responses to a previously extinguished conditioned stimulus. Learn Mem 17:86-96.

Lazzaro SC, Hou M, Cunha C, LeDoux JE, Cain CK (2010) Antagonism of lateral amygdala alpha1-adrenergic receptors facilitates fear conditioning and long-term potentiation. Learn Mem 17:489-493.

Ledgerwood L, Richardson R, Cranney J (2005) D-cycloserine facilitates extinction of learned fear: effects on reacquisition and generalized extinction. Biol Psychiatry 57:841-847.

Li WJ, Yu H, Yang JM, Gao J, Jiang H, Feng M, Zhao YX, Chen ZY (2010) Anxiolytic effect of music exposure on BDNFMet/Met transgenic mice. Brain Res 1347:71-79.

Likhtik E, Popa D, Apergis-Schoute J, Fidacaro GA, Pare D (2008) Amygdala intercalated neurons are required for expression of fear extinction. Nature 454:642-645.

Lin HC, Mao SC, Chen PS, Gean PW (2008) Chronic cannabinoid administration in vivo compromises extinction of fear memory. Learn Mem 15:876-884.

Lin HC, Mao SC, Su CL, Gean PW (2010) Alterations of excitatory transmission in the lateral amygdala during expression and extinction of fear memory. Int J Neuropsychopharmacol 13:335-345.

Lisboa SF, Reis DG, da Silva AL, Correa FM, Guimaraes FS, Resstel LB (2010) Cannabinoid CB1 receptors in the medial prefrontal cortex modulate the expression of contextual fear conditioning. Int J Neuropsychopharmacol 13:1163-1173.

Liu IY, Lyons WE, Mamounas LA, Thompson RF (2004) Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. J Neurosci 24:7958-7963.

Liu JL, Li M, Dang XR, Wang ZH, Rao ZR, Wu SX, Li YQ, Wang W (2009) A NMDA receptor antagonist, MK-801 impairs consolidating extinction of auditory conditioned fear responses in a Pavlovian model. PLoS One 4:e7548.

Liu Y, Formisano L, Savtchouk I, Takayasu Y, Szabo G, Zukin RS, Liu SJ (2010) A single fear-inducing stimulus induces a transcription-dependent switch in synaptic AMPAR phenotype. Nat Neurosci 13:223-231.

Lonsdorf TB, Weike AI, Golkar A, Schalling M, Hamm AO, Ohman A (2010) Amygdala-dependent fear conditioning in humans is modulated by the BDNFval66met polymorphism. Behav Neurosci 124:9-15.

Makkar SR, Zhang SQ, Cranney J (2010) Behavioral and neural analysis of GABA in the acquisition, consolidation, reconsolidation, and extinction of fear memory. Neuropsychopharmacology 35:1625-1652.

Maren S (2005) Synaptic mechanisms of associative memory in the amygdala. Neuron 47:783-786.

McCallum J, Kim JH, Richardson R (2010) Impaired extinction retention in adolescent rats: effects of D-cycloserine. Neuropsychopharmacology 35:2134-2142.

McKernan MG, Shinnick-Gallagher P (1997) Fear conditioning induces a lasting potentiation of synaptic currents in vitro. Nature 390:607-611.

McLaughlin KA, Conron KJ, Koenen KC, Gilman SE (2010) Childhood adversity, adult stressful life events, and risk of past-year psychiatric disorder: a test of the stress sensitization hypothesis in a population-based sample of adults. Psychol Med 40:1647-1658.

Mehta D, Gonik M, Klengel T, Rex-Haffner M, Menke A, Rubel J, Mercer KB, Putz B, Bradley B, Holsboer F, Ressler KJ, Muller-Myhsok B, Binder EB (2011) Using Polymorphisms in FKBP5 to Define Biologically Distinct Subtypes of Posttraumatic Stress Disorder: Evidence From Endocrine and Gene Expression Studies. Arch Gen Psychiatry.

Migues PV, Hardt O, Wu DC, Gamache K, Sacktor TC, Wang YT, Nader K (2010) PKMzeta maintains memories by regulating GluR2-dependent AMPA receptor trafficking. Nat Neurosci 13:630-634.

Milad MR, Orr SP, Lasko NB, Chang Y, Rauch SL, Pitman RK (2008) Presence and acquired origin of reduced recall for fear extinction in PTSD: results of a twin study. J Psychiatr Res 42:515-520.

Milliken CS, Auchterlonie JL, Hoge CW (2007) Longitudinal assessment of mental health problems among active and reserve component soldiers returning from the Iraq war. JAMA 298:2141-2148.

Mokin M, Zheng Z, Keifer J (2007) Conversion of silent synapses into the active pool by selective GluR1-3 and GluR4 AMPAR trafficking during in vitro classical conditioning. J Neurophysiol 98:1278-1286.

More L, Gravius A, Pietraszek M, Belozertseva I, Malyshkin A, Shekunova E, Barberi C, Schaefer D, Schmidt WJ, Danysz W (2007) Comparison of the mGluR1 antagonist A-841720 in rat models of pain and cognition. Behav Pharmacol 18:273-281.

Mueller D, Cahill SP (2010) Noradrenergic modulation of extinction learning and exposure therapy. Behav Brain Res 208:1-11.

Mueller D, Porter JT, Quirk GJ (2008) Noradrenergic signaling in infralimbic cortex increases cell excitability and strengthens memory for fear extinction. J Neurosci 28:369-375.

Mueller D, Bravo-Rivera C, Quirk GJ (2010) Infralimbic D2 receptors are necessary for fear extinction and extinction-related tone responses. Biol Psychiatry 68:1055-1060.

Musumeci G, Sciarretta C, Rodriguez-Moreno A, Al Banchaabouchi M, Negrete-Diaz V, Costanzi M, Berno V, Egorov AV, von Bohlen Und Halbach O, Cestari V, Delgado-Garcia JM, Minichiello L (2009) TrkB modulates fear learning and amygdalar synaptic plasticity by specific docking sites. J Neurosci 29:10131-10143.

Nedelescu H, Kelso CM, Lazaro-Munoz G, Purpura M, Cain CK, Ledoux JE, Aoki C (2010) Endogenous GluR1-containing AMPA receptors translocate to asymmetric synapses in the lateral amygdala during the early phase of fear memory formation: an electron microscopic immunocytochemical study. J Comp Neurol 518:4723-4739.

Ninan I, Bath KG, Dagar K, Perez-Castro R, Plummer MR, Lee FS, Chao MV (2010) The BDNF Val66Met polymorphism impairs NMDA receptor-dependent synaptic plasticity in the hippocampus. J Neurosci 30:8866-8870.

Norberg MM, Krystal JH, Tolin DF (2008) A meta-analysis of D-cycloserine and the facilitation of fear extinction and exposure therapy. Biol Psychiatry 63:1118-1126.

Ortiz O, Delgado-Garcia JM, Espadas I, Bahi A, Trullas R, Dreyer JL, Gruart A, Moratalla R (2010) Associative learning and CA3-CA1 synaptic plasticity are impaired in D1R null, Drd1a-/- mice and in hippocampal siRNA silenced Drd1a mice. J Neurosci 30:12288-12300.

Ota KT, Monsey MS, Wu MS, Schafe GE (2010) Synaptic plasticity and NO-cGMP-PKG signaling regulate pre- and postsynaptic alterations at rat lateral amygdala synapses following fear conditioning. PLoS One 5:e11236.

Ota KT, Pierre VJ, Ploski JE, Queen K, Schafe GE (2008) The NO-cGMP-PKG signaling pathway regulates synaptic plasticity and fear memory consolidation in the lateral amygdala via activation of ERK/MAP kinase. Learn Mem 15:792-805.

Otto MW, McHugh RK, Simon NM, Farach FJ, Worthington JJ, Pollack MH (2010a) Efficacy of CBT for benzodiazepine discontinuation in patients with panic disorder: Further evaluation. Behav Res Ther 48:720-727.

Otto MW, Tolin DF, Simon NM, Pearlson GD, Basden S, Meunier SA, Hofmann SG, Eisenmenger K, Krystal JH, Pollack MH (2010b) Efficacy of d-cycloserine for enhancing response to cognitive-behavior therapy for panic disorder. Biol Psychiatry 67:365-370.

Ou LC, Gean PW (2006) Regulation of amygdala-dependent learning by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol-3-kinase. Neuropsychopharmacology 31:287-296.

Ou LC, Yeh SH, Gean PW (2010) Late expression of brain-derived neurotrophic factor in the amygdala is required for persistence of fear memory. Neurobiol Learn Mem 93:372-382.

Pape HC, Pare D (2010) Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. Physiol Rev 90:419-463.

Parsons RG, Davis M (2011) Temporary disruption of fear-potentiated startle following PKMzeta inhibition in the amygdala. Nat Neurosci 14:295-296.

Paul C, Stratil C, Hofmann F, Kleppisch T (2010) cGMP-dependent protein kinase type I promotes CREB/CRE-mediated gene expression in neurons of the lateral amygdala. Neurosci Lett 473:82-86.

Peters J, Dieppa-Perea LM, Melendez LM, Quirk GJ (2010) Induction of fear extinction with hippocampal-infralimbic BDNF. Science 328:1288-1290.

Pole N, Neylan TC, Otte C, Henn-Hasse C, Metzler TJ, Marmar CR (2009) Prospective prediction of posttraumatic stress disorder symptoms using fear potentiated auditory startle responses. Biol Psychiatry 65:235-240.

Prado-Alcala RA, Haiek M, Rivas S, Roldan-Roldan G, Quirarte GL (1994) Reversal of extinction by scopolamine. Physiol Behav 56:27-30.

Quirk GJ, Mueller D (2008) Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology 33:56-72.

Quirk GJ, Repa C, LeDoux JE (1995) Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: parallel recordings in the freely behaving rat. Neuron 15:1029-1039.

Rattiner LM, Davis M, Ressler KJ (2004a) Differential regulation of brain-derived neurotrophic factor transcripts during the consolidation of fear learning. Learn Mem 11:727-731.

Rattiner LM, Davis M, French CT, Ressler KJ (2004b) Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. J Neurosci 24:4796-4806.

Raybuck JD, Lattal KM (2011) Double dissociation of amygdala and hippocampal contributions to trace and delay fear conditioning. PLoS One 6:e15982.

Rea K, Lang Y, Finn DP (2009) Alterations in extracellular levels of gamma-aminobutyric acid in the rat basolateral amygdala and periaqueductal gray during conditioned fear, persistent pain and fear-conditioned analgesia. J Pain 10:1088-1098.

Ressler KJ, Rothbaum BO, Tannenbaum L, Anderson P, Graap K, Zimand E, Hodges L, Davis M (2004) Cognitive enhancers as adjuncts to psychotherapy: use of D-cycloserine in phobic individuals to facilitate extinction of fear. Arch Gen Psychiatry 61:1136-1144.

Ressler KJ, Mercer KB, Bradley B, Jovanovic T, Mahan A, Kerley K, Norrholm SD, Kilaru V, Smith AK, Myers AJ, Ramirez M, Engel A, Hammack SE, Toufexis D, Braas KM, Binder EB, May V (2011) Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. Nature 470:492-497.

Rogan MT, Staubli UV, LeDoux JE (1997) Fear conditioning induces associative long-term potentiation in the amygdala. Nature 390:604-607.

Rogers MA, Yamasue H, Abe O, Yamada H, Ohtani T, Iwanami A, Aoki S, Kato N, Kasai K (2009) Smaller amygdala volume and reduced anterior cingulate gray matter density associated with history of post-traumatic stress disorder. Psychiatry Res 174:210-216.

Rudy JW, Matus-Amat P (2009) DHPG activation of group 1 mGluRs in BLA enhances fear conditioning. Learn Mem 16:421-425.

Rumpel S, LeDoux J, Zador A, Malinow R (2005) Postsynaptic receptor trafficking underlying a form of associative learning. Science 308:83-88.

Sah P, Westbrook RF, Luthi A (2008) Fear conditioning and long-term potentiation in the amygdala: what really is the connection? Ann N Y Acad Sci 1129:88-95.

Serrano P, Friedman EL, Kenney J, Taubenfeld SM, Zimmerman JM, Hanna J, Alberini C, Kelley AE, Maren S, Rudy JW, Yin JC, Sacktor TC, Fenton AA (2008) PKMzeta maintains spatial, instrumental, and classically conditioned long-term memories. PLoS Biol 6:2698-2706.

Shin LM, Liberzon I (2010) The neurocircuitry of fear, stress, and anxiety disorders. Neuropsychopharmacology 35:169-191.

Shin LMaH, K. (2009) Is posttraumatic stress disorder a stress-induced fear circuitry disorder? J Trauma Stress 22:6.

Shumyatsky GP, Malleret G, Shin RM, Takizawa S, Tully K, Tsvetkov E, Zakharenko SS, Joseph J, Vronskaya S, Yin D, Schubart UK, Kandel ER, Bolshakov VY (2005) stathmin, a gene enriched in the amygdala, controls both learned and innate fear. Cell 123:697-709.

Siegl S, Flor PJ, Fendt M (2008) Amygdaloid metabotropic glutamate receptor subtype 7 is involved in the acquisition of conditioned fear. Neuroreport 19:1147-1150.

Sierra-Mercado D, Padilla-Coreano N, Quirk GJ (2011) Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. Neuropsychopharmacology 36:529-538.

Sigurdsson T, Doyere V, Cain CK, LeDoux JE (2007) Long-term potentiation in the amygdala: a cellular mechanism of fear learning and memory. Neuropharmacology 52:215-227.

Soliman F, Glatt CE, Bath KG, Levita L, Jones RM, Pattwell SS, Jing D, Tottenham N, Amso D, Somerville LH, Voss HU, Glover G, Ballon DJ, Liston C, Teslovich T, Van Kempen T,

Lee FS, Casey BJ (2010) A genetic variant BDNF polymorphism alters extinction learning in both mouse and human. Science 327:863-866.

Suendermann O, Ehlers A, Boellinghaus I, Gamer M, Glucksman E (2010) Early heart rate responses to standardized trauma-related pictures predict posttraumatic stress disorder: a prospective study. Psychosom Med 72:301-308.

Takei S, Morinobu S, Yamamoto S, Fuchikami M, Matsumoto T, Yamawaki S (2011) Enhanced hippocampal BDNF/TrkB signaling in response to fear conditioning in an animal model of posttraumatic stress disorder. J Psychiatr Res 45:460-468.

Thomaes K, Dorrepaal E, Draijer N, de Ruiter MB, van Balkom AJ, Smit JH, Veltman DJ (2010) Reduced anterior cingulate and orbitofrontal volumes in child abuse-related complex PTSD. J Clin Psychiatry 71:1636-1644.

Tolin DF, Foa EB (2006) Sex differences in trauma and posttraumatic stress disorder: a quantitative review of 25 years of research. Psychol Bull 132:959-992.

True WR, Rice J, Eisen SA, Heath AC, Goldberg J, Lyons MJ, Nowak J (1993) A twin study of genetic and environmental contributions to liability for posttraumatic stress symptoms. Arch Gen Psychiatry 50:257-264.

Tsvetkov E, Carlezon WA, Benes FM, Kandel ER, Bolshakov VY (2002) Fear conditioning occludes LTP-induced presynaptic enhancement of synaptic transmission in the cortical pathway to the lateral amygdala. Neuron 34:289-300.

Tye KM, Prakash R, Kim SY, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature 471:358-362.

van Marle HJ, Hermans EJ, Qin S, Fernandez G (2009) From specificity to sensitivity: how acute stress affects amygdala processing of biologically salient stimuli. Biol Psychiatry 66:649-655.

van Wingen G, Rijpkema M, Franke B, van Eijndhoven P, Tendolkar I, Verkes RJ, Buitelaar J, Fernandez G (2010) The brain-derived neurotrophic factor Val66Met polymorphism affects memory formation and retrieval of biologically salient stimuli. Neuroimage 50:1212-1218.

Wessa M, Flor H (2007) Failure of extinction of fear responses in posttraumatic stress disorder: evidence from second-order conditioning. Am J Psychiatry 164:1684-1692.

Wilcox HC, Storr CL, Breslau N (2009) Posttraumatic stress disorder and suicide attempts in a community sample of urban american young adults. Arch Gen Psychiatry 66:305-311.

Wilhelm S, Buhlmann U, Tolin DF, Meunier SA, Pearlson GD, Reese HE, Cannistraro P, Jenike MA, Rauch SL (2008) Augmentation of behavior therapy with D-cycloserine for obsessive-compulsive disorder. Am J Psychiatry 165:335-341; quiz 409.

Wiltgen BJ, Godsil BP, Peng Z, Saab F, June HL, Linn ML, Cook JM, Houser CR, O'Dell TJ, Homanics GE, Fanselow MS (2009) The alpha1 subunit of the GABA(A) receptor modulates fear learning and plasticity in the lateral amygdala. Front Behav Neurosci 3:37.

Xie P, Kranzler HR, Poling J, Stein MB, Anton RF, Brady K, Weiss RD, Farrer L, Gelernter J (2009) Interactive effect of stressful life events and the serotonin transporter 5-HTTLPR genotype on posttraumatic stress disorder diagnosis in 2 independent populations. Arch Gen Psychiatry 66:1201-1209.

Yee BK, Zhu SW, Mohammed AH, Feldon J (2007) Levels of neurotrophic factors in the hippocampus and amygdala correlate with anxiety- and fear-related behaviour in C57BL6 mice. J Neural Transm 114:431-444.

Yehuda R, LeDoux J (2007) Response variation following trauma: a translational neuroscience approach to understanding PTSD. Neuron 56:19-32.

Yu H, Wang Y, Pattwell S, Jing D, Liu T, Zhang Y, Bath KG, Lee FS, Chen ZY (2009) Variant BDNF Val66Met polymorphism affects extinction of conditioned aversive memory. J Neurosci 29:4056-4064.

Zhang H, Ozbay F, Lappalainen J, Kranzler HR, van Dyck CH, Charney DS, Price LH, Southwick S, Yang BZ, Rasmussen A, Gelernter J (2006) Brain derived neurotrophic factor (BDNF) gene variants and Alzheimer's disease, affective disorders, posttraumatic stress disorder, schizophrenia, and substance dependence. Am J Med Genet B Neuropsychiatr Genet 141B:387-393.

Zhang S, Cranney J (2008) The role of GABA and anxiety in the reconsolidation of conditioned fear. Behav Neurosci 122:1295-1305.

Zimmerman JM, Maren S (2010) NMDA receptor antagonism in the basolateral but not central amygdala blocks the extinction of Pavlovian fear conditioning in rats. Eur J Neurosci 31:1664-1670.

Chapter 2

The Role of Homer1 in Posttraumatic Stress Disorder and Psychiatric Disease

2.1 Homer1 genetic association studies in Psychiatric Disease

As discussed in Chapter 1, genetic variability can contribute to an individual's vulnerability to the development of PTSD as well as other psychiatric illnesses. A majority of the evidence for such genetic vulnerability comes from genome wide association studies (Benke and Fallin, 2010; Cornelis et al., 2010; Van Winkel et al., 2010). These studies are generally conducted by taking DNA from cheek, blood or saliva and comparing polymorphisms across between separate cohorts of individuals with or without a specific psychiatric disease (Benke and Fallin, 2010; Cornelis et al., 2010; Van Winkel et al., 2010). While these studies are correlative in nature and one gene rarely associates completely with any psychiatric disorder, these studies may provide a list of potential genes to study mechanistically in animal models. One particular gene family, Homer1, has been associated with several psychiatric disorders (Figure 2.1) (Dahl et al., 2005; De Luca et al., 2009; Rietschel et al., 2010), and has also been demonstrated to be important in animal models of psychiatry (Szumlinski et al., 2006).

In one such study, two particular single nucleotide polymorphisms in the putative regulatory or promoter region of the Homer1 gene were associated with major depressive disorder (MDD) when compared to population based control subjects (Rietschel et al., 2010). One of the single nucleotide polymorphisms was also correlated with changes in prefrontal activity during executive cognition and in anticipation of reward (Rietschel et al., 2010). MDD is highly comorbid with PTSD and there is a lot of overlap in symptoms between the two disorders (Brady et al., 2000). In addition, altered prefrontal activity is a major neurological symptom found in trauma victims who go on to develop PTSD relative to trauma victims who do not develop PTSD (Etkin and Wager, 2007; Francati et

al., 2007; Quirk and Mueller, 2008; van Marle et al., 2009; de Carvalho et al., 2010). Thus this study, not only provides insight into the genetic underpinnings of major depression, but this may also suggest that Homer1 may be involved in related disorders such as PTSD.

Interestingly, the Homer1 gene has also been associated with the presentation of psychotic symptoms in individuals with Parkinson's disease (De Luca et al., 2009). Parkinson's disease is a neurodegenerative disorder that sometimes presents with psychotic side effects (Poewe, 2008a, b; Zahodne and Fernandez, 2008; Friedman, 2010). In one study they found that a single nucleotide polymorphism in the Homer1 promoter region was associated with the development of these psychotic symptoms relative to Parkinson's patients whom did not manifest these psychotic symptoms (De Luca et al., 2009). While typically considered a neurological disorder, Parkinson's is often comorbid with psychiatric symptoms such as psychosis (Poewe, 2008a; Poewe et al., 2008; Zahodne and Fernandez, 2008; Friedman, 2010) and depression (Chaudhuri and Odin, 2010; Kasten et al., 2010; Eskow Jaunarajs et al., 2011).

Lastly, a distinct Homer1 single nucleotide polymorphism has been associated with cocaine dependence in a specific African American population (De Luca et al., 2009). This single nucleotide polymorphism was located in the first exon of the Homer1 gene (De Luca et al., 2009). While the association is interesting in its own right, there is evidence suggesting that appetitive learning (such as what occurs with drugs of abuse) shares common molecular and synaptic mechanisms with aversive learning (such as what occurs with Pavlovian fear conditioning) (Quirk and Gehlert, 2003; Balleine and Killcross, 2006; Morrison and Salzman, 2010). It is thought that one commonality among

all of these disorders may be aberrant synaptic plasticity, (Quirk et al., 1995; McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Blair et al., 2001; Tsvetkov et al., 2002; Maren, 2005; Shumyatsky et al., 2005; Lanius et al., 2006; Francati et al., 2007; Sigurdsson et al., 2007; Sah et al., 2008; Pape and Pare, 2010), which is further supported by the common genetic association of Homer1, a molecule known to contribute to plasticity and NMDA/AMPA glutamate receptor function (Bertaso et al., 2010; Hu et al., 2010).

Finally, it is interesting that a majority of the published genetic association studies demonstrate single nucleotide polymorphisms in the putative regulatory or promoter region of Homer1. This could suggest that these individuals with increased susceptibility to major depression or cocaine dependence have altered regulation of Homer1 transcription. Understanding the mechanisms of homer 1 gene transcription in animal models of psychiatric disease might lead to an understanding of how homer1 leads to altered synaptic plasticity in the psychiatric diseases. While, the association studies only demonstrate a role for the Homer1 gene as a whole, homer1a is a likely variant of the Homer1 gene to study because it is the only gene variant of Homer1 dynamically regulated with synaptic activity in an immediate-early gene like fashion and would likely be implicated in plasticity related disorders.

2.2 The Homer1 gene family: Structure and Function

In the mammalian genome, there are three genes (Homer1-3) that transcribe the Homer family of proteins (Shiraishi-Yamaguchi and Furuichi, 2007). These proteins share two common functional domains: an EVH1 binding domain and a coiled coil domain (Shiraishi-Yamaguchi and Furuichi, 2007). The EVH1 binding domain

recognizes proline rich regions of various receptors and proteins in the postsynaptic density (Shiraishi-Yamaguchi and Furuichi, 2007; Peterson and Volkman, 2009), including Type 1 metabotropic glutamate receptors (Bertaso et al., 2010), SHANK (Bertaso et al., 2010), IP3 receptors (Tu et al., 1998; Yuan et al., 2003), TRPC receptors (Yuan et al., 2003), ryanodine channels (Pouliquin and Dulhunty, 2009), and Pax6 (Cooper and Hanson, 2005). The coiled coil domain is a leucine zipper structure that recognizes other coiled coil domains from other Homer molecules (Shiraishi-Yamaguchi and Furuichi, 2007). The Homer1 gene consists of 10 exons, the first five exons make up the EVH1 binding domain whereas the last five exons comprise the coiled coil domain (Shiraishi-Yamaguchi and Furuichi, 2007). Of the seven homer1 gene variants (homer1a-g), homer1b-g contain all 10 exons (Shiraishi-Yamaguchi and Furuichi, 2007). Homer1a, however, has an alternate poly-A stop site just past exon 5 resulting in a truncated protein product lacking the coiled coil domain (Niibori et al., 2007; Shiraishi-Yamaguchi and Furuichi, 2007).

Interestingly, homer1a is the only gene variant in the Homer family that is dynamically regulated during network activity (Shiraishi-Yamaguchi and Furuichi, 2007). Homer1a can be activated at the physiological level with synaptic stimulation (Shiraishi-Yamaguchi and Furuichi, 2007), at the neurotramsitter level with NMDA (Ango et al., 2000; Sato et al., 2001; Nielsen et al., 2002), BDNF (Sato et al., 2001), or PACAP (Nielsen et al., 2002; Girard et al., 2004; Kammermeier, 2008) activation and at the behavioral level with stress (Szumlinski et al., 2006), drug exposure (Szumlinski et al., 2006) or even exposure to a novel environment (Szumlinski et al., 2004; Lominac et al., 2005; Szumlinski et al., 2005a). This truncated version of the Homer1 protein is

unable to self-aggregate, but binds with equal affinity to proline-rich regions of the same proteins that homer1b-g bind (Shiraishi-Yamaguchi and Furuichi, 2007). The rapid upregulation of homer1a with synaptic plasticity suggests that homer1a might competitively bind with these receptors disrupting already established receptor clusters at the synapse between longer versions of homer and their binding partners (Shiraishi-Yamaguchi and Furuichi, 2007).

Experimental evidence suggests that homer1a can interact with type 1 metabotropic glutamate receptors (mGLURs) to enhance agonist independent inhibition of NMDA receptors (Bertaso et al., 2010). Type 1 mGLURs are physically linked to NMDA glutamate receptors through protein interactions with homer and shank (Figure 2.2) (Bertaso et al., 2010). Expression of homer1a in primary cortical cell culture can result in a disruption of the physical link between mGLURs and NMDA receptors (Bertaso et al., 2010). Furthermore, this disruption allows for agonist-independent inhibition of NMDA by mGLURs (Bertaso et al., 2010).

AMPA receptors are another ionotropic glutamate receptor crucial for synaptic plasticity (Turrigiano and Nelson, 2000). Interestingly, expression of homer 1a in cortical neurons appears to decrease surface expression of AMPA receptors (Hu et al., 2010). This effect also appears to be dependent on agonist independent metabotropic glutamate signaling in that when you inhibit mGLURS, homer1a expression does not decrease AMPA receptor surface expression (Hu et al., 2010). AMPA receptor surface expression is increased in neurons from Homer1a KO mice (Hu et al., 2010). Furthermore replacing homer1a in homer1a KO neurons reduces the elevated surface expression of AMPA

receptors (Hu et al., 2010). Finally homeostatic scaling of AMPA receptors is impaired in homer1a KO mice (Hu et al., 2010).

Homeostatic scaling is a non-Hebbian form of neuronal plasticity thought to help maintain neuronal excitability and informational content in the face of changes in neuronal activity (Turrigiano and Nelson, 2000). One mechanism of homeostatic plasticity is a cell wide reduction in surface expression of AMPA receptors at excitatory synapses which allows the cells to maintain relative strength at synapses (Turrigiano and Nelson, 2000). Given the diversity in binding partners (Peterson and Volkman, 2009), homer1a appears to have many other roles at the synapse including calcium signaling (Westhoff et al., 2003; Sala et al., 2005; Worley et al., 2007; Gasperini et al., 2009), structural plasticity (Sala et al., 2001; de Bartolomeis and Iasevoli, 2003; Sala et al., 2003; Sala et al., 2005) and signal transduction (Sala et al., 2001; de Bartolomeis and Iasevoli, 2003; Cooper and Hanson, 2005; Hu et al., 2010; Roloff et al., 2010). In the remaining sections we describe how homer1a has been implicated in animal models of psychiatric disease and Pavlovian fear conditioning.

2.3 Role of Homer1a in animal models of psychiatry

Interestingly, homer1a appears to play a role in animal models of drug addiction, schizophrenia, depression and stress/anxiety disorders. Exposure to drugs of abuse causes a rapid increase in *homer1a* mRNA in the neocortex (Fujiyama et al., 2003; Ghasemzadeh et al., 2009), the striatum (Zhang et al., 2007) and the prefrontal cortex (Cochran et al., 2002; Nichols and Sanders-Bush, 2002), and results in decreases in mRNA in the nucleus accumbens (Kane et al., 2005). Homer1 knock out mice show impaired cocaine induced locomotor activity (Szumlinski et al., 2004; Lominac et al.,

2005; Szumlinski et al., 2005a), impaired cocaine induced release of glutamate in the nucleus accumbens (Szumlinski et al., 2004; Lominac et al., 2005; Szumlinski et al., 2005a) and prefrontal cortex (Szumlinski et al., 2004; Lominac et al., 2005; Szumlinski et al., 2005a).

Homer1a upregulation has been seen in animal models of schizophrenia as well. Antipsychotic drugs such as haloperidol, which are commonly given to treat the symptoms of schizophrenia, increase *homer1a* mRNA expression in the striatum and nucleus accumbens. Furthermore, Homer1 knockout mice show impaired prepulse inhibition (an animal model of schizophrenia) and are hypersensitive to psychomotor-activating effects of both stimulant and dissociative anesthetic drugs (Szumlinski et al., 2004; Szumlinski et al., 2005a; Szumlinski et al., 2005b). These effects can be reversed by an AAV-mediated restoration of homer1a in the prefrontal cortex (Lominac et al., 2005).

Animal models of anxiety, including novel environment exposure (Vazdarjanova et al., 2002; Igaz et al., 2004), a single foot shock stress (Igaz et al., 2004) and contextual fear conditioning (Hashikawa et al., 2011), all produce an increase in *homer1a* mRNA expression. In addition, Homer1 knockout mice have altered exploration of novel objects (Szumlinski et al., 2005a) and novel environments (Szumlinski et al., 2004; Lominac et al., 2005; Szumlinski et al., 2005a) as well as increased spontaneous motor activity, (Szumlinski et al., 2004; Lominac et al., 2005; Szumlinski et al., 2005a) all of which are animal models of anxiety.

Notably, very few of these studies show protein level changes. In addition, most transgenic studies were done in Homer1 knockout mice, for which all variants of the

Homer1 gene deleted, but it is clear that homer1a has unique cellular effects to the other gene variants, making it difficult to pinpoint the effects of a particular gene variant on these changes in behavior. Nonetheless, this family of proteins has been highly implicated in animal models of drug addiction, schizophrenia and anxiety.

2.4 Homer1a and Pavlovian Fear conditioning

There is limited evidence connecting *homer1a* gene expression with Pavlovian fear conditioning. However, that evidence is consistent in supporting the fact that *homer1a* is upregulated and necessary for consolidation of Pavlovian fear conditioning. Using compartmental analysis fluorescence in situ hybridization (CatFISH) Hashikawa et al. (2011) demonstrated that after contextual fear conditioning, *homer1a* was rapidly upregulated in the nucleus of lateral amygdala neurons (Hashikawa et al., 2011). Homer1a KO mice demonstrate deficits in contextual fear conditioning (Inoue et al., 2009). Finally, over-expressing *homer1a* in the hippocampus enhances contextual fear memories and is dependent upon mGLUR signaling (Tronson et al., 2010). While there are only three publications to-date assessing the role of homer1a in Pavlovian fear conditioning, the evidence is enough to suggest that homer1a may play a critical role in synaptic plasticity during Pavlovian fear conditioning in both the amygdala and hippocampus. Furthermore, given the immediately early gene-like fashion of *homer1a* transcription, studying mechanisms of its transcription will provide molecular insight into the mechanisms of Pavlovian fear conditioning.

Generating a homer1a specific KO mouse was challenging in that there are only 11 amino acids differentiating the homer1a and homer1b/c proteins. To generate a short-form specific gene targeting mouse, genomic DNA corresponding to the 11 amino acids

was replaced by cDNA encoding the long gene variant of Homer (homer1b/c) specific C-terminal acids (Inoue et al., 2009). The mice produced Homer1b/c but not the Homer1a proteins. These mice, lacking the Homer1a protein, demonstrated normal acquisition and short-term memory for contextual fear conditioning (Inoue et al., 2009). However these mice did have impairments in long-term memory consolidation as well as memory retention (Inoue et al., 2009).

The interpretations of the results from this study are obscured by the fact that Homer1a KO mice showed decreased shock reactivity. Therefore the impairments in consolidation of fear may be due to the fact that the unconditioned stimulus was perceived as less intense by the Homer1a KO mice. This study made no attempt to correct for differences in pain perception. In addition, the deletion of homer1a was global; in other words the homer1a gene was deleted in all cell types and brain regions within the mouse. Therefore one is unable to interpret 1) Which brain regions are responsible for this phenotype and 2) Whether or not this is a developmental effect. While it is clear that homer1a is important for contextual fear memories, the interpretations from this study are limited.

Instead of genetically deleting homer1a, Tronson et al (2010) examined the effects of over-expressing homer1a during contextual fear conditioning and homer1a interaction with mGluRs during contextual fear conditioning. After contextual fear conditioning, homer1a is increasingly bound to type 1 metabotropic glutamate receptors (mGLUR), and homer1b/*c* was decreasingly bound to type 1 mGLURs. In addition, using an adenovirus construct, over-expressing homer1a in the hippocampus enhanced consolidation of contextual fear conditioning. This effect was reversed with MPEP, an

mGLUR antagonist. Thus the authors concluded that the effects of homer1a over-expression were due to its actions on Type 1 mGLUR receptors. This study was interesting because it goes beyond showing that homer1a is important for fear conditioning in that it provides some insight into a possible mechanism for how homer1a may act at the synapse.

2.5 Conclusions

Homer1a is a synaptic protein involved in homeostatic synaptic plasticity (Hu et al., 2010), calcium signaling (Sala et al., 2005), mGLUR-NMDA interactions (Bertaso et al., 2010) as well as surface expression of AMPA receptors (Hu et al., 2010) during synaptic plasticity. The regulatory region of this gene is highly associated with multiple psychiatric disorders in humans, and there is further supporting evidence for homer1a in animal models of psychiatric disease (Dahl et al., 2005; De Luca et al., 2009; Rietschel et al., 2010). Finally, homer1a has been shown to be essential for contextual fear conditioning (Inoue et al., 2009; Tronson et al., 2010) through its interactions with Type 1 mGLURs (Tronson et al., 2010). Given the association of Homer1 with psychiatric disease, the role of homer1a in Pavlovian fear conditioning, and the immediate early gene-like expression of *homer1a* mRNA during synaptic activity, examining the mechanism by which *homer1a* mRNA is upregulated during Pavlovian fear conditioning may provide valuable insight into molecular mechanisms underlying posttraumatic stress disorder.

2.6 Figures

Figure 2.1 Summary of human genetic association studies demonstrating a role for the Homer1 gene in psychiatric disease.

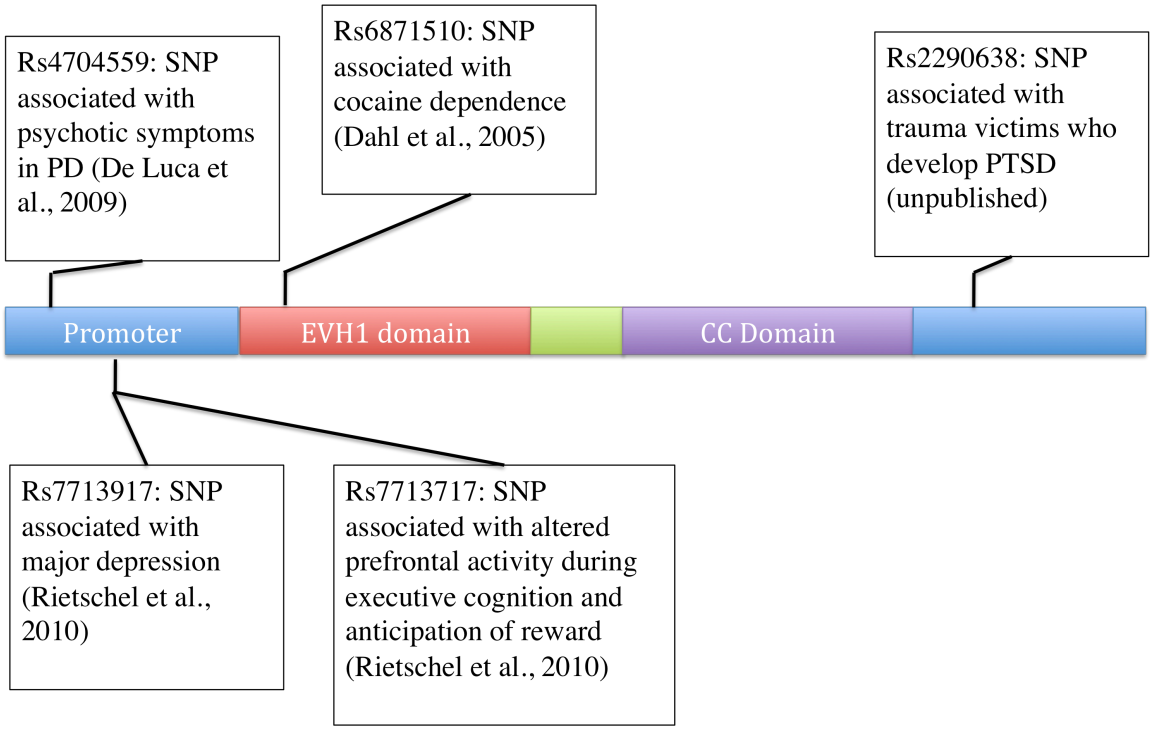
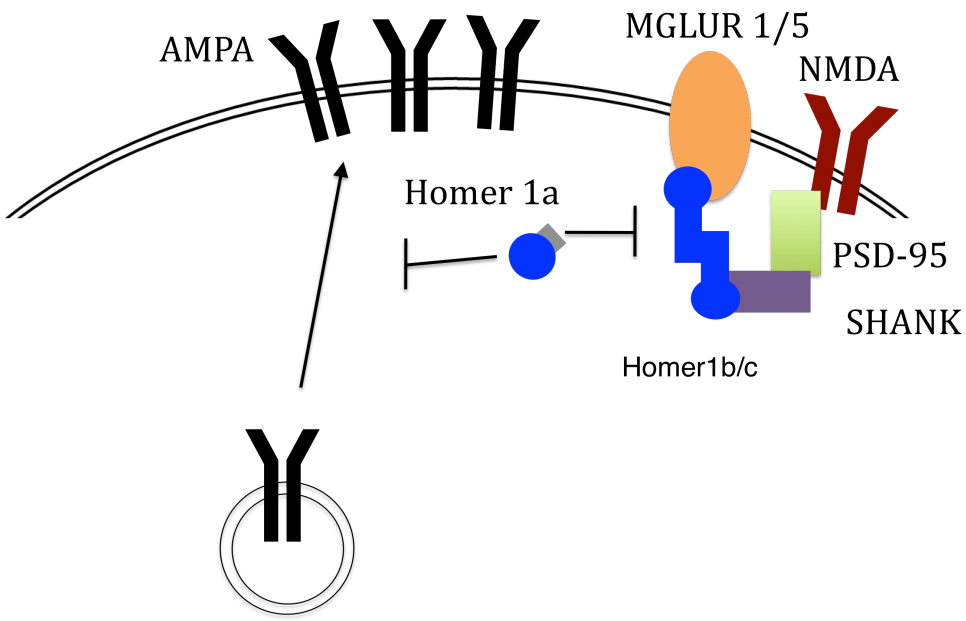

Rs4704559: SNP associated with psychotic symptoms in PD (De Luca et al., 2009)
Rs6871510: SNP associated with cocaine dependence (Dahl et al., 2005)
Rs2290638: SNP associated with trauma victims who develop PTSD (unpublished)
Promoter
EVH1 domain
CC Domain
Rs7713917: SNP associated with major depression (Rietschel et al., 2010)
Rs7713717: SNP associated with altered prefrontal activity during executive cognition and anticipation of reward (Rietschel et al., 2010)

Figure 2.2 Interactions of *homer1a* and glutamate receptors. Homer1 proteins physically bind with Type 1 metabotropic glutamate receptors (mGLURs) as well as SHANK proteins. These interactions physically link NMDA receptors with MGLURs. Over-expression of *homer1a* results in a disruption in the physical link of metabotropic glutamate receptors and NMDA receptors. Furthermore, homer1a expression results in agonist independent inhibition of NMDA receptors by mGLURs. Homer1a also results in a decrease in surface expression of AMPA receptors through mGLUR agonist independent signaling.


AMPA
MGLUR 1/5
NMDA
Homer 1a
PSD-95
SHANK
Homer1b/c

2.7 References

Ango F, Pin JP, Tu JC, Xiao B, Worley PF, Bockaert J, Fagni L (2000) Dendritic and axonal targeting of type 5 metabotropic glutamate receptor is regulated by homer1 proteins and neuronal excitation. J Neurosci 20:8710-8716.

Balleine BW, Killcross S (2006) Parallel incentive processing: an integrated view of amygdala function. Trends Neurosci 29:272-279.

Benke KS, Fallin MD (2010) Methods: genetic epidemiology. Psychiatr Clin North Am 33:15-34.

Bertaso F, Roussignol G, Worley P, Bockaert J, Fagni L, Ango F (2010) Homer1a-dependent crosstalk between NMDA and metabotropic glutamate receptors in mouse neurons. PLoS One 5:e9755.

Blair HT, Schafe GE, Bauer EP, Rodrigues SM, LeDoux JE (2001) Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning. Learn Mem 8:229-242.

Brady KT, Killeen TK, Brewerton T, Lucerini S (2000) Comorbidity of psychiatric disorders and posttraumatic stress disorder. J Clin Psychiatry 61 Suppl 7:22-32.

Chaudhuri KR, Odin P (2010) The challenge of non-motor symptoms in Parkinson's disease. Prog Brain Res 184:325-341.

Cochran SM, Fujimura M, Morris BJ, Pratt JA (2002) Acute and delayed effects of phencyclidine upon mRNA levels of markers of glutamatergic and GABAergic neurotransmitter function in the rat brain. Synapse 46:206-214.

Cooper ST, Hanson IM (2005) A screen for proteins that interact with PAX6: C-terminal mutations disrupt interaction with HOMER3, DNCL1 and TRIM11. BMC Genet 6:43.

Cornelis MC, Nugent NR, Amstadter AB, Koenen KC (2010) Genetics of post-traumatic stress disorder: review and recommendations for genome-wide association studies. Curr Psychiatry Rep 12:313-326.

Dahl JP, Kampman KM, Oslin DW, Weller AE, Lohoff FW, Ferraro TN, O'Brien CP, Berrettini WH (2005) Association of a polymorphism in the Homer1 gene with cocaine dependence in an African American population. Psychiatr Genet 15:277-283.

de Bartolomeis A, Iasevoli F (2003) The Homer family and the signal transduction system at glutamatergic postsynaptic density: potential role in behavior and pharmacotherapy. Psychopharmacol Bull 37:51-83.

de Carvalho MR, Rozenthal M, Nardi AE (2010) The fear circuitry in panic disorder and its modulation by cognitive-behaviour therapy interventions. World J Biol Psychiatry 11:188-198.

De Luca V, Annesi G, De Marco EV, de Bartolomeis A, Nicoletti G, Pugliese P, Muscettola G, Barone P, Quattrone A (2009) HOMER1 promoter analysis in Parkinson's disease: association study with psychotic symptoms. Neuropsychobiology 59:239-245.

Eskow Jaunarajs KL, Angoa-Perez M, Kuhn DM, Bishop C (2011) Potential mechanisms underlying anxiety and depression in Parkinson's disease: consequences of l-DOPA treatment. Neurosci Biobehav Rev 35:556-564.

Etkin A, Wager TD (2007) Functional neuroimaging of anxiety: a meta-analysis of emotional processing in PTSD, social anxiety disorder, and specific phobia. Am J Psychiatry 164:1476-1488.

Francati V, Vermetten E, Bremner JD (2007) Functional neuroimaging studies in posttraumatic stress disorder: review of current methods and findings. Depress Anxiety 24:202-218.

Friedman JH (2010) Parkinson's disease psychosis 2010: a review article. Parkinsonism Relat Disord 16:553-560.

Fujiyama K, Kajii Y, Hiraoka S, Nishikawa T (2003) Differential regulation by stimulants of neocortical expression of mrt1, arc, and homer1a mRNA in the rats treated with repeated methamphetamine. Synapse 49:143-149.

Gasperini R, Choi-Lundberg D, Thompson MJ, Mitchell CB, Foa L (2009) Homer regulates calcium signalling in growth cone turning. Neural Dev 4:29.

Ghasemzadeh MB, Windham LK, Lake RW, Acker CJ, Kalivas PW (2009) Cocaine activates Homer1 immediate early gene transcription in the mesocorticolimbic circuit: differential regulation by dopamine and glutamate signaling. Synapse 63:42-53.

Girard BM, Keller ET, Schutz KC, May V, Braas KM (2004) Pituitary adenylate cyclase activating polypeptide and PAC1 receptor signaling increase Homer 1a expression in central and peripheral neurons. Regul Pept 123:107-116.

Hashikawa K, Matsuki N, Nomura H (2011) Preferential Arc transcription at rest in the active ensemble during associative learning. Neurobiol Learn Mem 95:498-504.

Hu JH, Park JM, Park S, Xiao B, Dehoff MH, Kim S, Hayashi T, Schwarz MK, Huganir RL, Seeburg PH, Linden DJ, Worley PF (2010) Homeostatic scaling requires group I mGluR activation mediated by Homer1a. Neuron 68:1128-1142.

Igaz LM, Bekinschtein P, Izquierdo I, Medina JH (2004) One-trial aversive learning induces late changes in hippocampal CaMKIIalpha, Homer 1a, Syntaxin 1a and ERK2 protein levels. Brain Res Mol Brain Res 132:1-12.

Inoue N, Nakao H, Migishima R, Hino T, Matsui M, Hayashi F, Nakao K, Manabe T, Aiba A, Inokuchi K (2009) Requirement of the immediate early gene vesl-1S/homer-1a for fear memory formation. Mol Brain 2:7.

Kammermeier PJ (2008) Endogenous homer proteins regulate metabotropic glutamate receptor signaling in neurons. J Neurosci 28:8560-8567.

Kane JK, Hwang Y, Konu O, Loughlin SE, Leslie FM, Li MD (2005) Regulation of Homer and group I metabotropic glutamate receptors by nicotine. Eur J Neurosci 21:1145-1154.

Kasten M, Kertelge L, Bruggemann N, van der Vegt J, Schmidt A, Tadic V, Buhmann C, Steinlechner S, Behrens MI, Ramirez A, Binkofski F, Siebner H, Raspe H, Hagenah J, Lencer R, Klein C (2010) Nonmotor symptoms in genetic Parkinson disease. Arch Neurol 67:670-676.

Lanius RA, Bluhm R, Lanius U, Pain C (2006) A review of neuroimaging studies in PTSD: heterogeneity of response to symptom provocation. J Psychiatr Res 40:709-729.

Lominac KD, Oleson EB, Pava M, Klugmann M, Schwarz MK, Seeburg PH, During MJ, Worley PF, Kalivas PW, Szumlinski KK (2005) Distinct roles for different

Homer1 isoforms in behaviors and associated prefrontal cortex function. J Neurosci 25:11586-11594.

Maren S (2005) Synaptic mechanisms of associative memory in the amygdala. Neuron 47:783-786.

McKernan MG, Shinnick-Gallagher P (1997) Fear conditioning induces a lasting potentiation of synaptic currents in vitro. Nature 390:607-611.

Morrison SE, Salzman CD (2010) Re-valuing the amygdala. Curr Opin Neurobiol 20:221-230.

Nichols CD, Sanders-Bush E (2002) A single dose of lysergic acid diethylamide influences gene expression patterns within the mammalian brain. Neuropsychopharmacology 26:634-642.

Nielsen HS, Georg B, Hannibal J, Fahrenkrug J (2002) Homer-1 mRNA in the rat suprachiasmatic nucleus is regulated differentially by the retinohypothalamic tract transmitters pituitary adenylate cyclase activating polypeptide and glutamate at time points where light phase-shifts the endogenous rhythm. Brain Res Mol Brain Res 105:79-85.

Niibori Y, Hayashi F, Hirai K, Matsui M, Inokuchi K (2007) Alternative poly(A) site-selection regulates the production of alternatively spliced vesl-1/homer1 isoforms that encode postsynaptic scaffolding proteins. Neurosci Res 57:399-410.

Pape HC, Pare D (2010) Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. Physiol Rev 90:419-463.

Peterson FC, Volkman BF (2009) Diversity of polyproline recognition by EVH1 domains. Front Biosci 14:833-846.

Poewe W (2008a) Non-motor symptoms in Parkinson's disease. Eur J Neurol 15 Suppl 1:14-20.

Poewe W (2008b) When a Parkinson's disease patient starts to hallucinate. Pract Neurol 8:238-241.

Poewe W, Gauthier S, Aarsland D, Leverenz JB, Barone P, Weintraub D, Tolosa E, Dubois B (2008) Diagnosis and management of Parkinson's disease dementia. Int J Clin Pract 62:1581-1587.

Pouliquin P, Dulhunty AF (2009) Homer and the ryanodine receptor. Eur Biophys J 39:91-102.

Quirk GJ, Gehlert DR (2003) Inhibition of the amygdala: key to pathological states? Ann N Y Acad Sci 985:263-272.

Quirk GJ, Mueller D (2008) Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology 33:56-72.

Quirk GJ, Repa C, LeDoux JE (1995) Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: parallel recordings in the freely behaving rat. Neuron 15:1029-1039.

Rietschel M et al. (2010) Genome-wide association-, replication-, and neuroimaging study implicates HOMER1 in the etiology of major depression. Biol Psychiatry 68:578-585.

Rogan MT, Staubli UV, LeDoux JE (1997) Fear conditioning induces associative long-term potentiation in the amygdala. Nature 390:604-607.

Roloff AM, Anderson GR, Martemyanov KA, Thayer SA (2010) Homer 1a gates the induction mechanism for endocannabinoid-mediated synaptic plasticity. J Neurosci 30:3072-3081.

Sah P, Westbrook RF, Luthi A (2008) Fear conditioning and long-term potentiation in the amygdala: what really is the connection? Ann N Y Acad Sci 1129:88-95.

Sala C, Roussignol G, Meldolesi J, Fagni L (2005) Key role of the postsynaptic density scaffold proteins Shank and Homer in the functional architecture of Ca2+ homeostasis at dendritic spines in hippocampal neurons. J Neurosci 25:4587-4592.

Sala C, Piech V, Wilson NR, Passafaro M, Liu G, Sheng M (2001) Regulation of dendritic spine morphology and synaptic function by Shank and Homer. Neuron 31:115-130.

Sala C, Futai K, Yamamoto K, Worley PF, Hayashi Y, Sheng M (2003) Inhibition of dendritic spine morphogenesis and synaptic transmission by activity-inducible protein Homer1a. J Neurosci 23:6327-6337.

Sato M, Suzuki K, Nakanishi S (2001) NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. J Neurosci 21:3797-3805.

Shiraishi-Yamaguchi Y, Furuichi T (2007) The Homer family proteins. Genome Biol 8:206.

Shumyatsky GP, Malleret G, Shin RM, Takizawa S, Tully K, Tsvetkov E, Zakharenko SS, Joseph J, Vronskaya S, Yin D, Schubart UK, Kandel ER, Bolshakov VY

(2005) stathmin, a gene enriched in the amygdala, controls both learned and innate fear. Cell 123:697-709.

Sigurdsson T, Doyere V, Cain CK, LeDoux JE (2007) Long-term potentiation in the amygdala: a cellular mechanism of fear learning and memory. Neuropharmacology 52:215-227.

Szumlinski KK, Kalivas PW, Worley PF (2006) Homer proteins: implications for neuropsychiatric disorders. Curr Opin Neurobiol 16:251-257.

Szumlinski KK, Lominac KD, Kleschen MJ, Oleson EB, Dehoff MH, Schwarz MK, Seeburg PH, Worley PF, Kalivas PW (2005a) Behavioral and neurochemical phenotyping of Homer1 mutant mice: possible relevance to schizophrenia. Genes Brain Behav 4:273-288.

Szumlinski KK, Lominac KD, Oleson EB, Walker JK, Mason A, Dehoff MH, Klugmann M, Cagle S, Welt K, During M, Worley PF, Middaugh LD, Kalivas PW (2005b) Homer2 is necessary for EtOH-induced neuroplasticity. J Neurosci 25:7054-7061.

Szumlinski KK, Dehoff MH, Kang SH, Frys KA, Lominac KD, Klugmann M, Rohrer J, Griffin W, 3rd, Toda S, Champtiaux NP, Berry T, Tu JC, Shealy SE, During MJ, Middaugh LD, Worley PF, Kalivas PW (2004) Homer proteins regulate sensitivity to cocaine. Neuron 43:401-413.

Tronson NC, Guzman YF, Guedea AL, Huh KH, Gao C, Schwarz MK, Radulovic J (2010) Metabotropic glutamate receptor 5/Homer interactions underlie stress effects on fear. Biol Psychiatry 68:1007-1015.

Tsvetkov E, Carlezon WA, Benes FM, Kandel ER, Bolshakov VY (2002) Fear conditioning occludes LTP-induced presynaptic enhancement of synaptic transmission in the cortical pathway to the lateral amygdala. Neuron 34:289-300.

Tu JC, Xiao B, Yuan JP, Lanahan AA, Leoffert K, Li M, Linden DJ, Worley PF (1998) Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. Neuron 21:717-726.

Turrigiano GG, Nelson SB (2000) Hebb and homeostasis in neuronal plasticity. Curr Opin Neurobiol 10:358-364.

van Marle HJ, Hermans EJ, Qin S, Fernandez G (2009) From specificity to sensitivity: how acute stress affects amygdala processing of biologically salient stimuli. Biol Psychiatry 66:649-655.

Van Winkel R, Esquivel G, Kenis G, Wichers M, Collip D, Peerbooms O, Rutten B, Myin-Germeys I, Van Os J (2010) REVIEW: Genome-wide findings in schizophrenia and the role of gene-environment interplay. CNS Neurosci Ther 16:e185-192.

Vazdarjanova A, McNaughton BL, Barnes CA, Worley PF, Guzowski JF (2002) Experience-dependent coincident expression of the effector immediate-early genes arc and Homer 1a in hippocampal and neocortical neuronal networks. J Neurosci 22:10067-10071.

Westhoff JH, Hwang SY, Duncan RS, Ozawa F, Volpe P, Inokuchi K, Koulen P (2003) Vesl/Homer proteins regulate ryanodine receptor type 2 function and intracellular calcium signaling. Cell Calcium 34:261-269.

Worley PF, Zeng W, Huang G, Kim JY, Shin DM, Kim MS, Yuan JP, Kiselyov K, Muallem S (2007) Homer proteins in Ca2+ signaling by excitable and non-excitable cells. Cell Calcium 42:363-371.

Yuan JP, Kiselyov K, Shin DM, Chen J, Shcheynikov N, Kang SH, Dehoff MH, Schwarz MK, Seeburg PH, Muallem S, Worley PF (2003) Homer binds TRPC family channels and is required for gating of TRPC1 by IP3 receptors. Cell 114:777-789.

Zahodne LB, Fernandez HH (2008) Pathophysiology and treatment of psychosis in Parkinson's disease: a review. Drugs Aging 25:665-682.

Zhang GC, Mao LM, Liu XY, Parelkar NK, Arora A, Yang L, Hains M, Fibuch EE, Wang JQ (2007) In vivo regulation of Homer1a expression in the striatum by cocaine. Mol Pharmacol 71:1148-1158.

Chapter 3

Homer 1a transcription is upregulated by Pavlovian Fear conditioning and BDNF-TrkB signaling in the amygdala and Hippocampus

3.1 Abstract

The consolidation of fear conditioning involves upregulation of genes necessary for long-term memory formation. We examined whether *homer1a*, which is required for memory formation, is downstream of BDNF - TrkB activation. We initially found that *homer1a* mRNA 1) increases after fear conditioning *in vivo* within both amygdala and hippocampus, 2) increases after BDNF application to primary hippocampal and amygdala cultures *in vitro,* and 3) these increases are dependent on transcription and MAPK signaling. Lastly we show that a trkB agonist, 7,8-DHF enhances long-term memory for Pavlovian fear conditioning in vivo as well as *homer1a,* in vitro, and that inhibiting trkB signaling impairs *homer1a* expression during Pavlovian conditioning in the amygdala and hippocampus. These data provide evidence for dynamic regulation of *homer1a* following BDNF-induced plasticity or during a BDNF-dependent learning process.

3.2 Introduction

Understanding the molecular mechanisms of fear conditioning is crucial for the development of novel treatments for fear-related disorders such as posttraumatic stress disorder. Numerous genes are rapidly upregulated during the consolidation, retrieval and extinction of fear conditioning including immediate-early genes (arc, Zif268, c-fos and JunB) ((Hall et al., 2001; Radwanska et al., 2002; Strekalova et al., 2003; Lonergan et al., 2010), neurotrophic factors (BDNF (Ressler et al., 2002; Ou and Gean, 2007) and NGFI-B (von Hertzen and Giese, 2005)), and synaptic structural proteins (Lonergan et al., 2010). Many of these genes are upregulated through calcium, extracellular-regulated kinase (ERK) /mitogen-activated protein kinase kinase (MAPKK or MEK) (Ploski et al., 2010) and cAMP response element-binding (CREB)-activation (Hall et al., 2001). The

downstream effect of increased gene transcription is diverse. Some genes act as transcription factors further activating other genes (Tischmeyer and Grimm, 1999), whereas BDNF has widespread effects on synaptic growth and signal transduction, and upregulation of other genes results in structural changes at the synapse.

In the present study we look at regulation of the gene variant, homer1a (also known as vesl-1S), during BDNF-induced plasticity and fear conditioning. Homer1a belongs to a family of scaffolding proteins that localize at the postsynaptic density (Shiraishi-Yamaguchi and Furuichi, 2007; Foa and Gasperini, 2009). The Homer1 gene family is highly conserved across species (Foa and Gasperini, 2009) and plays a role in intracellular calcium homeostasis, clustering/trafficking of receptors, gene transcription, and signal transduction (Shiraishi-Yamaguchi and Furuichi, 2007; Foa and Gasperini, 2009). The long forms of Homer (homer1g-f) are constitutively expressed and contain two functional domains: the EVH1 binding domain which recognizes proline rich regions of various receptors (including mGluR1/5, IP3, ryanoide receptors and shank), and a coiled-coil structure with a leucine zipper motif that aggregates with coiled-coil domains of other Homer molecules (Shiraishi-Yamaguchi and Furuichi, 2007). Multimers of long Homer proteins are thought to form protein clusters with other postsynaptic density proteins (Shiraishi-Yamaguchi and Furuichi, 2007).

The shorter variant of the Homer1 family (homer1a) lacks the coiled-coil domain and is expressed in an activity dependent manner (Shiraishi-Yamaguchi and Furuichi, 2007) (Figure 3.1a). This suggests that homer1a might act to disrupt homer-protein clusters by competitively binding to target proteins (Shiraishi-Yamaguchi and Furuichi, 2007). Furthermore it has been shown that homer1a can disrupt the physical link between

mGluR and NMDA, altering mGluR1/5's ability to modulate NMDA functioning (Bertaso et al., 2010), and also plays a role in synaptic scaling of AMPA receptors through agonist-indepenent mGLUR activity. Homer1a knockout mice appear to show impaired memory consolidation (Inoue et al., 2009). Overexpressing homer1a in the hippocampus enhances contextual fear conditioning (Tronson et al., 2010). These data suggest that homer1a is important for the consolidation of fear learning.

Here we examine mechanisms of transcription during Pavlovian fear conditioning and BDNF induced plasticity of a specific gene product, *homer1a*, which may have a specific functional role in regulating synapse structural reorganization during the synaptic plasticity that mediates the consolidation of fear memory. The TrkB pathway has been heavily implicated in Pavlovian fear conditioning. BDNF mRNA and protein is upregulated during consolidation of Pavlovian fear conditioning (Rattiner et al., 2004a). Moreover inhibiting BDNF impairs consolidation of fear conditioning in the amygdala (Rattiner et al., 2004b) and hippocampus as well as impairing extinction of cued fear conditioning in the amygdala. Thus examining BDNF induced plasticity in primary amygdala and hippocampal cell culture may provide mechanistic insight into regulation of *homer1a* mRNA expression in Pavlovian fear conditioning.

2.3 Methods

2.3.1 Animals

All experiments were performed on adult (6-8 weeks old) wild-type strain C57BL/6J male mice from Jackson Laboratory (Bar Harbor, ME.). All procedures were approved by the Institutional Animal Care and Use Committee of Emory University and were in compliance with National Institutes of Health guidelines. Separate cohorts of

animals were used for each experiment. The Erk inducible knockout mice contain loxP sites flanking exon 2 of the *Erk2* gene (Samuels et al., 2008). These mice have an intact Erk1 gene, but deletion of the Erk2 exon 2 prevents translation and protein production of the Erk2 protein product in cells expressing Cre recombinase (Samuels et al., 2008).

2.3.2 Behavior

Fear conditioning was conducted in nonrestrictive acrylic cylinders (SR-LAB startle response system, San Diego Instruments) located in a ventilated, sound-attenuated chamber. The foot shock (unconditioned stimulus) was delivered through a stainless steel grid floor. Shock reactivity was defined as the peak activity (measured with a piezoelectric accelerometer) that occurred during the 200 msec after the onset of the unconditioned stimulus. The tone-conditioned stimulus was generated by a Tektronix function generator audio oscillator and delivered through a high frequency speaker. One day prior to training, mice were preexposed to the tone through a 5 tone alone presentation program to both habituate them to handling and to the tone, but to get baseline fear responses to the tone presentation. Preexposure was done in a separate context. During cued fear conditioning, mice received five trials of a conditioned stimulus tone (30 seconds, 6 kHz, 70 dB) coterminating with an unconditioned stimulus foot shock (500 msec, 1 mA) with a variable inter-trial-interval between 60 and 180 seconds. The expression of fear was assessed 24 hours after fear conditioning and consisted of 3 minutes in the same context for which the training occurred (contextual memory) with no stimulus and 5 conditioned stimulus tone presentations of 30 second each with a 1.5-minute inter-trial interval in a different context (see figure 3.1 for schematic). Stimulus presentation and data acquisition were controlled and digitized by,

and stored in, an interfacing desktop computer using SR-LAB and analyzed with the Freeze View software program (Coulbourn Instruments, Whitehall, Pa.).

2.3.3 Cell Culture:

Primary cultures of postnatal hippocampal neurons were described previously (Brewer, 1997) with modifications, C57BL/6J mice (21 days postnatal) were decapitated and the hippocampus and amygdala were removed and immersed in ice-cold dissection buffer consisting of Hibernate-A medium (BrainBits, Springfield, IL, USA), B27 supplement and gentamycin (Invitrogen) (12g/ml) for the preparation of separate hippocampal and amygdala neuronal cell cultures. The hippocampus and amygdala tissues were sliced and then enzymatically digested with papain (Worthington, Lakewood, NJ, USA) in Hibernate-A medium at 32°C for 30 minutes. Cells were dissociated by triturating with Pasteur pipets fired on the tips to narrow openings. Neurons were purified in a density gradient media including Hibernate-A and OptiPrep (Sigma, St. Louis, MO, USA) by centrifugation. The density gradient media consisted of four layers. The first was 1 mL dissection buffer containing 35% OptiPrep; the second 1 mL dissection buffer contained 25% Optiprep and the third 1 ml dissection buffer contained 20% OptiPrep and the fourth 1 mL dissection buffer contained 15% OptiPrep. They were added on the top of each other carefully, resulting in clear layer separation. Then cells were added on the top of the density gradient media. After centrifugation, the densest layer with a cream color, located at the middle of the tube, could be seen. This layer of neurons was taken out by using a sterile transfer pipette and put into a new tube. After washing with dissection buffer, neuronal cells were plated onto Poly-D-Lysine (Sigma) coated plates or glass coverslips at the density of 2.5×10^5 cells/cm^2 in culture

media consisting of Neurobasal A medium (Invitrogen) with 2% B27 supplement, 2 mM glutamax and gentamycin (5 g/ml). Thereafter, the cultures were kept in a humidified incubator at 37˚C and 5% CO_2 and media were changed every 5 days until used for experiments. After 2-3 weeks in vitro, the cells were used for the experiments reported in the present study.

3.3.4 Drugs:

Recombinant human BDNF was purchased from Cell Sciences (Canton, MA, USA) and reconstituted in sterile PBS as 100 mg/ml stock. The aliquots of stock were stored at -30˚ C and final drugs and concentrations for cell culture experiments were as following: BDNF (100 ng/ml), 7, 8-DHF (500 nM), U0126 (Tocris biosciences Ellisvile, MO, USA, 10 μM), ActD (25 μM Sigma, St Louis, MO, USA).

3.3.6 RNA Preparation

Total RNA was prepared from frozen amygdala and hippocampal dissections in mice. Brains were extracted using rapid decapitation 2 hours after training. Amygdala and hippocampal tissue was rapidly dissected in ice-cold PBS and then frozen immediately on dry ice and stored at -80˚C until ready to use. Briefly, tissue samples were homogenized and centrifuged at 13,000g for 3 minutes. RNA was washed with 70% ETOH and purified using RNeasy columns (Qiagen). RNA amount and quality were determined using a nanodrop spectrophotometer.

3.3.7 Quantitative RT-PCR

140 micrograms of total RNA were reverse transcribed using the RT2-First Strand Kit (C-03, SA Biosciences). Quantitative PCR was performed using the Applied Biosystems 7500 Fast. Online detection of reaction products was carried out using the

SybrGreen Gene Assay with custom made primers for *homer1a*, *homer1c* and *GAPDH*. SybrGreen mastermix was obtained from SA biosciences, and manufacturer's instructions were followed. Calculated values are presented as mean +/- SEM to indicate accuracy of measurement. Homer1a and Homer1c values were normalized for measurements of GADPH. PCR conditions were 2 minutes at 50 ˚C, 10 minutes at 95 ˚C and 40 cycles with 15s 95 ˚C, 60 s 60 ˚C.

3.3.8 Primer Design

Primers were designed and confirmed by Primer blast. There sequence is as follows: Homer1a – FWD – 5'- GAAGTCGCAGGAGAAGATG-3'; Homer1a – REV – 5'- TGATTGCTGAATTGAATGTGTACC-3'; Homer 1c – FWD – 5'-ACACCCGATGTGACACAGAACT-3; Homer 1c – REV - 5'-TCAACCTCCCAGTGGTTGCT-3'; Primers for GAPDH were obtained from SA Biosciences.

3.3.9 Statistical Analysis

Statistically significant differences were determined by Student's t-test or by between subjects two-way ANOVA. The results were presented as means +/- s.e.m. For all mRNA data, fold changes relative to control were determined using the ΔΔCt method; a mean fold change value along with an s.e.m. value were determined; the ΔΔCt values from each data set were used in two-tailed paired t-tests (which were adjusted for multiple comparisons) to determine statistical significance ($*P < 0.05$). All values included in the figure legends represent mean +/- s.e.m.

3.4 Results

3.4.1 Pavlovian Fear conditioning results in an increase in *Homer1a* mRNA

Homer1a was dynamically regulated during consolidation of Pavlovian fear conditioning. Homer1a contains a unique stop site at the end of exon 5 that makes its sequence unique to the longer gene variants of homer such as *homer1c*. Primers for RTPCR were designed based on this sequence to differentiate between *homer1a* and *homer1c* expression (Figure 3.1b). For all of the experiments described amygdala and hippocampal tissue was extracted during consolidation of fear, 2 hours after training (Figure 3.2a). As illustrated in figure 3.2a, all animals were measured for baseline freezing to presentation of a tone in one context (Context A). One day later, animals were presented with 5 tone-shock pairings or 5 tones without any shock in a novel context (Context B). On the third day, animals were tested in Context B without any tones or shocks for 3 minutes as an assessment of contextual fear conditioning and then immediately placed into context A where freezing in response to 5 tone-alone trials is assessed. This paradigm demonstrated that with one training paradigm we are able to achieve both retention of contextual and cued fear conditioning (Figures 3.2b and c) ($p < 0.05$). In a separate cohort of animals quantitative PCR demonstrated an increase in *homer1a* mRNA in the hippocampus (Fig. 3.3a and b) ($p < 0.05$) and in the amygdala (Figures 3.3c and d) ($p < 0.05$) 2 hours after fear conditioning. RNA for *homer1c* (a longer gene variant of the Homer1 gene family) was not increased during Pavlovian fear conditioning in either brain region (Figures 3.3a and c) (hippocampus $p = 0.91$; amygdala $p = 0.91$). No changes in *homer1a* mRNA levels were seen in the striatum (Figure 3.3e) ($p = 0.79$).

3.4.2 TrkB agonist, 7,8-DHF, enhances consolidation Pavlovian Fear Conditioning

Animals were trained as described previously with five tone-shock pairings.

Immediately following training, animals were injected with 7,8-DHF and then returned to their home cage. 24 hours later, animals were tested in a novel context but were presented with 5 tones. Freezing in response to the tone was quantified. Relative to untrained controls, fear conditioned animals had an increased level of freezing in during the presentation of the tone. Fear conditioned animals that received 7,8-DHF had further enhanced freezing relative to vehicle treated fear-conditioned animals (Figure 3.4a). In a separate cohort, mice were given either 5 tone shock pairings or five tone-alone pairings and training was followed by an injection of either 7,8-DHF or vehicle. 2 hours later the amygdala and hippocampal tissue was dissected and analyzed for homer1a mRNA. Hippocampal (Figure 3.4b) tissue showed no interaction between 7,8-DHF treatment and fear conditioning in homer1a mRNA relative to controls ($F(1,8) = 0.20$, $n = 6$). While in amygdala tissue it appeared that 7,8-DHF was trending towards significantly decreasing *homer1a* expression after fear conditioning this interaction was not significant (Figure 3.4c) ($F(1,8) = 1.06$, $n = 6$).

3.4.3 BDNF causes an upregulation of homer 1a in primary amygdala and hippocampal cultures, which is transcription dependent, MEK dependent and ERK dependent.

To assess differential regulation of *homer1a* through TrkB signaling, primary hippocampal and amygdala cell cultures were used. Much like in fear conditioning, BDNF-induced plasticity increased *homer1a* mRNA levels in both hippocampal and amygdala cell culture (hippocampus $p < 0.05$, $n = 6$; amygdala $p < 0.05$, $n = 6$) but not *homer1c* levels (hippocampus $p = 0.68$, $n = 6$; amygdala $p = 0.06$, $n = 6$) (Figure 3.5a and e). The trkB-specific agonist (7, 8-DHF) upregulated *homer1a* in cell culture (Figure 3.5b

and f) (hippocampus $p < 0.05$, $n = 6$, amygdala $p < 0.05$, $n = 6$). Figures 3.5 c and d are representative pictures of immunostainting for CamKII in both hippocampal (c) and amygdala (g) primary neuronal cultures. Figure 3.5 d and h are representative pictures of immunostaining for parvalbumin in both hippocampal (d) and amgydala (h) primary neuronal cell cultures.

Blocking transcription with Actinomysin D (ActD) inhibited BDNF-induced upregulation of *homer1a* in both amygdala and hippocampus cell cultures (Fig. 3.6a and e) (a: $F(1,8) = 5.92$, $p < 0.05$, $n = 6$; e: $F(1,8)=9.45$, $p < 0.05$, $n = 6$). In addition MEK inhibition by U0126 blocked BDNF induced increases in *homer1a* in both amygdala and hippocampal cells (Fig. 3.6b and f) (b: $F(1,8)= 12.45$, $p < 0.01$, $n = 6$, f: $F(1,8) = 16.37$, $p < 0.01$, $n = 6$). We next utilized primary cell culture from floxed-ERK knockout mice, in which we transfected cells with a Cre Recombinase expressing lentivirus to delete the *ERK* gene. We found that genetically deleting ERK impaired BDNF induced upregulation of *homer1a* in both amygdala and hippocampal cells as well (Fig. 3.6c and g) (c: $F(1,8) = 11.27$, $p < 0.01$, $n = 6$, g: $F(1,8) = 11.27$, $p < 0.01$, $n = 6$) . Thus, BDNF appears to upregulate *homer1a* in a transcriptionally dependent manner, and through MEK and ERK signaling mechanisms. None of these manipulations had any effect on *homer1c* mRNA levels (table 3.1). Genetic deletion of ERK was demonstrated through QT-PCR in figures 3.6d ($p = 0.046$, $n = 6$) and h ($p = 0.039$, $n = 6$).

3.6 Discussion

In this chapter we demonstrate that the *homer1a* mRNA is increased during the consolidation of Pavlovian cued fear conditioning in the amygdala and hippocampus, as well as during BDNF-induced plasticity in amygdala and hippocampal primary cell

culture. As mentioned previously, BDNF has been shown to play a critical role in PTSD, fear conditioning and fear extinction in both the amygdala and hippocampus. We chose to use a primary cell culture model as a way to more directly address the molecular mechanisms of *homer1a* gene transcription and potentially tease apart any differences in hippocampal and amgydala neurons separate from their functional connectivity seen in the brain. While we did not demonstrate any significant differences in TrkB regulation of *homer1a* during Pavlovian fear conditioning or BDNF induced plasticity, in future chapters we discuss differential epigenetic regulation of *homer1a* that replicate both *in vivo* and *in vitro* suggesting a difference molecularly that is separate from the functional connectivity of the hippocampus versus the amygdala.

In this chapter we fail to rule out the possibility that other receptor-ligand systems play a role in *homer1a* transcription. There are several potential signaling pathways that could result in upregulation of *homer1a* during Pavlovian fear conditioning, including NMDA activation (Ango et al., 2000; Sato et al., 2001). However, we have demonstrated that BDNF induced plasticity in cell culture is one mechanism for Homer1a upregulation. Given the role of BDNF in the amygdala and in the hippocampus during consolidation of fear conditioning (Rattiner et al., 2004b; Rattiner et al., 2004a; Heldt et al., 2007; Musumeci et al., 2009), it is plausible that an increase in BDNF in the hippocampus and amygdala might result in an increase in homer1a signaling during the consolidation of Pavlovian fear conditioning.

We also show that TrkB agonists enhance both *homer1a in vitro*, and enhance long-term fear memories *in vivo*, although, 7,8-DHF did not further enhance homer1a expression. However, we did demonstrate that 7,8-DHF could induce homer1a

transcription in cell culture. It is possible that our training paradigm produces maximal levels of homer1a transcription and that any additional effects of 7,8-DHF are either due to prolonged levels of homer1a upregulation, or due to alternate mechanisms. It would be interesting to see if homer1a mRNA levels stayed elevated longer in 7,8-DHF treated animals compared to vehicle treated animals. However, we did not rule out the possibility that the effects of 7,8-DHF were due to other downstream effects of trkB activation.

There is substantial evidence suggesting that *homer1a* plays a role in consolidation of fear memories (Inoue et al., 2009; Tronson et al., 2010; Hashikawa et al., 2011). However, future *in vivo* studies inhibiting BDNF signaling would have to be done to determine whether BDNF is necessary for *homer1a* transcription during Pavlovian fear conditioning. Furthermore it would be interesting to examine the effect of trkB agonists in Homer1a knockout mice, to determine whether or not *homer1a* transcription is important for trkB induced enhancements in long term fear memories. Given the widespread effects of BDNF in the brain, it is likely that genetically deleting *homer1a* would not completely abolish BDNF and trkB agonist effects on fear conditioning. However even a small diminishment in this enhancement would be potentially interesting and argue for at least a partial role for homer1a in Pavlovian fear conditioning.

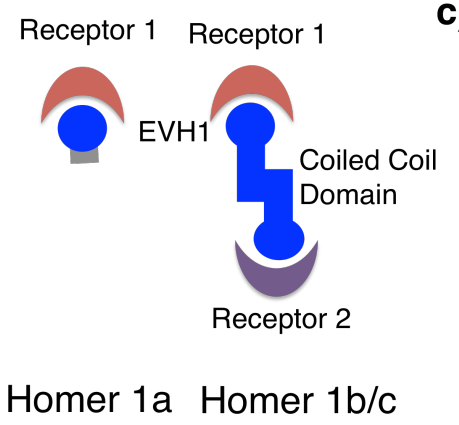
While we know that fear learning requires long term potentiation and synaptic plasticity, very little direct evidence links the increases in gene transcription with increases in LTP seen in fear conditioning. Homer1a, a known regulator of both Pavlovian fear conditioning and physiological plasticity, is epigenetically regulated after learning, and may provide a useful connection between gene expression and physiological synaptic plasticity. Furthermore, studying the role of *homer1a* transcription

and activity in the consolidation of fear may help better understand the connection between epigenetics, gene transcription, LTP and the consolidation of memory formation. In addition, it may provide insight into future drug targets for the enhancement of extinction learning as well as behavioral therapy for PTSD and other fear-related disorders.

3.7 Figures

Figure 3.1 The Homer1 gene family. a) Functional domains of *homer1a* and *1c*. The EVH1 binding domain recognizes proline rich regions of synaptic receptors and scaffolding proteins. The coiled coil domain recognizes other coiled-coil domain (CC) domains of other homer molecules. *Homer1a* lacks the CC domain but competitively binds with other receptors with its EVH1 binding domain. b) Schematic of the *homer1a* and *homer1c* gene variants. Both genes contain similar EVH1 binding domains. *Homer1c* however, contains an additional CC domain. *Homer1a* and *1c* contain different transitional domains as well, which were used to design RTPCR primers for quantifying mRNA expression levels. c) The promoter region of the Homer1 gene family. The promoter region contains several CRE binding sites, suggesting CREB mediation of gene expression. Primers for chromatin immunoprecipitation experiments were designed to recognize this region about 1kb upstream of the transcription start site.

a)



c)

AGCTCTCCTCAGACTGTCTCCGGCGCCTTGGCCTTTAAGGAGAAGCGAGAGAACCGG

GAGCAGGTGGGCCCGGAGCAGCTCGCTCAGCCCGCCTCCCCCAGCTCTTTCCCTAATT

CRE CRE

TAGCAGTACTTCCGGCCGCGT***GACATCAT***CCCCCGCACAAGCTGACGTGAGCGGAG***G***

CRE

GTGACGTATGTGCGGAGAGGAGCGCGCT***GACGTTG***CTATTTAAAGGTCCTTCTGCGG

GGAGGATGGAGACACAGCGCGAGCACCCGGCGTGCGGAGCGGACGAGGCCGTGGCGG

CCCAGAGCCAGCGCAGGAGCCGAGCGCACTGCCCGTCCGCCGCCGGCCGCTAGGGGA

GGGGAAGGAAAAAGGGACGGACCGACGGACCGGCCAGCTCATCCCCTCCGGTGTTGT

TCCTCAGCGCCCGGTCCGAGCCCGGGCCCCGCCTCCGCCGCGCCCCACCTCCTCCGCC

GGCCTGCGGCGGCGGCAGCGCTCCGCCCCTCGAGCCGGGAGGGCCGCGCTCTCCGCCC

Transcription Start Site

CGCCCCGCCCCGCCCCGCCCCCTCTCCTGCTCCGC***TGCCGTGCTCGTCTCA***CGCCGGC

GGCGCGGACCGGGAAGCGGCGCCCTCGGCTGCGCTCGGTTCGGAGCCCGCTCCGTGG

AGTCAGAGGCAGCGACGGGCGCCAGGCCGCCCGGGCCGACGGAGTCAGCCGCTGAGG

GGAGTGTAAGGAGGCAGCGGCAGCCCCGGGAATGTGAGGCGGCGCGGAGCGGGAGA

GACGAGCCGGGGTGGG

b)

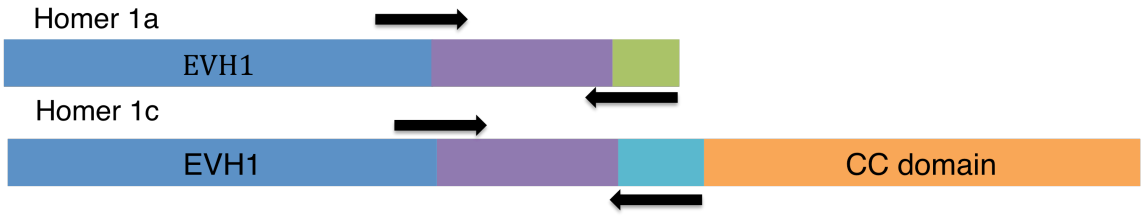


Figure 3.2 Pavlovian Cued and Contextual fear conditioning. a) Schematic diagram of behavioral experiments. On day 1, animals are presented with 5 tones and baseline freezing is recorded. On day 2, in a novel context, the 5 tones co-terminate with a mild foot shock. 2 hours after training, for the molecular experiments, hippocampal and amygdala tissues were dissected. For behavioral experiments, on day 3, animals are first placed into the day 2 context, and contextual freezing is measured. Then they are placed into the context from day 1 and presented with the tone. Cued freezing is then measured. b) Percent freezing during acquisition of fear conditioning. Freezing was measured during presentation of the tone immediately prior to foot shock. c) Animals that received tone-shock pairs had enhanced freezing in response to the final tone during acquisition ($p=0.0003$, $n = 10$); enhanced freezing when placed in the same context 24 hrs later ($p = 0.0005$, $n = 10$), and enhanced freezing when presented with the cue (tone) test in a novel context ($p = 0.02$, $n = 10$).

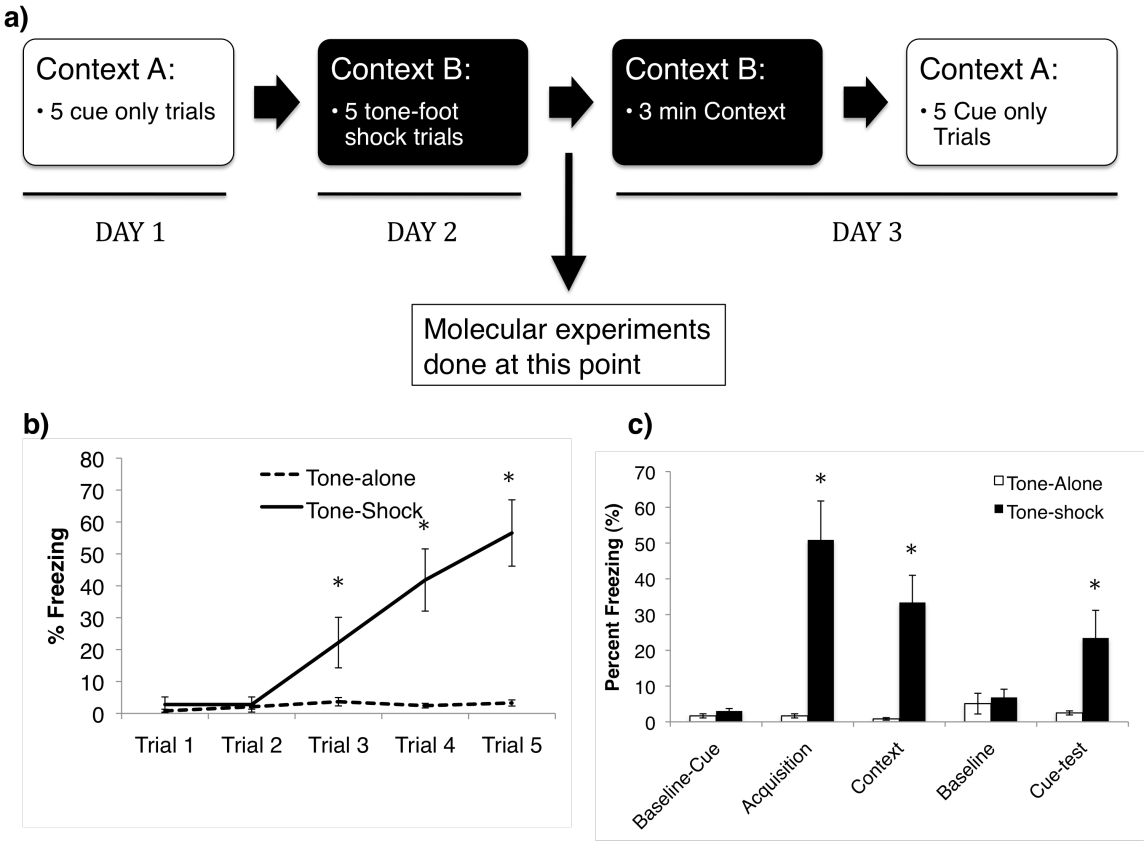

a)
Context A:
• 5 cue only trials
Context B:
• 5 tone-foot shock trials
Context B:
• 3 min Context
Context A:
• 5 Cue only Trials
DAY 1
DAY 2
DAY 3
Molecular experiments done at this point
b)
% Freezing
Tone-alone
Tone-Shock
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
c)
Percent Freezing (%)
Tone-Alone
Tone-shock
Baseline-Cue
Acquisition
Context
Baseline
Cue-test

Figure 3.3 Expressional analysis of *homer1a* and *homer1c* after Pavlovian fear conditioning in the hippocampus and amygdala. The mRNA levels of *homer1a* and *1c* in the amygdala and hippocampus were measured by QT-PCR in fear conditioned and non-fear conditioned mice. a) *Homer1a* mRNA ($p = 0.004$) but not *homer1c* ($p = 0.91$, $n = 10$) mRNA in the hippocampus was increased in the fear conditioned group 2 hours after training. b) Average QT-PCR spectra of *homer1a* and *GAPDH* levels in the hippocampus 2 hours after fear conditioning. c) *Homer1a* mRNA ($p = 0.007$, $n = 10$) but not *homer1c* mRNA ($p = 0.85$, $n = 10$) was upregulated in the amygdala 2 hours after Pavlovian fear conditioning. d) Average QT-PCR spectra of *homer1a* and *GAPDH* levels in the amygdala 2 hours after fear conditioning. e) Expressional analysis of Homer1a after Pavlovian fear conditioning in the striatum. No significant differences were found in striatal homer1a mRNA levels after fear conditioning ($p = 0.79$, $n = 10$).

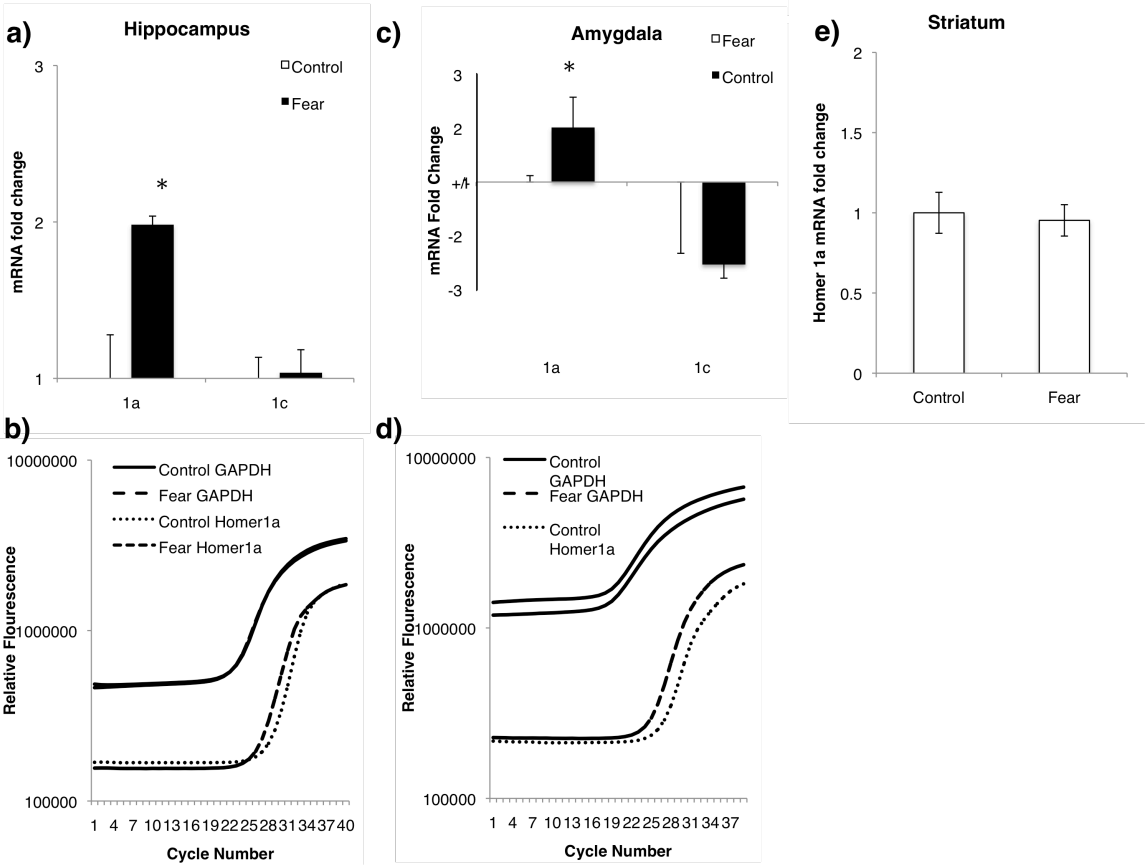
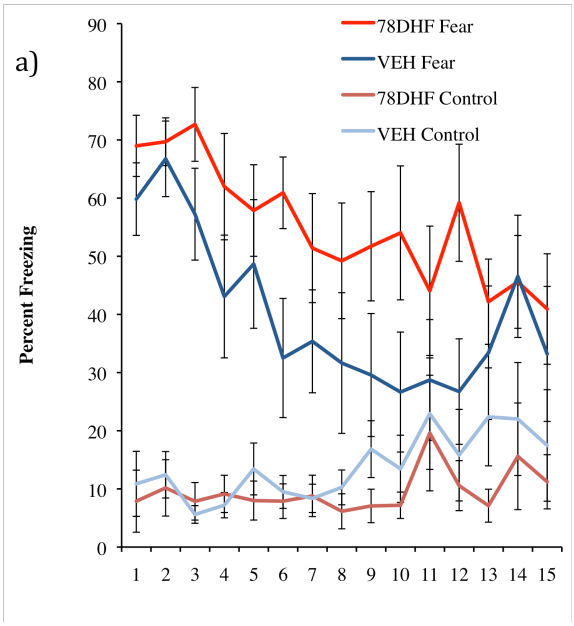
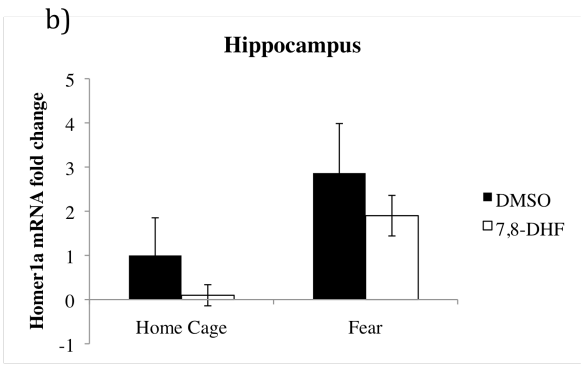

a)
Hippocampus
Control
Fear
mRNA fold change
3
2
1
*
1a
1c
c)
Amygdala
Fear
Control
mRNA Fold Change
3
2
+/-
-2
-3
*
1a
1c
e)
Striatum
Homer 1a mRNA fold change
2
1.5
1
0.5
0
Control
Fear
b)
10000000
1000000
100000
Relative Flourescence
Control GAPDH
Fear GAPDH
Control Homer1a
Fear Homer1a
1 4 7 10 13 16 19 22 25 28 31 34 37 40
Cycle Number
d)
10000000
1000000
100000
Relative Flourescence
Control GAPDH
Fear GAPDH
Control Homer1a
1 4 7 10 13 16 19 22 25 28 31 34 37
Cycle Number

Figure 3.4 TrkB agonist, 7,8-DHF enhances Pavlovian Fear Conditioning a)Prior to training, animals were injected either with vehicle (17% DMSO in 1x PBS) or 7,8-DHF. Animals were trained with 5 tone-shock pairings and were subsequently tested 24 hours later with 15 tone presentations. Animals that had received both 7,8 –DHF and fear conditioning showed enhanced freezing in response to the tone, indicating enhances long-term memory for the fear association. b,c) In a separate cohort, mice were given either 5 tone shock pairings or five tone-alone pairings and training was followed by an injection of either 7,8-DHF or vehicle. 2 hours later the amygdala and hippocampal tissue was dissected and analyzed for *homer1a* mRNA. Hippocampal (b) tissue showed any differences in *homer1a* relative to controls, where as amygdala tissue (c) showed decreases in *homer1a* mRNA with 7,8-DHF.


a)
78DHF Fear
VEH Fear
78DHF Control
VEH Control
Percent Freezing
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


b)
Hippocampus
Homer1a mRNA fold change
5
4
3
2
1
0
-1
Home Cage
Fear
DMSO
7,8-DHF

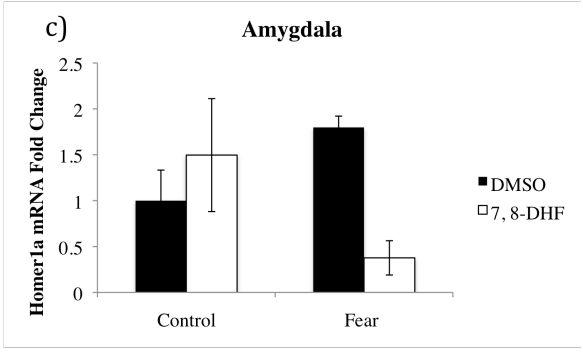

c)
Amygdala
Homer1a mRNA Fold Change
2.5
2
1.5
1
0.5
0
Control
Fear
DMSO
7, 8-DHF

Figure 3.5 *Homer1a* is upregulated through the BDNF-trkB signaling pathway in cultured amygdala and hippocampal neurons a,e) BDNF application to primary hippocampal (a) and amygdala (e) cells resulted in an increase in *homer1a* (a, $p = 0.0004$; e, $p < 0.05$, $n = 6$) but not *homer1c* (a, $p=0.68$, F, $p=0.06$, $n = 6$). b,f) TrkB-selective agonist (7,8-DHF) increased *homer1a* when applied to hippocampal (b, $p = 0.04$, $n = 6$) or amygdala (f, $p = 0.04$, $n = 6$) primary cells. c,g) Hippocampal (c) and amygdala (g) neurons expressed d,h) Hippocampal (d) and amygdala (h) neurons expressed Parvalbumin

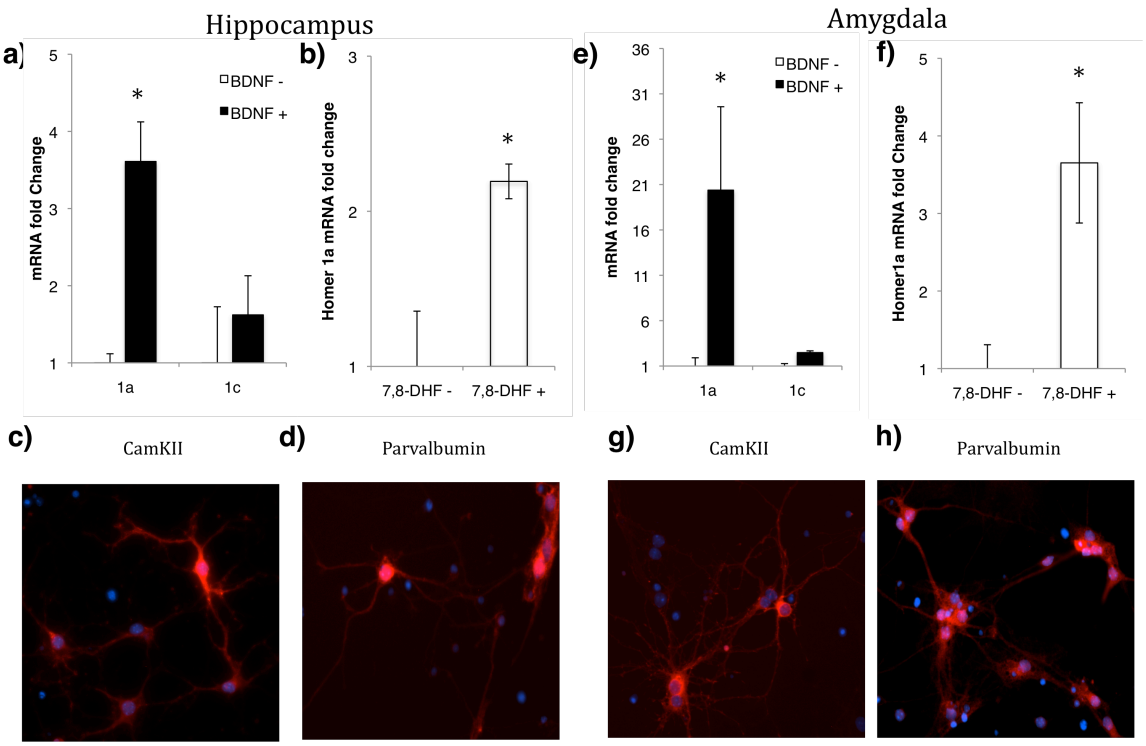

Hippocampus
Amygdala
a)
b)
e)
f)
BDNF -
BDNF +
mRNA fold Change
Homer 1a mRNA fold change
mRNA fold change
Homer1a mRNA fold Change
1a
1c
7,8-DHF -
7,8-DHF +
c)
CamKII
d)
Parvalbumin
g)
CamKII
h)
Parvalbumin

Figure 3.6. Homer1a upregulation by BDNF is transcription dependent, MEK dependent, and ERK dependent. a,e) BDNF-induced upregulation of *homer1a* was blocked by transcriptional inhibitor actinomysin D (ActD) in hippocampal (a: $F(1,8) = 5.92$, $p < 0.05$, $n = 6$) or amygdala (e: $F(1,8) = 9.45$, $p < 0.05$, $n = 6$) primary cells. b,f) BDNF-induced upregulation of *homer1a* was blocked by MEK inhibitor, U0126 in hippocampal (b: $F(1,8) = 12.45$, $p < 0.01$, $n = 6$) and amygdala (f: $F(1,8) = 16.37$, $p < 0.01$, $n = 6$) primary cell culture. c,g) BDNF-induced upregulation of *homer1a* was blocked by genetic deletion of ERK in hippocampal (c: $F(1,8) = 11.23$, $p < 0.05$, $n = 6$) and amygdala primary cell culture (g: $F(1,8) = 11.27$, $p < 0.01$, $n = 6$). d,h) Genetic knock down of ERK expression in cell culture. Hippocampal and amygdalar cell culture, in which ERK was genetically deleted, demonstrated significantly reduced ERK expression in hippocampal ($p = 0.046$, $n = 6$) and amygdala ($p = 0.039$, $n = 6$).

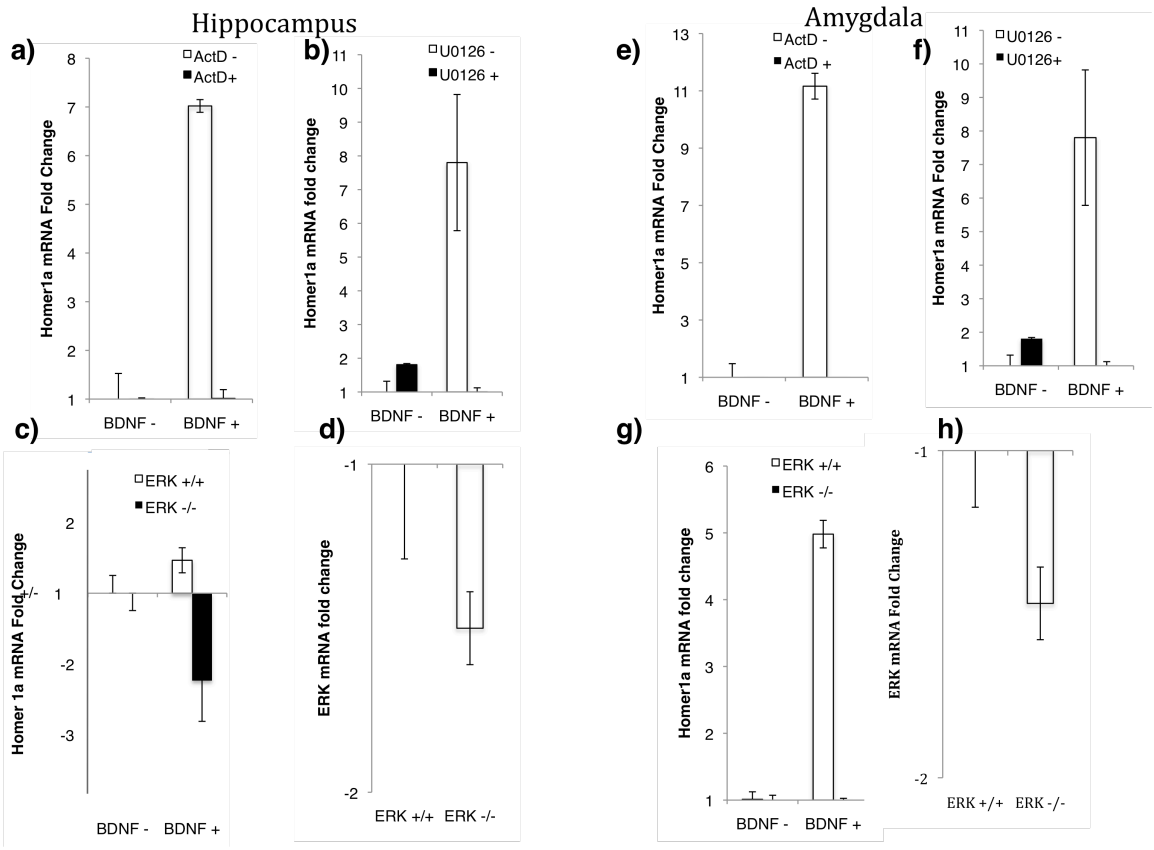

Hippocampus
Amygdala
a)
ActD -
ActD+
Homer1a mRNA Fold Change
BDNF -
BDNF +
b)
U0126 -
U0126 +
Homer1a mRNA fold change
BDNF -
BDNF +
e)
ActD -
ActD +
Homer1a mRNA Fold Change
BDNF -
BDNF +
f)
U0126 -
U0126+
Homer1a mRNA Fold change
BDNF -
BDNF +
c)
ERK +/+
ERK -/-
Homer 1a mRNA Fold Change
+/-
BDNF -
BDNF +
d)
ERK mRNA fold change
ERK +/+
ERK -/-
g)
ERK +/+
ERK -/-
Homer1a mRNA fold change
BDNF -
BDNF +
h)
ERK mRNA Fold Change
ERK +/+
ERK -/-

Table 3.1: *Homer1c* values during BDNF induced plasticity

Brain Region	Experiment	Treatment	Delta CT	Statistics
Hippocampus	78DHF	78DHF Control	10.89 11.21	P = 0.70
	Actinomysin D	BDNF BDNF+ACTD ACTD Control	12.44 13.43 13.26 12.93	BDNF: F(1,8) = 1.09 ActD: F(1,8) = .083 BDNF x ACTD: F(1,8) = 0.22
	U0126	BDNF BDNF+U0126 U0126 Control	11.99 15.87 15.94 15.72	BDNF: F(1,8) = 0.13 U0126: F(1,8) = 0.065 BDNF x U0126:F(1,8) = 0.0031
	ERK	BDNF BDNF + ERK -/- ERK -/- WT	-5.38 -1.91 -1.52 7.87	BDNF:F(1,8) = 11.13 ERK:F (1,8) = 16.94 BDNFxERK: F (1,8) = 0.087
Amygdala	78DHF	78DHF Control	11.08 13.82	P = 0.0037
	Actinomysin D	BDNF BDNF+ACTD ACTD Control	11.26 12.16 13.67 11.99	BDNF:F(1,8) = 0.9988 ActD: F(1,8) = 2.83 BDNFx ActD: F(1,8) = 0.017
	U0126	BDNF BDNF+U0126 U0126 Control	12.79 14.89 16.94 15.60	BDNF: F(1,8) = 0.246 U0126: F(1,8) = 0.127 BDNF x U0126: F(1,8) = 0.093
	ERK	BDNF BDNF + ERK -/- ERK -/- WT	-4.35 -1.54 -3.81 -1.92	BDNF: F (1,8) = 0.018 ERK: F (1,8) = 0.43 BDNF x ERK: F (1,8) = 0.096

3.8 References

Ango F, Pin JP, Tu JC, Xiao B, Worley PF, Bockaert J, Fagni L (2000) Dendritic and axonal targeting of type 5 metabotropic glutamate receptor is regulated by homer1 proteins and neuronal excitation. J Neurosci 20:8710-8716.

Bertaso F, Roussignol G, Worley P, Bockaert J, Fagni L, Ango F (2010) Homer1a-dependent crosstalk between NMDA and metabotropic glutamate receptors in mouse neurons. PLoS One 5:e9755.

Brewer GJ (1997) Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods 71:143-155.

Foa L, Gasperini R (2009) Developmental roles for Homer: more than just a pretty scaffold. J Neurochem 108:1-10.

Hall J, Thomas KL, Everitt BJ (2001) Fear memory retrieval induces CREB phosphorylation and Fos expression within the amygdala. Eur J Neurosci 13:1453-1458.

Hashikawa K, Matsuki N, Nomura H (2011) Preferential Arc transcription at rest in the active ensemble during associative learning. Neurobiol Learn Mem 95:498-504.

Heldt SA, Stanek L, Chhatwal JP, Ressler KJ (2007) Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. Mol Psychiatry 12:656-670.

Inoue N, Nakao H, Migishima R, Hino T, Matsui M, Hayashi F, Nakao K, Manabe T, Aiba A, Inokuchi K (2009) Requirement of the immediate early gene vesl-1S/homer-1a for fear memory formation. Mol Brain 2:7.

Lonergan ME, Gafford GM, Jarome TJ, Helmstetter FJ (2010) Time-dependent expression of Arc and zif268 after acquisition of fear conditioning. Neural Plast 2010:139891.

Musumeci G, Sciarretta C, Rodriguez-Moreno A, Al Banchaabouchi M, Negrete-Diaz V, Costanzi M, Berno V, Egorov AV, von Bohlen Und Halbach O, Cestari V, Delgado-Garcia JM, Minichiello L (2009) TrkB modulates fear learning and amygdalar synaptic plasticity by specific docking sites. J Neurosci 29:10131-10143.

Ou LC, Gean PW (2007) Transcriptional regulation of brain-derived neurotrophic factor in the amygdala during consolidation of fear memory. Mol Pharmacol 72:350-358.

Ploski JE, Park KW, Ping J, Monsey MS, Schafe GE (2010) Identification of plasticity-associated genes regulated by Pavlovian fear conditioning in the lateral amygdala. J Neurochem 112:636-650.

Radwanska K, Nikolaev E, Knapska E, Kaczmarek L (2002) Differential response of two subdivisions of lateral amygdala to aversive conditioning as revealed by c-Fos and P-ERK mapping. Neuroreport 13:2241-2246.

Rattiner LM, Davis M, Ressler KJ (2004a) Differential regulation of brain-derived neurotrophic factor transcripts during the consolidation of fear learning. Learn Mem 11:727-731.

Rattiner LM, Davis M, French CT, Ressler KJ (2004b) Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. J Neurosci 24:4796-4806.

Ressler KJ, Paschall G, Zhou XL, Davis M (2002) Regulation of synaptic plasticity genes during consolidation of fear conditioning. J Neurosci 22:7892-7902.

Samuels IS, Karlo JC, Faruzzi AN, Pickering K, Herrup K, Sweatt JD, Saitta SC, Landreth GE (2008) Deletion of ERK2 mitogen-activated protein kinase identifies its key roles in cortical neurogenesis and cognitive function. J Neurosci 28:6983-6995.

Sato M, Suzuki K, Nakanishi S (2001) NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. J Neurosci 21:3797-3805.

Shiraishi-Yamaguchi Y, Furuichi T (2007) The Homer family proteins. Genome Biol 8:206.

Strekalova T, Zorner B, Zacher C, Sadovska G, Herdegen T, Gass P (2003) Memory retrieval after contextual fear conditioning induces c-Fos and JunB expression in CA1 hippocampus. Genes Brain Behav 2:3-10.

Tischmeyer W, Grimm R (1999) Activation of immediate early genes and memory formation. Cell Mol Life Sci 55:564-574.

Tronson NC, Guzman YF, Guedea AL, Huh KH, Gao C, Schwarz MK, Radulovic J (2010) Metabotropic glutamate receptor 5/Homer interactions underlie stress effects on fear. Biol Psychiatry 68:1007-1015.

von Hertzen LS, Giese KP (2005) Memory reconsolidation engages only a subset of immediate-early genes induced during consolidation. J Neurosci 25:1935-1942.

Chapter 4

Epigentetic Regulation of Homer1a during Pavlovian fear conditioning and BDNF induced plasticity

4.1 Introduction

In the previous chapters we have described the importance of gene expression in the consolidation of fear, and that one such gene, *homer1a,* is dynamically regulated during Pavlovian fear conditioning and during BDNF induced synaptic plasticity. In the neuron, DNA is packaged densely into the nucleus in the form of chromatin. Each strand of DNA is wrapped around eight protein subunits called histones. Together histones and DNA form nucleosomes, which are further condensed into chromatin in order to fit into the nucleus. In order for gene expression to occur specific regions of the genome must be made accessible to transcriptional machinery. This is done by posttranslational modifications of the histone tails, which regulate to accessibility of transcription factors and polymerases to the surrounding DNA sequence. Modifications include acetylation, methylation and phosphorylation of histone tails, which can in turn affect the chromatin structure and promoter region accessibility. In general, acetylation of histone tails, relaxes chromatin structure enhancing gene transcription, and in general, methylation of histone tails, condenses chromatin structure repressing gene transcription. Phosphorylation has also been shown to enhance gene transcription but is the least studied of the three major modifications (Cheung and Lau, 2005; Gelato and Fischle, 2008; Bannister and Kouzarides, 2011).

These histone modifications have been shown to regulate fear conditioning (Chwang et al., 2006; Lattal et al., 2007; Miller et al., 2008; Sweatt, 2009; Gupta et al., 2010; Peleg et al., 2010). Global levels of histone methylation, acetylation and phosphorylation are increased after contextual fear conditioning in the hippocampus (Levenson et al., 2004; Chwang et al., 2006). Drugs that increase histone acetylation such as histone deacetylase (HDAC) inhibitors enhance long term memory for contextual fear conditioning when given systemically or intra-hippocampally (Levenson et al., 2004). HDAC inhibitors also enhance cued fear conditioning when infused into the amygdala(Monsey et al., 2011). All together this suggests that epigenetic mechanisms are linked extensively to associative fear conditioning and gene transcription

(Levenson et al., 2004).

While it is likely that histone acetylation and methylation during Pavlovian fear conditioning is specific to a subset of genes involved with learning and memory, a majority of these studies, examine global acetylation and methylation levels. During contextual fear conditioning, for example, we know that there is an increase in H3K4 and H3K9 methylation as well as an increase in global H3 and H4 acetylation in the hippocampus. H3K4 methylation and H3/H4 acetylation are transcriptional enhancers whereas H3K9 methylation modifications are typically thought of as transcriptional repressors (Cheung and Lau, 2005; Gelato and Fischle, 2008; Bannister and Kouzarides, 2011). Therefore it is likely that these modifications are not occurring globally but at specific and distinct region of the genome. However, it is unclear which regions of the genome are specifically being upregulated.

There is preliminary evidence that there is specific acetylation of H3 tails around the P4 promoter region of the BDNF gene (Gupta et al., 2010) as well as around the promoter region of zif268 (Gupta et al., 2010) after fear conditioning. However, this data is relatively sparse considering the vast amounts of genes that are upregulated during synaptic plasticity and fear conditioning, as well as the large number of potential posttranslational histone modifications that are known to occur in the cell (Cheung and Lau, 2005; Gelato and Fischle, 2008; Bannister and Kouzarides, 2011).

While most of the current behavioral epigenetic literature tends to focus on global histone modifications and alterations in gene transcription, here we demonstrate how such mechanisms regulate a specific gene, *homer1a*, which may have a functional role in regulating synapse structural organization during the synaptic plasticity that mediates the consolidation of fear memory. This study highlights specific chromatin regulation of a specific gene in the amygdala and hippocampus with Pavlovian fear conditioning and within amygdala and hippocampal cell culture models of BDNF-induced plasticity.

4.2 Methods

4.2.1 Animals

All experiments were performed on adult (6-8 weeks old) wild-type strain C57BL/6J male mice from Jackson Laboratory (Bar Harbor, ME.). All procedures were approved by the Institutional Animal Care and Use Committee of Emory University and were in compliance with National Institutes of Health guidelines. Separate cohorts of animals were used for each experiment.

4.2.2 Behavior

Fear conditioning was conducted in nonrestrictive acrylic cylinders (SR-LAB startle response system, San Diego Instruments) located in a ventilated, sound-attenuated chamber. The foot shock (unconditioned stimulus) was delivered through a stainless steel grid floor. Shock reactivity was defined as the peak activity (measured with a piezoelectric accelerometer) that occurred during the 200 milliseconds after the onset of the unconditioned stimulus. The tone-conditioned stimulus was generated by a Tektronix function generator audio oscillator and delivered through a high frequency speaker. One day prior to training, mice were preexposed to the tone through a '5 tone-alone' presentation program to both habituate them to handling and to the tone, but to also get baseline fear responses to the tone presentation. Preexposure was done in a separate context. During cued fear conditioning, mice received five trials of a conditioned stimulus tone (30 seconds, 6 kHz, 70 dB) coterminating with an unconditioned stimulus foot shock (500 msec, 1 mA) with a variable inter-trial-interval between 60 and 180 seconds. The expression of fear was assessed 24 hours after fear conditioning. It consisted of 3 minutes in the same context in which the training occurred (contextual memory) with no stimulus and 5 conditioned stimulus tone presentations of 30 second each with a 1.5-minute inter-trial interval in a different context (see figure 1 for schematic). Stimulus presentation and data acquisition were controlled and digitized by, and stored in, an interfacing desktop computer using SR-LAB and analyzed with the Freeze View software program (Coulbourn Instruments, Whitehall, Pa.).

4.2.3 Cell Culture:

Primary cultures of postnatal hippocampal neurons were described previously (Brewer, 1997) with modifications, C57BL/6J mice (21 days postnatal) were decapitated and the hippocampus and amygdala were removed and immersed in ice-cold dissection buffer consisting of Hibernate-A medium (BrainBits, Springfield, IL, USA), B27 supplement and gentamycin (Invitrogen) (12g/ml) for the preparation of separate hippocampal and amygdala neuronal cell cultures. The hippocampus and amygdala tissues were sliced and then enzymatically digested with papain (Worthington, Lakewood, NJ, USA) in Hibernate-A medium at 32°C for 30 minutes. Cells were dissociated by triturating with Pasteur pipets fired on the tips to narrow openings. Neurons were purified in a density gradient media including Hibernate-A and OptiPrep (Sigma, St. Louis, MO, USA) by centrifugation. The density gradient media consisted of four layers. The first was 1 mL dissection buffer containing 35% OptiPrep; the second 1 mL dissection buffer contained 25% Optiprep and the third 1 ml dissection buffer contained 20% OptiPrep and the fourth 1 mL dissection buffer contained 15% OptiPrep. They were added on the top of each other carefully, resulting in clear layer separation. Then cells were added on the top of the density gradient media. After centrifugation, the densest layer with a cream color, located at the middle of the tube, could be seen. This layer of neurons was taken out by using a sterile transfer pipette and put into a new tube. After washing with dissection buffer, neuronal cells were plated onto Poly-D-Lysine (Sigma) coated plates or glass coverslips at the density of 2.5 x 10^5 cells/cm^2 in culture media consisting of Neurobasal A medium (Invitrogen) with 2% B27 supplement, 2 mM glutamax and gentamycin (5 g/ml). Thereafter, the cultures were kept in a humidified incubator at 37˚C and 5% CO_2 and media were changed every 5 days until used for experiments. After 2-3 weeks in vitro, the cells were used for the experiments reported in the present study.

4.2.4 Drugs:

Recombinant human BDNF was purchased from Cell Sciences (Canton, MA, USA) and reconstituted in sterile PBS as 100 mg/ml stock. The aliquots of stock were stored at -30ºC and

final drugs and concentrations for cell culture experiments were as following: BDNF (100 ng/ml), 7, 8-DHF (500 nM), U0126 (Tocris biosciences Ellisvile, MO, USA, 10 uM), ActD (25 uM Sigma, St Louis, MO, USA). Mice received intraperitoneal injections of 1.2 g/kg sodium butyrate (NaB, Sigma Aldrich, B5887) dissolved in distilled water or an equal volume of distilled water alone (vehicle). This dose has been shown previously to enhance contextual fear memories (Levenson et al., 2004). The injections occurred immediately after training. For in vitro studies a concentration of 10 uM dissolved in distilled water was used.

4.2.5 Chromatin Immunoprecipitation

Tissue samples were treated using an EpiQuik tissue ChIP kit (Epigentek Group Inc. Brooklyn, NY). Brains were extracted using rapid decapitation 2 hours after training. Amygdala and hippocampal tissue was rapidly dissected in ice-cold PBS and then frozen immediately on dry ice and stored at -80˚C until ready to use. Cells/tissues were harvested and mixed with formaldehyde at a final concentration of 1.0% for 10 min at 37°C to cross-link protein to DNA. Cells/tissue then were suspended in 0.2 mL of SDS lysis buffer and allowed to settle on ice for 10 minutes. DNA cross-linked with protein was then sonicated into fragments of 200-1000 bp. One-tenth of the sample was set aside as an input control, and the rest was then immunoprecipitated 1.5 h at room temperature with 5 g of primary antibody in the ChIP kit strip wells. As a control samples were immunoprecipitated with 5g nonimmune rabbit IgG. After immunoprecipitaiton, the DNA-protein complex was eluted and the proteins were digested with DNA release buffer and proteinase K. DNA was dissociated at 65ºC for 1.5 hours under reverse buffer. The DNA, associated with antibody of interest (pan-H3-acetylated, pan-H4 acetylated, H3K9 dimethylation, and H3K27 dimethylation) was extracted with binding buffer, precipitated with 70% and 90% ethanol and finally elutes DNA by elution buffer. Quantitative real-time PCR was performed with primers specific to the Homer1 promoter and for the GAPDH promoter regions.

4.2.6 RNA Preparation

Total RNA was prepared from frozen amygdala and hippocampal dissections in mice. Brains were extracted using rapid decapitation 2 hours after training. Amygdala and hippocampal tissue was rapidly dissected in ice-cold PBS and then frozen immediately on dry ice and stored at -80˚C until ready to use. Briefly, tissue samples were homogenized and centrifuged at 13,000g for 3 minutes. RNA was washed with 70% ETOH and purified using RNeasy columns (Qiagen). RNA amount and quality were determined using a nanodrop spectrophotometer.

4.2.7 Quantitative RT-PCR

140 micrograms of total RNA were reverse transcribed using the RT2-First Strand Kit (C-03, SA Biosciences). Quantitative PCR was performed using the Applied Biosystems 7500 Fast. Online detection of reaction products was carried out using the SybrGreen Gene Assay with custome made primers for *homer1a*, *homer1c* and *GAPDH*. SybrGreen mastermix was obtained from SA biosciences, and manufacturer's instructions were followed. Calculated values are presented as mean +/- SEM to indicate accuracy of measurement. *Homer1a* and *homer1c* values were normalized for measurements of *GADPH*. PCR conditions were 2 minutes at 50ºC, 10 minutes at 95ºC and 40 cycles with 15s 95 ºC, 60 s 60 ºC.

4.2.8 Primer Design

Primers were designed and confirmed by Primer blast. There sequence is as follows: Homer1a – FWD – 5'- GAAGTCGCAGGAGAAGATG-3'; Homer1a – REV – 5'-TGATTGCTGAATTGAATGTGTACC-3'; Homer 1c – FWD – 5'-ACACCCGATGTGACACAGAACT-3; Homer 1c – REV - 5'-TCAACCTCCCAGTGGTTGCT-3'; Homer1 Promoter FWD – 5'- GGTGACGTATGTGCGGAGAGGA-3'; Homer1 Promoter – REV – 5'- GGTCCGTCGGTCCGTCCCTTT-3'; Primers for GAPDH and GAPHD promoter region were obtained from SA Biosciences. Referece to chapter 3, figure 3.1 for location of primers on the promoter region.

4.2.9 Statistical Analysis

Statistically significant differences were determined by Student's t-test or by between subjects

two-way ANOVA. The results were presented as means **+/-** s.e.m. For all ChIP and mRNA data, fold changes relative to control were determined using the ΔΔCt method; a mean fold change value along with an s.e.m. value were determined; the ΔΔCt values from each data set were used in two-tailed paired t-tests (which were adjusted for multiple comparisons) to determine statistical significance (* = $P < 0.05$). All values included in the figure legends represent mean +/- s.e.m. The RTPCR ChIP data was analyzed identically to the mRNA data using the ΔΔCt method, except that ChIP data were normalized to 'input' rather than GAPHD.

4.3 Results

4.3.1 BDNF application onto amygdala cells results in histone modifications along the Homer1 promoter.

In order to determine the epigenetic role of BDNF signaling on homer1a expression we examined histone modifications around the *homer1*a promoter region after BDNF induced plasticity. Chromatin immunoprecipitation (ChIP) assays were performed to measure the levels of several histone modifications around the Homer1 promoter after BDNF-induced plasticity. Levels of promoter enrichment were quantified by QT-PCR. We found that BDNF application had distinct effects in hippocampal and amygdala primary cell culture. In the hippocampal cell cultures, there was a significant increase in H3 acetylation ($p < 0.05$, $n = 6$) following BDNF application, but no changes were apparent in H4 acetylation, H3K9 methylation or H3K27 methylation (Figure 4.1a). In amygdala cell cultures, however, there appears to be a decrease in H3K9 methylation ($p < 0.05$, $n = 6$) following BDNF application, but no changes in H3 acetylation, H4 acetylation or H3K27 methylation (Figure 4.1c). Significant changes in acetylation or methylation were not detected at the GAPDH promoter region.

4.3.2 Pavlovian fear conditioning induces epigenetic modifications of histones along the Homer1 promoter

We next examined the effect of Pavlovian fear conditioning on epigenetic modifications of the Homer1 promoter region. In the hippocampus there was a significant increase in H3

acetylation ($p < 0.05$, $n = 10$), but no difference in H4 acetylation, H3K9 methylation or H3K27 methylation (Figure 4.1b). In the amygdala, however, there was a significant decrease in H3K9 methylation ($p < 0.05$, $n = 10$), but no changes in H3 acetylation, H4 acetylation, or H3K27 methylation (Figure 4.1d). Significant changes in acetylation or methylation were not detected at the GAPDH promoter region. Notably, these *in vivo* results parallel the histone modification-specific findings seen in amygdala and hippocampal primary cell culture.

4.3.4 HDAC inhibition enhances fear conditioning, Homer1a expression, and modifications of the Homer1 promoter

HDAC inhibitors have been shown to enhance contextual fear conditioning (Levenson et al., 2004). In this experiment, we examined the effect of the HDAC inhibitor, sodium butyrate (NaB) on fear conditioning. We showed that IP administration of NaB can induce increases in contextual fear memories ($p < 0.05$, $n = 10$) but did not appear to cause an increase in cued fear conditioning (Figure 4.2a). NaB also appeared to enhance hippocampal *homer1a* mRNA expression (Figure 4.2b; $F(1,16) = 5.01$, $p < 0.05$, $n = 10$), but seemed to reverse the mRNA increase in amygdala tissue (Figure 4.2c, $F(1,16) = 5.45$, $p < 0.05$, $n = 10$). Sodium butyrate enhanced H3 acetylation in hippocampal tissue (Figure 4.2d, $F(1,16) = 9.54$, $p < 0.01$, $n = 10$) but reversed fear conditioned induced decreases in H3K9 methylation in amygdala tissue (Figure 4.2.e, $F(1,16) = 4.58$, $p < 0.05$, $n = 10$).

4.4 Discussion

In this chapter we demonstrate that Pavlovian fear conditioning results in an increase in H3 acetylation around the Homer1 promoter in the hippocampus, and a decrease in H3K9 methylation around the Homer1 promoter in the amygdala. However, we saw no differences in H4 acetylation or H3K27 methylation. Given that there is no existing evidence for histone posttranslational modifications regulating homer1a and very limited data for specific modifications regulating the expression of other genes during Pavlovian fear conditioning (Fuchikami et al., 2010; Gupta et al., 2010) we began this work using a representative sample of

well characterized histone-specific antibodies. There is evidence for H3K9 global methylation and H3 and H4 global acetylation during Pavlovian fear conditioning (Gupta et al., 2010). In addition there is data looking at specific H3 acetylation around the promoter region of BDNF (Fuchikami et al., 2010; Gupta et al., 2010; Takei et al., 2011). For future studies, it may be informative to look at a wider array of posttranslational modifications involved in *homer1a* transcription during Pavlovian fear conditioning. However, with our very limited selection, we were able to find distinct epigenetic regulation of *homer1a* in hippocampal and amygdala tissue during Pavlovian fear conditioning and cells during BDNF induced plasticity.

Interestingly, during BDNF induced plasticity, we also demonstrate an increase in H3 acetylation around the Homer1 promoter in hippocampal primary cell culture and a decrease in H3K9 methylation in amygdala primary cell culture. Despite the fact that homer1a mRNA was upregulated in both the hippocampus and amygdala, histone modifications around the Homer1 promoter were distinct between the two brain regions. The hippocampus primarily exhibited increases in histone H3 acetylation, which is associated with enhanced gene transcription, within the Homer1 promoter region. The amygdala, however, primarily exhibited decreases in histone H3K9 methylation, a repressive marker of transcription, in the Homer1 promoter region. Notably, although the specific histone regulation was different in these two brain regions, and in primary cell culture from these regions, both histone tail modifications would result in enhanced transcription. These differences were seen in vivo after fear conditioning and in vitro after BDNF – induced plasticity suggesting that these differences are not due to the unique functional connectivity of the hippocampus and amygdala but due to intrinsic molecular properties of the neurons themselves.

Moreover, histone deacetylase (HDAC) inhibition with sodium butyrate (NaB) had differential effects on hippocampal and amygdala tissues. As expected, NaB enhanced hippocampal H3 acetylation around the Homer1 promoter in fear-conditioned mice but not control mice. NaB did not induce global increases in acetylation in that the GAPDH promoter

showed no enhancement in H3 or H4 acetylation, nor did NaB induce an increase in histone H4 acetylation around the Homer1 promoter in the hippocampus. This result is straightforward in that NaB prevents the removal of acetyl groups from histone tails. In order for there to be a resulting increase in acetylation there would already have to be an initial addition of acetyl groups, which only would occur around already activated genes. If histone H3 but not H4 is increasingly acetylated around the Homer1 promoter, then NaB should only enhance H3 acetylation. This may also explain why NaB did not enhance acetylation of H3/H4 tails around the Homer1 promoter in amygdala tissues. NaB did however reduce H3K9 methylation around the Homer1 promoter in control mice and reverse the decrease in H3K9 methylation seen in fear conditioned mice. While it has been demonstrated that HDAC inhibition can decrease H3K9 methylation (Gupta et al., 2010), the mechanism is less clear and the mechanism underlying NaB effects on H3K9 methylation in the amygdala is not so obvious.

Dynamic regulation of *homer1a* directly modifies mGluR1/5 and NMDA interactions during synaptic plasticity (Bertaso et al., 2010). Type 1 mGluRs and NMDA receptors are physically linked through Homer1 proteins and Shank scaffolding protein interactions. Type I mGLUR agonists inhibit EPSC potentials generated by NMDA receptors. Overexpression of homer1a disrupts the physical link between receptors, allowing agonist independent inhibition of NMDA current by mGluR agonists (Bertaso et al., 2010). Homer1a upregulation also increases network activity and evokes agonist-independent signaling of group I mGluRs, which in turn scales down expression of synaptic AMPA receptors (Hu et al., 2010). Overexpression of Homer1a in the hippocampus prior to contextual fear conditioning both enhances contextual memory and disrupts Homer1b/c binding with mGluR (Tronson et al., 2010), presumably disrupting the physical and functional link between type 1 mGluR and NMDA (Tronson et al., 2010). Understanding the mechanisms by which *homer1a* is upregulated could directly tie epigenetic mechanisms of gene transcription to physiological plasticity seen with Pavlovian fear conditioning.

Understanding the molecular mechanisms of Pavlovian fear conditioning will give us an understanding of the mechanism and potential treatments for PTSD and other fear-related disorders. Histone modifying drugs have been shown to enhance fear conditioning as well as fear extinction (Kaplan and Moore 2011). Our data suggest a distinct epigenetic signature for Homer1a gene expression in hippocampal and amygdala cells/tissues separate from the two regions' functional connectivity. These differences may explain why HDAC inhibitors such as sodium butyrate only enhance hippocampal dependent contextual memories and mainly enhance hippocampal histone modifications.

While we know that fear learning requires long term potentiation and synaptic plasticity, very little direct evidence links the increases in gene transcription with increases in LTP seen in fear conditioning. Homer1a, a know regulator of both Pavlovian fear conditioning and physiological plasticity, is epigenetically regulated after learning, and may provide a useful connection between gene expression and physiological synaptic plasticity. Furthermore, studying the role of *homer1a* transcription and activity in the consolidation of fear may help better understand the connection between epigenetics, gene transcription, LTP and the consolidation of memory formation. In addition, it may provide insight into future drug targets for the enhancement of extinction learning as well as behavioral therapy for PTSD and other fear-related disorders.

4.5 Figures

Figure 4.1 BDNF-induced and fear conditioning-induced histone modifications around the Homer1 promoter. Chromatin immunoprecipitation (ChIP) assays were performed to measure the levels of several histone modifications around the Homer1 promoter after fear conditioning and BDNF-induced plasticity. Levels of promoter enrichment were quantified by QT-PCR. a) Histone H3 acetylation, an enhancive marker of transcription was strongly increased at the Homer1 promoter after BDNF induced plasticity in hippocampal cells ($p = 0.0046$, $n = 6$) b) Histone H3 acetylation was also increased at the Homer1 promoter in the hippocampus after fear conditioning ($p = 0.047$, $n = 10$). Histone H4 acetylation, H3K9 methylation and H3K27 methylation were not altered after BDNF induced plasticity or fear conditioning. c) Histone H3K9 methylation, a repressive marker of transcription was strongly decreased after BDNF induced plasticity at the Homer1 promoter in amygdala cells ($p = 0.035$, $n = 6$). d) Histone H3K9 methylation was also decreased after fear conditioning in the amygdala. Significant changes in acetylation or methylation were not detected at the GAPDH promoter region (data not shown).

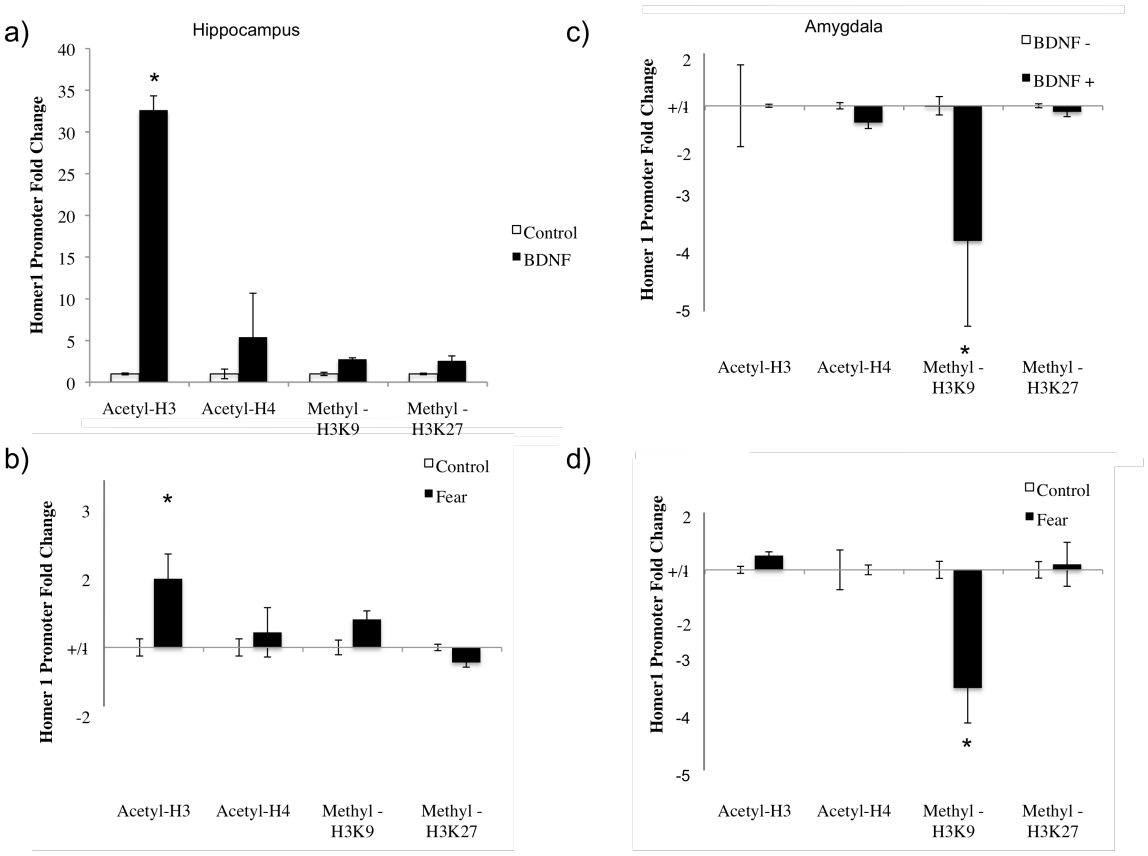
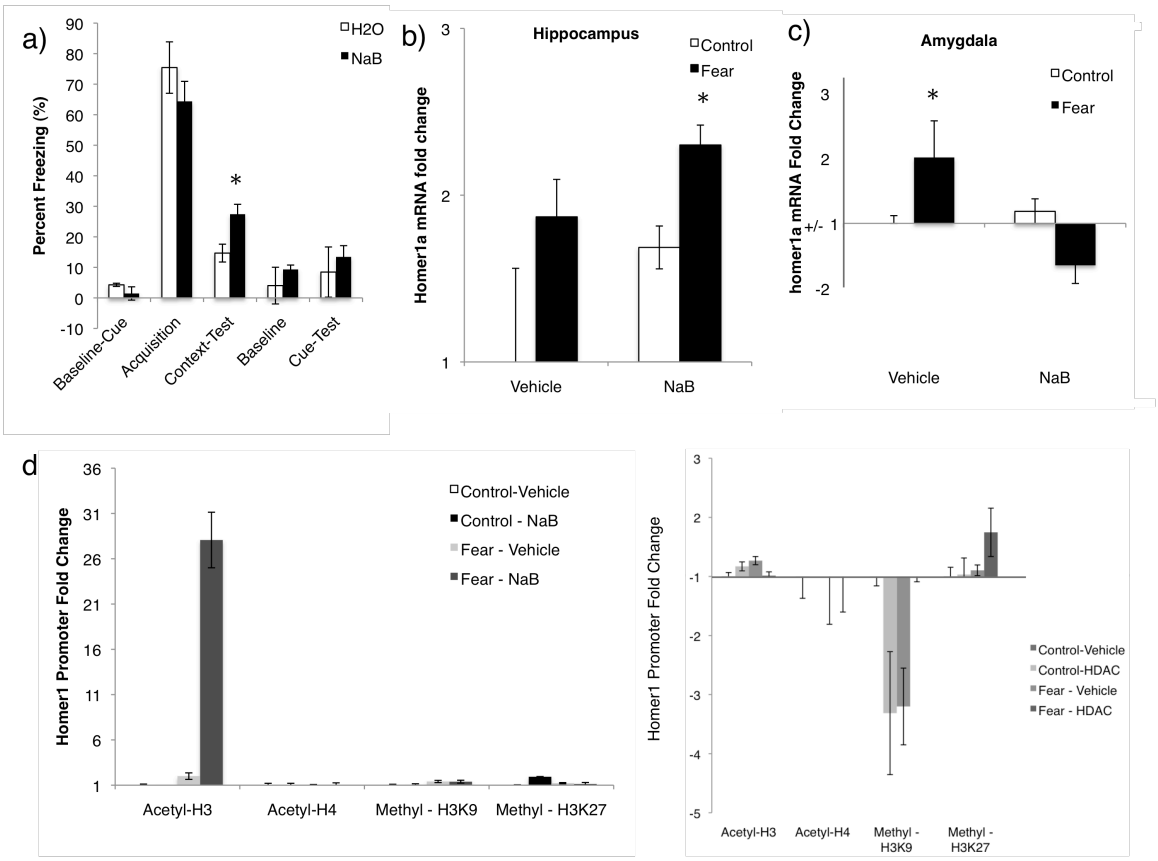

a)
Hippocampus
Homer1 Promoter Fold Change
40
35
30
25
20
15
10
5
0
*
Control
BDNF
Acetyl-H3
Acetyl-H4
Methyl - H3K9
Methyl - H3K27
c)
Amygdala
BDNF -
BDNF +
Homer 1 Promoter Fold Change
2
+/1
-2
-3
-4
-5
*
Acetyl-H3
Acetyl-H4
Methyl - H3K9
Methyl - H3K27
b)
Control
Fear
Homer 1 Promoter Fold Change
3
2
+/1
-2
*
Acetyl-H3
Acetyl-H4
Methyl - H3K9
Methyl - H3K27
d)
Control
Fear
Homer1 Promoter Fold Change
2
+/1
-2
-3
-4
-5
*
Acetyl-H3
Acetyl-H4
Methyl - H3K9
Methyl - H3K27

Figure 4.2. Effects of histone deacetylase inhibition on fear conditioning, *homer1a* mRNA levels, and histone modifications around the Homer1 promoter. a) Sodium Butyrate (NaB), a histone deacetylase inhibitor, had no effect on baseline freezing, acquisition or cued fear memory expression. However, NaB significantly enhanced contextual freezing ($p < 0.05$, $n = 10$). b) NaB enhanced *homer1a* mRNA in the hippocampus ($F(1,16) = 5.01$, $p < 0.05$, $n = 10$). c) NaB appeared to inhibit fear induced upregulation of Homer1a mRNA in the amygdala ($F(1,16) = 5.45$, $p < 0.05$, $n = 10$). d) NaB enhanced H3 acetylation in the hippocampus around the Homer1 promoter after fear conditioning ($F(1,16) = 9.54$, $p < 0.01$, $n = 10$) e) There was a significant interaction of NaB treatment with fear conditioning on H3K9 methylation in the amygdala ($F(1,16) = 4.58$, $p < 0.05$, $n = 10$). While NaB treatment in the absence of fear significantly reduced H3K9 methylation in the amgydala, as did fear in the absence of NaB, NaB and Fear combined showed no changes in H3K9 methylation relative to control.


a)
Percent Freezing (%)
H2O
NaB
Baseline-Cue
Acquisition
Context-Test
Baseline
Cue-Test
b)
Hippocampus
Homer1a mRNA fold change
Control
Fear
Vehicle
NaB
c)
Amygdala
homer1a mRNA Fold Change
Control
Fear
Vehicle
NaB
d)
Homer1 Promoter Fold Change
Control-Vehicle
Control - NaB
Fear - Vehicle
Fear - NaB
Acetyl-H3
Acetyl-H4
Methyl - H3K9
Methyl - H3K27
Homer1 Promoter Fold Change
Control-Vehicle
Control-HDAC
Fear - Vehicle
Fear - HDAC
Acetyl-H3
Acetyl-H4
Methyl - H3K9
Methyl - H3K27

4.6 References

Bannister AJ, Kouzarides T (2011) Regulation of chromatin by histone modifications. Cell Res 21:381-395.

Bertaso F, Roussignol G, Worley P, Bockaert J, Fagni L, Ango F (2010) Homer1a-dependent crosstalk between NMDA and metabotropic glutamate receptors in mouse neurons. PLoS One 5:e9755.

Brewer GJ (1997) Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods 71:143-155.

Cheung P, Lau P (2005) Epigenetic regulation by histone methylation and histone variants. Mol Endocrinol 19:563-573.

Chwang WB, O'Riordan KJ, Levenson JM, Sweatt JD (2006) ERK/MAPK regulates hippocampal histone phosphorylation following contextual fear conditioning. Learn Mem 13:322-328.

Fuchikami M, Yamamoto S, Morinobu S, Takei S, Yamawaki S (2010) Epigenetic regulation of BDNF gene in response to stress. Psychiatry Investig 7:251-256.

Gelato KA, Fischle W (2008) Role of histone modifications in defining chromatin structure and function. Biol Chem 389:353-363.

Gupta S, Kim SY, Artis S, Molfese DL, Schumacher A, Sweatt JD, Paylor RE, Lubin FD (2010) Histone methylation regulates memory formation. J Neurosci 30:3589-3599.

Lattal KM, Barrett RM, Wood MA (2007) Systemic or intrahippocampal delivery of histone deacetylase inhibitors facilitates fear extinction. Behav Neurosci 121:1125-1131.

Levenson JM, O'Riordan KJ, Brown KD, Trinh MA, Molfese DL, Sweatt JD (2004) Regulation of histone acetylation during memory formation in the hippocampus. J Biol Chem 279:40545-40559.

Miller CA, Campbell SL, Sweatt JD (2008) DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. Neurobiol Learn Mem 89:599-603.

Monsey MS, Ota KT, Akingbade IF, Hong ES, Schafe GE (2011) Epigenetic alterations are critical for fear memory consolidation and synaptic plasticity in the lateral amygdala. PLoS One 6:e19958.

Peleg S, Sananbenesi F, Zovoilis A, Burkhardt S, Bahari-Javan S, Agis-Balboa RC, Cota P, Wittnam JL, Gogol-Doering A, Opitz L, Salinas-Riester G, Dettenhofer M, Kang H, Farinelli L, Chen W, Fischer A (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. Science 328:753-756.

Sweatt JD (2009) Experience-dependent epigenetic modifications in the central nervous system. Biol Psychiatry 65:191-197.

Takei S, Morinobu S, Yamamoto S, Fuchikami M, Matsumoto T, Yamawaki S (2011) Enhanced hippocampal BDNF/TrkB signaling in response to fear conditioning in an animal model of posttraumatic stress disorder. J Psychiatr Res 45:460-468.

Chapter 5

Cyclic AMP Response Element Binding Protein (CREB) and CREB Binding Protein (CBP) activity Mediated Transcription of Homer1a during Pavlovian fear conditioning and BDNF induced plasticity.

5.1 Abstract

Homer1a is rapidly upregulated with synaptic plasticity and Pavlovian fear conditioning. Numerous studies suggest that homer1a may play a mediating role in Pavlovian fear conditioning. Homer 1a is also dynamically regulated with BDNF induced synaptic plasticity. Both fear conditioning and BDNF induced plasticity result in rapid changes in histone modifications around the Homer1 promoter. Here we show that *homer1a* gene transcription and changes in histone modifications during fear conditioning are mediated through CREB binding around the Homer1 promoter. We first demonstrate that CREB and CBP are increasingly bound to the Homer1 promoter after fear conditioning. We next demonstrate that inhibiting CREB inhibits *homer1a* expression, CBP binding and histone modifications after fear conditioning. Together these data suggest that CREB and CBP are critical for regulation of *homer1a* expression.

5.2 Introduction

Cyclic-amp response element binding protein (CREB) is a critical mediator of gene transcription and has been shown to be necessary in long-term memory in behavioral paradigms including the Pavlovian fear conditioning (Bourtchuladze et al., 1994). During consolidation of fear, CREB is rapidly phosphorylated in the basolateral amygdala (BLA) and in the hippocampus (Viosca et al., 2009a) as well as other regions of the brain responsible for processing fear (Han et al., 2009). Over-expressing CREB in the BLA can enhance fear memories (Josselyn et al., 2001; Zhou et al., 2009). Inactivating CREB expressing neurons in the BLA disrupts expression of fear conditioning in a persistent manner. This suggests that long term memories are held specifically in the CREB activated neurons (Han et al., 2009; Zhou et al., 2009). CREB

dependent gene expression appears to be necessary and sufficient for long-term consolidation and stability of fear memories (Viosca et al., 2009a) but not for encoding, storage or retrieval of these memories (Kida et al., 2002).

Within the amygdala, only a subset of neurons demonstrates CREB activation after fear conditioning. CREB activated neurons following Pavlovian fear conditioning appear to show increased neuronal excitability relative to non-CREB activated neurons (Zhou et al., 2009). Enhancing CREB-dependent gene expression appears to increase excitability of neurons in the BLA (Viosca et al., 2009b). Furthermore, CREB mutants, with impaired CREB functioning show impaired long-term potentiation (LTP) (Bourtchuladze et al., 1994). LTP is considered the neural/cellular correlate of learning and memory, and is crucial for consolidation and persistence of fear memories (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Maren, 2005; Pape and Pare, 2010). Though the connection between CREB dependent gene expression and LTP is vague, understanding this connection will be important to our understanding of consolidation of fear. More directly, CREB is a transcription factor, and its role in gene transcription is well understood. CREB is activated via phosphorylation and translocated to the nucleus where it binds to CRE sites within the promoter regions of various genes (Montminy et al., 1990).

Phosphorylated CREB proteins bound to the promoter can recruit phosphorylation and binding of CBP/P300 to the promoter complex (Bedford et al., 2010). CBP proteins can promote transcription both by recruitment of transcriptional machinery and through histone acetyl-transferase activity. The addition of acetyl groups to histone tails relaxes chromatin, making DNA more accessible to transcriptional machinery (Bedford et al.,

2010). CBP has been implicated in Pavlovian fear conditioning as well. Deficits in contextual fear conditioning as well as other hippocampal dependent memory were seen in transgenic mice expressing a truncated form of CBP (Wood et al., 2005). These mice also showed impaired LTP in hippocampal slices indicating altered physiological plasticity (Wood et al., 2005). While CBP impairment results in long-term memory deficits, short-term memory appears to be spared (Korzus et al., 2004). Behavioral deficits can be rescued with a histone deacetylase inhibitor indicating that it is CBP's histone acetyl transferase activity that may be crucial for long-term memory (Korzus et al., 2004).

In the present chapter we examine epigenetic regulation of the gene variant, *homer1a* (also known as vesl-1S), during BDNF-induced plasticity and fear conditioning. The Homer1 promoter contains several CRE sites making CREB a potential mediator of its transcription (Bottai et al., 2002). While CREB and CBP affect transcription of a variety of genes, here we demonstrate how such mechanisms regulate a specific gene, *homer1a*, which may have a functional role in regulating synapse structural organization during synaptic plasticity, which mediates the consolidation of fear memory. Here we show that phosphorylated CREB and CBP are increasingly bound to the Homer1 promoter after both Pavlovian fear conditioning and BDNF induced plasticity in cell culture models. In addition, inhibiting CREB expression impairs BDNF induced increases in *homer1a* mRNA expression as well as CBP binding and histone modifications around the homer1 promoter. Together these data suggest that CREB is a critical mediator of *homer1a* expression in BDNF and fear induced plasticity.

5.3 Methods

5.3.1 Calcium Phosphate Transfection

Transfections were performed as described previously (Xia et al., 1996). Culture media was replaced with plain pre-warmed DMEM for transfection. Cells were washed twice with plain DMEM transfection medium. Plates were incubated at 5% CO_2 for 30-60 minutes, while precipitate was prepared. Calcium phosphate/DNA precipitate was composed of 4 μg of DNA (CREB siRNA or GFP plasmid) and 0.25 M $CaCl_2$. Calcium phosphate/DNA mixture was added dropwise to 2x HEPES-Buffered Saline (HeBS). Mixture was allowed to sit for 25 minutes at room temperature and then added to the cells for 1 hour. Transfection was stopped by washing twice with warmed plain DMEM and then adding back the original media to the cell culture. A CREB siRNA plasmid was obtained from Cell Signaling (Cat No. 6588). Control groups were transfected with a plasmid expressing GFP.

5.3.2 Animals

All experiments were performed on adult (6-8 weeks old) wild-type strain C57BL/6J male mice from Jackson Laboratory (Bar Harbor, ME.). All procedures were approved by the Institutional Animal Care and Use Committee of Emory University and were in compliance with National Institutes of Health guidelines. Separate cohorts of animals were used for each experiment.

5.3.4 Behavior

Fear conditioning was conducted in nonrestrictive acrylic cylinders (SR-LAB startle response system, San Diego Instruments) located in a ventilated, sound-attenuated chamber. The foot shock (unconditioned stimulus) was delivered through a stainless steel

grid floor. Shock reactivity was defined as the peak activity (measured with a piezoelectric accelerometer) that occurred during the 200 msec after the onset of the unconditioned stimulus. The tone-conditioned stimulus was generated by a Tektronix function generator audio oscillator and delivered through a high frequency speaker. One day prior to training, mice were preexposed to the tone through a 5 "tone-alone" presentation program to both habituate them to handling and to the tone, but to get baseline fear responses to the tone presentation. Preexposure was done in a separate context. During cued fear conditioning, mice received five trials of a conditioned stimulus tone (30 seconds, 6 kHz, 70 dB) coterminating with an unconditioned stimulus foot shock (500 msec, 1 mA) with a variable inter-trial-interval between 60 and 180 seconds. Stimulus presentation and data acquisition were controlled and digitized by, and stored in, an interfacing desktop computer using SR-LAB and analyzed with the Freeze View software program (Coulbourn Instruments, Whitehall, Pa.).

5.2.3 Cell Culture:

Primary cultures of postnatal hippocampal neurons were described previously (Brewer, 1997) with modifications. C57BL/6J mice (21 days postnatal) were decapitated and the hippocampus and amygdala were removed and immersed in ice-cold dissection buffer consisting of Hibernate-A medium (BrainBits, Springfield, IL, USA), B27 supplement and gentamycin (Invitrogen) (12g/ml) for the preparation of separate hippocampal and amygdala neuronal cell cultures. The hippocampus and amygdala tissues were sliced and then enzymatically digested with papain (Worthington, Lakewood, NJ, USA) in Hibernate-A medium at 32°C for 30 minutes. Cells were dissociated by triturating with Pasteur pipets fired on the tips to narrow openings.

Neurons were purified in a density gradient media including Hibernate-A and OptiPrep (Sigma, St. Louis, MO, USA) by centrifugation. The density gradient media consisted of four layers. The first was 1 mL dissection buffer containing 35% OptiPrep; the second 1 mL dissection buffer contained 25% Optiprep and the third 1 ml dissection buffer contained 20% OptiPrep and the fourth 1 mL dissection buffer contained 15% OptiPrep. They were added on the top of each other carefully, resulting in clear layer separation. Then cells were added on the top of the density gradient media. After centrifugation, the densest layer with a cream color, located at the middle of the tube, could be seen. This layer of neurons was taken out by using a sterile transfer pipette and put into a new tube. After washing with dissection buffer, neuronal cells were plated onto Poly-D-Lysine (Sigma) coated plates or glass coverslips at the density of 2.5 x 10^5 cells/cm^2 in culture media consisting of Neurobasal A medium (Invitrogen) with 2% B27 supplement, 2 mM glutamax and gentamycin (5 g/ml). Thereafter, the cultures were kept in a humidified incubator at 37˚C and 5% CO_2 and media were changed every 5 days until used for experiments. After 2-3 weeks in vitro, the cells were used for the experiments reported in the present study.

5.2.4 Drugs:

Recombinant human BDNF was purchased from Cell Sciences (Canton, MA, USA) and reconstituted in sterile PBS as 100 mg/ml stock. The aliquots of stock were stored at -30 deg C and final drugs and concentrations for cell culture experiments were as following: BDNF (100 ng/ml)

5.2.5 Chromatin Immunoprecipitation

Tissue samples were treated using an EpiQuik tissue ChIP kit (Epigentek Group

Inc. Brooklyn, NY). Brains were extracted using rapid decapitation 2 hours after training. Amygdala and hippocampal tissue was rapidly dissected in ice-cold PBS and then frozen immediately on dry ice and stored at -80˚C until ready to use. Cells/tissues were harvested and mixed with formaldehyde at a final concentration of 1.0% for 10 minutes at 37° C to cross-link protein to DNA. Cells/tissue then were suspended in 0.2 mL of SDS lysis buffer and settle on ice for 10 minutes. DNA cross-linked with protein was then sonicated into fragments of 200-1000 bp. One-tenth of the sample was set aside as an input control, and the rest was then immunoprecipitated 1.5 h at room temperature with 5 g of primary antibody in the ChIP kit strip wells. As a control samples were immunoprecipitated with 5g non-immune rabbit IgG. After immunoprecipitaiton, the DNA-protein complex was eluted and the proteins were digested with DNA release buffer and proteinase K. DNA was dissociated at 65ºC for 1.5 hours under reverse buffer. The DNA, associated with antibody of interest (pan-H3-acetylated, pan-H4 acetylated, H3K9 dimethylation, and H3K27 dimethylation) was extracted with binding buffer, precipitated with 70% and 90% ethanol and DNA was finally eluted with elution buffer. Quantitative real-time PCR was performed with primers specific to the Homer1 promoter and for the GAPDH promoter regions.

5.2.6 RNA Preparation

Total RNA was prepared from frozen amygdala and hippocampal dissections in mice. Brains were extracted using rapid decapitation 2 hours after training. Amygdala and hippocampal tissue was rapidly dissected in ice-cold PBS and then frozen immediately on dry ice and stored at -80˚C until ready to use. Briefly, tissue samples were homogenized and centrifuged at 13,000g for 3 minutes. RNA was washed with 70%

ETOH and purified using RNeasy columns (Qiagen). RNA amount and quality were determined using a nanodrop spectrophotometer.

5.2.7 Quantitative RT-PCR

140 micrograms of total RNA were reverse transcribed using the RT2-First Strand Kit (C-03, SA Biosciences). Quantitative PCR was performed using the Applied Biosystems 7500 Fast. Online detection of reaction products was carried out using the SybrGreen Gene Assay with custom made primers for *homer1a*, *homer1c* and *GAPDH*. SybrGreen mastermix was obtained from SA biosciences, and manufacturer's instructions were followed. Calculated values are presented as mean +/- SEM to indicate accuracy of measurement. *Homer1a* and *homer1c* values were normalized for measurements of GAPDH. PCR conditions were 2 min at 50 deg C, 10 min at 95 deg C and 40 cycles with 15s 95 deg C, 60 s 60 deg C.

5.2.8 Primer Design

Primers were designed and confirmed by Primer blast. There sequence is as follows: Homer1a – FWD – 5'- GAAGTCGCAGGAGAAGATG-3'; Homer1a – REV – 5'- TGATTGCTGAATTGAATGTGTACC-3'; Homer 1c – FWD – 5'-ACACCCGATGTGACACAGAACT-3; Homer 1c – REV - 5'-TCAACCTCCCAGTGGTTGCT-3'; Homer1 Promoter FWD – 5'-GGTGACGTATGTGCGGAGAGGA-3'; Homer1 Promoter – REV – 5'-GGTCCGTCGGTCCGTCCCTTT-3'; Primers for GAPDH and GAPDH promoter region were obtained from SA Biosciences.

5.2.9 Statistical Analysis

Statistically significant differences were determined by a Student's t-test or by a between

subjects two-way ANOVA. The results were presented as means +/- SEM. For all ChIP and mRNA data, fold changes relative to control were determined using the ΔΔCt method; a mean fold change value along with an SEM value were determined; the ΔΔCt values from each data set were used in two-tailed paired t-tests (which were adjusted for multiple comparisons) to determine statistical significance (* = $P < 0.05$). All values included in the figure legends represent mean +/- SEM. The RTPCR ChIP data were analyzed identically to the mRNA data using the ΔΔCt method, except that ChIP data were normalized to 'input' rather than GAPDH level.

5.3 Results

5.3.1 Homer 1a is dynamically regulated during the consolidation period following Pavlovian fear conditioning

Homer 1a was rapidly upregulated following Pavlovian fear conditioning. Mice were preexposed to the tone 1 day prior to training. On day one of training animals were presented with 5 tone-shock pairings. Freezing in response to the tone increased significantly to each tone presentation ($p < 0.05$). Previous chapters have demonstrated that this paradigm is sufficient to produce long-term memory (LTM) of fear for up to 24 hours after training. Following training, animals were sacrificed at specified time intervals after fear conditioning. In the hippocampus, *homer1a* significantly increased 30 minutes ($p < 0.05$), 60 minutes ($p < 0.05$) and 120 minutes ($p < 0.05$) after fear conditioning and remained significantly elevated for up to 2 hours post training in the hippocampus (figure 5.1a). In the amygdala, *homer1a* significantly increased at 0 minutes ($p < 0.05$), 30 minutes ($p < 0.05$), 60 minutes ($p < 0.05$) and 120 minutes ($p < 0.05$) after

fear conditioning and remained significantly elevated for up to 2 hours post training in both the hippocampus (figure 5.1b). No changes were seen in *homer1a* in the striatum (figure 5.1c) and no changes in regulation of homer1c were seen in the hippocampus, amygdala or striatum (figures 5.2 a-c).

5.3.2 pCREB is increasingly bound to Homer1 after fear conditioning in cell culture

To determine the relative occupancy of phosphorylated CREB bound at the Homer1 promoter, we used chromatin immunoprecipitation followed by QT-PCR. We found that pCREB was increasingly bound to the Homer1 promoter in the hippocampus at 0 minutes ($p < 0.05$), 30 minutes ($p < 0.05$) and 60 minutes ($p < 0.05$), but not after 2 hours (Figure 5.3a). In the amygdala, we also found that pCREB was increasingly bound to the Homer1 promoter at 0 minutes ($p < 0.05$), 30 minutes ($p < 0.05$), and 60 minutes ($p < 0.05$) but not 2 hours after Pavlovian fear conditioning (Figure 5.3b). There was not a significant increase in CREB binding to the GAPDH promoter in the amygdala or hippocampus at anytime after fear conditioning.

5.3.3 CBP is increasingly bound to Homer1 after fear conditioning and BDNF induced plasticity

To determine the relative occupancy of CREB binding protein (CBP) at the Homer1 promoter, we used chromatin immunoprecipitation followed by QT-PCR. We found that CBP was increasingly bound to the Homer1 promoter in the hippocampus at 0 minutes ($p < 0.05$), and 30 minutes ($p < 0.05$), but not after 2 hours (Figure 5.4a). In the amygdala, we also found that CBP was increasingly bound to the Homer1 promoter at 0 minutes ($p < 0.05$), 30 minutes ($p < 0.05$), and 60 minutes ($p < 0.05$) but not 2 hours after Pavlovian fear conditioning (Figure 5.4b). There was not a significant increase in CBP

binding to the GAPDH promoter in the amygdala or hippocampus at anytime after fear conditioning.

5.3.4 Inhibition of CREB function impairs BDNF induced increases in *homer1a* mRNA in the hippocampus and amygdala

To determine whether or not CREB plays a mediating role in transcription of *homer1a* mRNA during BDNF induced plasticity, a plasmid expressing CREB siRNA was transfected into primary amgydala and hippocampal neurons. In our model, CREB siRNA transfection successfully decreased *CREB* mRNA levels ($p < 0.05$), thereby reducing CREB activity(Figure 5.4). CREB siRNA inhibited BDNF induced upregulation of *homer1a* when compared with cells treated with a plasmid expressing GFP in both amgydala (Figure 5.5a) ($F(1,8) = 5.28$, $n = 6$) and hippocampal ($F(1,8) = 6.63$, $n = 6$) (Figure 5.5b) cell culture. Neither CREB siRNA nor BDNF had any effect on levels of *homer1c* in hippocampal, nor amygdala primary cell culture.

5.3.5 Inhibition of CREB function impairs CBP binding to Homer1a

To determine whether or not CREB plays a mediating role in recruitment of CBP to the Homer1 promoter during BDNF induced plasticity, a plasmid expressing CREB siRNA was transfected into primary amgydala and hippocampal neurons. CREB siRNA inhibited BDNF induced recruitment of CBP to the Homer1 promoter when compared with cells treated with a plasmid expressing GFP in both amgydala ($F(1,8) = 6.51$, $n = 6$) (Figure 5.6a) and hippocampal ($F(1,8) = 8.72$, $n = 6$) (Figure 5.6b) cell culture. Neither CREB siRNA nor BDNF had any effect on recruitment of CBP to the GAPDH promoter in hippocampal nor amygdala primary cell culture.

5.3.6 Inhibition of CREB function impairs histone modifications in hippocampal and amgydala cells

To determine whether or not CREB plays a mediating role in H3 acetylation around the Homer1 promoter during BDNF induced plasticity, a plasmid expressing CREB siRNA was transfected into primary amgydala and hippocampal neurons. CREB siRNA inhibited BDNF induced H3 acetylation around the Homer1 promoter when compared with cells treated with a plasmid expressing GFP in both hippocampal (Figure 5.7a) ($F(1,8) = 5.63$, $n = 6$) but not amygdala (Figure 5.7b) cell culture. Neither CREB siRNA nor BDNF had any effect on H3 acetylation around the GAPDH promoter in hippocampal or amygdala primary cell culture.

5.4 Discussion

In this study, we found that the mRNA encoding the *homer1a* but not *1c* gene variant was rapidly upregulated after Pavlovian fear conditioning in both amygdala and hippocampal tissue. In previous chapters we had shown that *homer1a* mRNA was elevated at 2 hours post fear conditioning. We originally chose to look at 2 hours, because this is generally thought to be the time window in which consolidation and the molecular changes associated with consolidation of fear occur. However, here we show that *homer1a* transcription can happen much faster than 2 hours after the last trial. It is important to note however that tissue was collected at the specified period after the last trial. However, the training paradigm lasted approximately 30 minutes and learning/consolidation may begin to occur as soon as the first training trial. Some fear training paradigms, especially for contextual fear conditioning, are much shorter. This

shorter training session may account for prior reports of molecular changes after 2 hours when we encountered them much quicker relative to the end of our longer training session.

Over a slightly faster time course, phospho-CREB and CBP were increasingly bound to the Homer1 promoter region. pCREB and CBP both increased very rapidly after fear conditioning and returned to baseline by 2 hours. This is not surprising given the time course of action for pCREB (within 30 minutes) as seen in synaptic plasticity and other learning and memory paradigms (Viosca et al., 2009a).

Inhibition of CREB signaling through siRNA resulted in decreased *homer1a* mRNA expression in the hippocampal and amygdala cell culture. While it is not surprising that CREB binds to the promoter region (there are several CRE recognition sites in the Homer1 promoter), the Homer1 promoter regulates transcription of the entire Homer1 gene family, not just the *homer1a* variant. Therefore it is interesting that CREB siRNA only appears to affect *homer1a* mRNA regulation during BDNF induced plasticity. It is currently unknown how factors in the promoter region of Homer1 may differentially regulate the different gene variants of Homer1 and clarifying these mechanisms is an interesting area for future research.

In addition, CREB siRNA inhibited BDNF induced CBP binding to the Homer1 promoter in primary hippocampal and amygdala cell culture. Finally CREB siRNA inhibited BDNF induced H3 acetylation around the Homer1 promoter in hippocampal primary cell culture. There was also an increase in CBP binding at the Homer1 promoter in the amygdala after Pavlovian fear conditioning. This might be slightly unexpected in that we have shown that there is no increase in acetylation at the Homer1 promoter with

fear conditioning or BDNF induced plasticity. However, CBP also plays a role in recruitment of polymerases to the promoter region and could be having this effect on the Homer1 promoter. In addition, acetylation could be happening in the amygdala, just at a different time course then we originally expected. We only examined H3 acetylation around the Homer1 promoter 2 hours after Pavlovian fear conditioning. It could be that acetylation in the amygdala follows a different time course and that we simply did not see any effects because we looked at the wrong time point. Finally, we confirmed in the final experiment that BDNF did not increase H3 acetylation at 2 hours after fear conditioning in the amygdala. This is consistent with our finding that CREB siRNA did not further change H3 acetylation levels in amygdala cultures.

CREB is a transcription factor that likely activates many genes in the hippocampus and amygdala, which are involved in Pavlovian fear conditioning and synaptic plasticity. Here we demonstrate CREB physically binds to Homer1 during Pavlovian fear conditioning and is essential to regulation of histone modifications around the Homer1 promoter and subsequent transcription of Homer1a. It will be interesting to get a better idea of all the genes activated by CREB binding and how these genes and protein products interact with each other. Furthermore, future studies will be necessary to determine how CREB binding after Pavlovian fear conditioning can result in an increase in *homer1a* mRNA but not *homer1c* mRNA when both transcripts are produced from the same putative promoter region. Finally future studies will also need to address how the protein Homer1a mechanistically regulates synaptic plasticity during Pavlovian fear conditioning through homeostatic mechanisms.

5.5 Figures

Figure 5.1 *Homer 1a* is dynamically regulated shortly following Pavlovian fear conditioning Mice were preexposed to the tone 1 day prior to training. On day of training animals were presented with 5 tone-shock pairings. Previous chapters have demonstrated that this paradigm is sufficient to produce LTM of fear memories for up to 24 hours after training. Following training animals were sacrificed at specified time intervals after fear conditioning. a,b) Homer1a significantly increased 30 minutes after fear conditioning and remained significantly elevated for up to 2 hours post training in both the hippocampus (a) and amygdala (b). c) No changes were seen in homer1a in the striatum. * $p < 0.05$

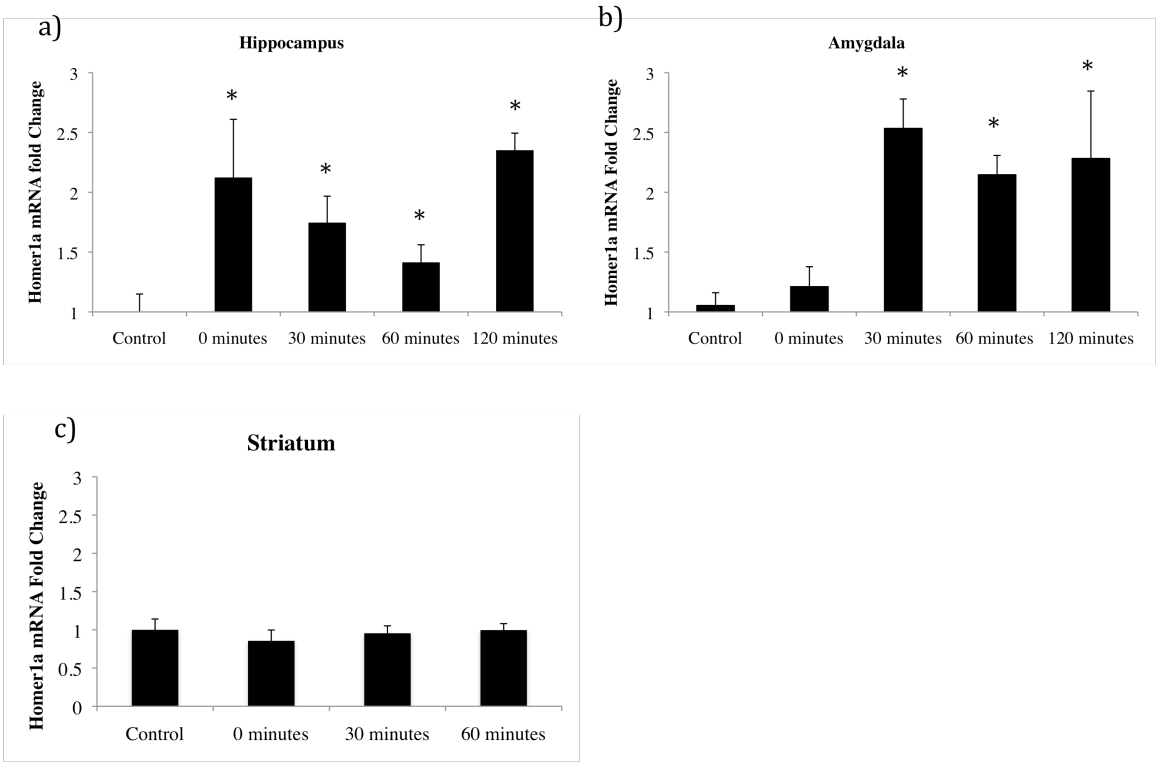
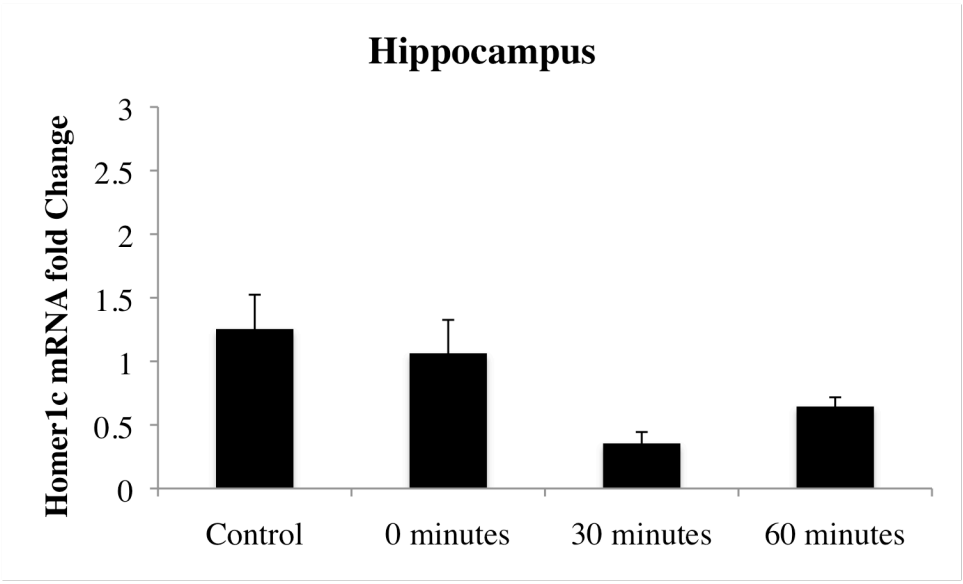

a)
Hippocampus
Homer1a mRNA fold Change
3
2.5
2
1.5
1
*
*
*
*
Control
0 minutes
30 minutes
60 minutes
120 minutes
b)
Amygdala
Homer1a mRNA Fold Change
3
2.5
2
1.5
1
*
*
*
Control
0 minutes
30 minutes
60 minutes
120 minutes
c)
Striatum
Homer1a mRNA Fold Change
3
2.5
2
1.5
1
0.5
0
Control
0 minutes
30 minutes
60 minutes

Figure 5.2 Expressional analysis of *homer1c* levels after Pavlovian fear conditioning in the amygdala, and hippocampus. No changes in regulation of homer1c were seen in the hippocampus (a), or amygdala (b).

a)


Hippocampus
Homer1c mRNA fold Change
3
2.5
2
1.5
1
0.5
0
Control
0 minutes
30 minutes
60 minutes

b)

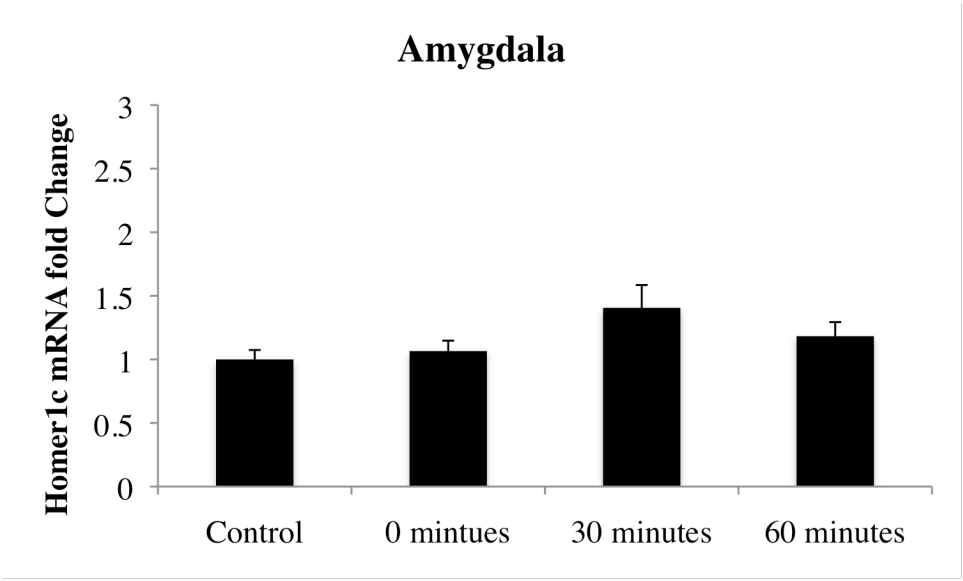

Amygdala
Homer1c mRNA fold Change
3
2.5
2
1.5
1
0.5
0
Control
0 mintues
30 minutes
60 minutes

Figure 5.3 Analysis of phosphorylated CREB bound to the Homer1a promoter during Pavlovian fear conditioning. To determine the relative occupancy of phosphorylated CREB at the Homer1 promoter, we used chromatin immunoprecipitation followed by QT-PCR. A,B) We found that pCREB was increasingly bound to the Homer1 promoter as soon as 30 minutes after Pavlovian fear conditioning and returns to baseline at 2 hours in both the amygdala (a) and hippocampus (b) * $p < 0.05$.

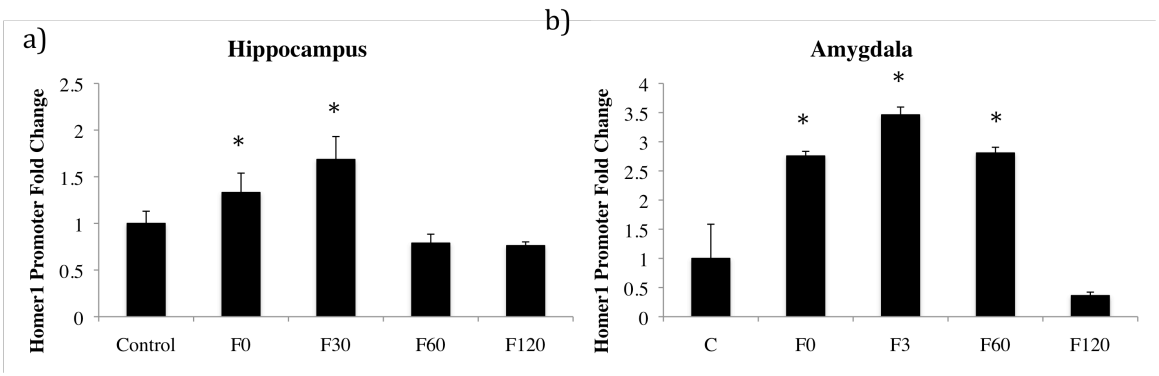
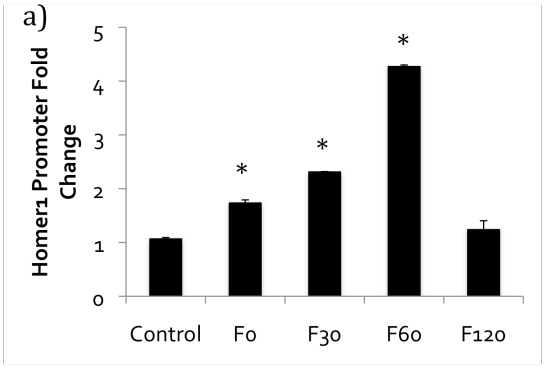

a)
Hippocampus
Homer1 Promoter Fold Change
2.5
2
1.5
1
0.5
0
Control
F0
F30
F60
F120
b)
Amygdala
Homer1 Promoter Fold Change
4
3.5
3
2.5
2
1.5
1
0.5
0
C
F0
F3
F60
F120

Figure 5.4 Analysis of CREB Binding Protein (CBP) bound to the Homer1a promoter during Pavlovian fear conditioning. To determine the relative occupancy of CREB binding protein (CBP) at the Homer1 promoter, we used chromatin immunoprecipitation followed by QT-PCR. A,B) We found that CBP was increasingly bound to the Homer1 promoter as soon as 30 minutes after Pavlovian fear conditioning and returns to baseline at 2 hours in both the amygdala (a) and hippocampus (b). * $p < 0.05$.


a)
Homer1 Promoter Fold Change
0
1
2
3
4
5
*
*
*
Control
Fo
F30
F60
F120

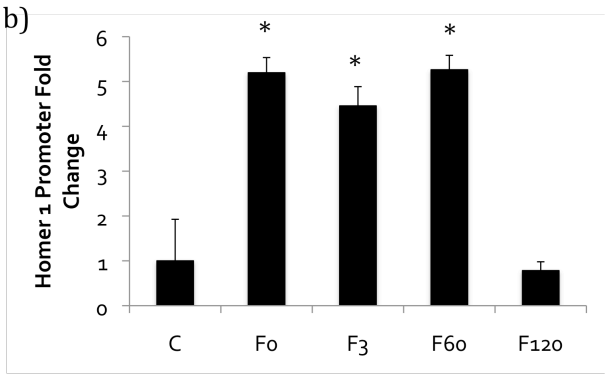

b)
Homer 1 Promoter Fold Change
0
1
2
3
4
5
6
*
*
*
C
Fo
F3
F60
F120

Figure 5.5 Expressional analysis of CREB mRNA after calcium phosphate tranfection of CREB siRNA *in vitro*. To assess the effectiveness of calcium phosphate transfections with siRNA, levels of CREB mRNA with or without CREB siRNA and with or without added BDNF were quantified. There was a significant reduction in CREB mRNA after transfection of siRNA when compared to control GFP plasmid transfections. However there was no effect of BDNF application on CREB mRNA levels.

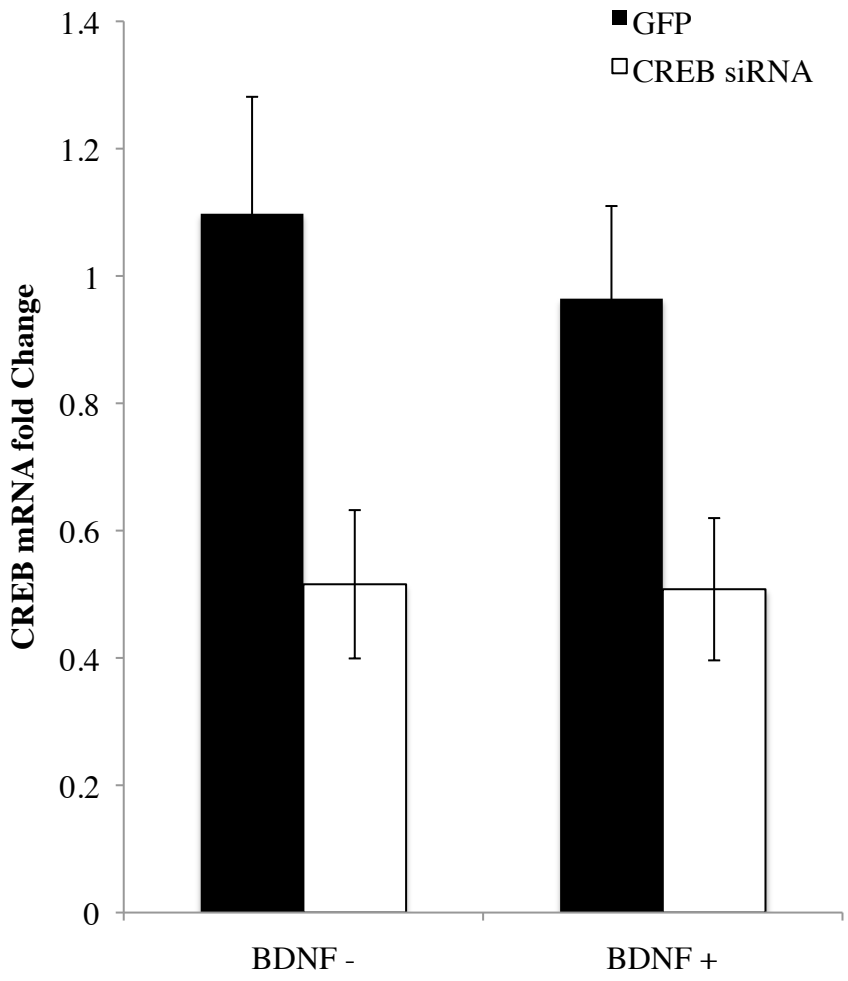
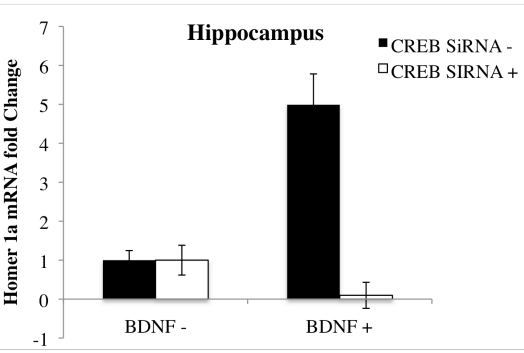

GFP
CREB siRNA
CREB mRNA fold Change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BDNF -
BDNF +

Figure 5.6 Effect of CREB siRNA on Homer1a upregulation during BDNF induced plasticity. To determine whether or not CREB plays a mediating role in transcription of *homer1a* mRNA during BDNF induced plasticity, a plasmid expressing CREB siRNA was transfected into primary amgydala and hippocampal neurons. a, b) CREB siRNA inhibited BDNF induced upregulation of *homer1a* mRNA when compared with cells treated with a plasmid expressing GFP in both amgydala (a) and hippocampal (b) cell culture. Neither CREB siRNA nor BDNF had any effect on levels of *homer1c* in hippocampal nor amygdala primary cell culture.

a)


Hippocampus
CREB SiRNA -
CREB SIRNA +
Homer 1a mRNA fold Change
7
6
5
4
3
2
1
0
-1
BDNF -
BDNF +

b)

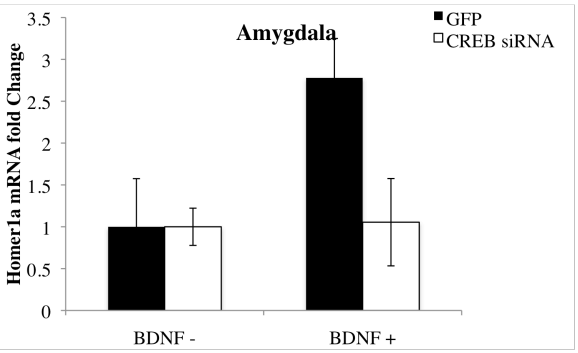

Amygdala
GFP
CREB siRNA
Homer1a mRNA fold Change
3.5
3
2.5
2
1.5
1
0.5
0
BDNF -
BDNF +

Figure 5.7 Effect of CREB siRNA on CBP binding to the Homer1 promoter during BDNF induced plasticity. a,b) BDNF resulted in an increase in CBP binding to the Homer1 promoter in both hippocampal (a) and amygdala (b) primary cell culture. BDNF nor CREB siRNA did not result in an increase in CBP binding to the GAPDH promoter in hippocampal or amygdala primary cell culture (data not shown).

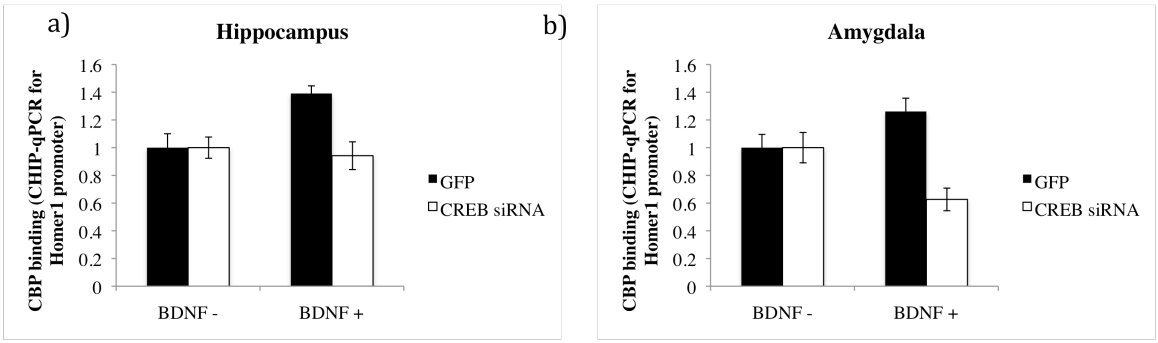
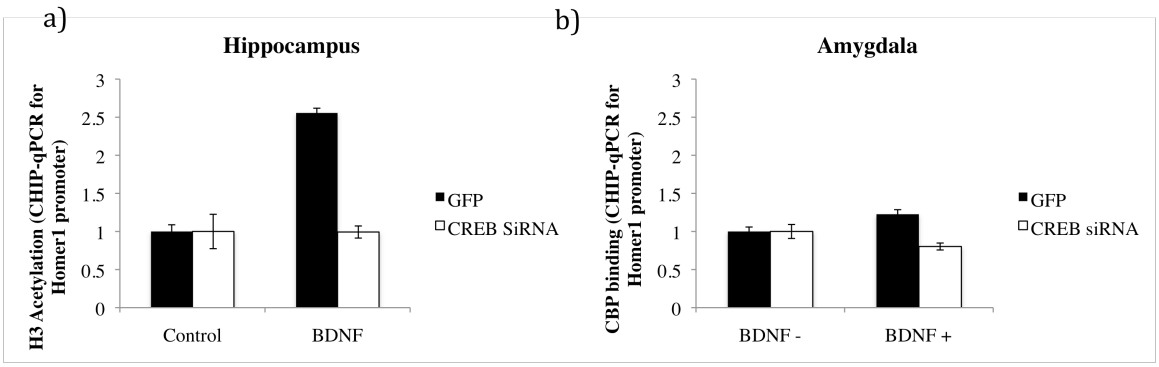

a)
Hippocampus
CBP binding (CHIP-qPCR for Homer1 promoter)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BDNF -
BDNF +
GFP
CREB siRNA
b)
Amygdala
CBP binding (CHIP-qPCR for Homer1 promoter)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BDNF -
BDNF +
GFP
CREB siRNA

Figure 5.8 Effect of CREB siRNA on H3 Acetylation during BDNF induced plasticity. To determine whether or not CREB plays a mediating role in H3 acetylation around the Homer1 promoter during BDNF induced plasticity, a plasmid expressing CREB siRNA was transfected into primary amygdala and hippocampal neurons. a,b) CREB siRNA inhibited BDNF induced H3 acetylation around the Homer1 promoter when compared with cells treated with a plasmid expressing GFP in both hippocampal (a) but not amygdala (b) cell culture.


a)
Hippocampus
H3 Acetylation (CHIP-qPCR for Homer1 promoter)
3
2.5
2
1.5
1
0.5
0
Control
BDNF
GFP
CREB SiRNA
b)
Amygdala
CBP binding (CHIP-qPCR for Homer1 promoter)
3
2.5
2
1.5
1
0.5
0
BDNF -
BDNF +
GFP
CREB siRNA

5.7 References:

Bedford DC, Kasper LH, Fukuyama T, Brindle PK (2010) Target gene context influences the transcriptional requirement for the KAT3 family of CBP and p300 histone acetyltransferases. Epigenetics 5:9-15.

Bottai D, Guzowski JF, Schwarz MK, Kang SH, Xiao B, Lanahan A, Worley PF, Seeburg PH (2002) Synaptic activity-induced conversion of intronic to exonic sequence in Homer 1 immediate early gene expression. J Neurosci 22:167-175.

Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G, Silva AJ (1994) Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell 79:59-68.

Brewer GJ (1997) Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods 71:143-155.

Han JH, Kushner SA, Yiu AP, Hsiang HL, Buch T, Waisman A, Bontempi B, Neve RL, Frankland PW, Josselyn SA (2009) Selective erasure of a fear memory. Science 323:1492-1496.

Josselyn SA, Shi C, Carlezon WA, Jr., Neve RL, Nestler EJ, Davis M (2001) Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. J Neurosci 21:2404-2412.

Kida S, Josselyn SA, Pena de Ortiz S, Kogan JH, Chevere I, Masushige S, Silva AJ (2002) CREB required for the stability of new and reactivated fear memories. Nat Neurosci 5:348-355.

Korzus E, Rosenfeld MG, Mayford M (2004) CBP histone acetyltransferase activity is a critical component of memory consolidation. Neuron 42:961-972.

Maren S (2005) Synaptic mechanisms of associative memory in the amygdala. Neuron 47:783-786.

McKernan MG, Shinnick-Gallagher P (1997) Fear conditioning induces a lasting potentiation of synaptic currents in vitro. Nature 390:607-611.

Montminy MR, Gonzalez GA, Yamamoto KK (1990) Regulation of cAMP-inducible genes by CREB. Trends Neurosci 13:184-188.

Pape HC, Pare D (2010) Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. Physiol Rev 90:419-463.

Rogan MT, Staubli UV, LeDoux JE (1997) Fear conditioning induces associative long-term potentiation in the amygdala. Nature 390:604-607.

Viosca J, Lopez de Armentia M, Jancic D, Barco A (2009a) Enhanced CREB-dependent gene expression increases the excitability of neurons in the basal amygdala and primes the consolidation of contextual and cued fear memory. Learn Mem 16:193-197.

Viosca J, Malleret G, Bourtchouladze R, Benito E, Vronskava S, Kandel ER, Barco A (2009b) Chronic enhancement of CREB activity in the hippocampus interferes with the retrieval of spatial information. Learn Mem 16:198-209.

Wood MA, Kaplan MP, Park A, Blanchard EJ, Oliveira AM, Lombardi TL, Abel T (2005) Transgenic mice expressing a truncated form of CREB-binding protein (CBP) exhibit deficits in hippocampal synaptic plasticity and memory storage. Learn Mem 12:111-119.

Xia Z, Dudek H, Miranti CK, Greenberg ME (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J Neurosci 16:5425-5436.

Zhou Y, Won J, Karlsson MG, Zhou M, Rogerson T, Balaji J, Neve R, Poirazi P, Silva AJ (2009) CREB regulates excitability and the allocation of memory to subsets of neurons in the amygdala. Nat Neurosci 12:1438-1443.

Chapter 6

General Discussion/Conclusion

Summary of Findings, Future Directions, and Clinical Implications

6.1 Summary of Findings

This body of work examines an underlying molecular mechanism of consolidation of Pavlovian fear conditioning (figure 6.1). We have argued that Pavlovian fear conditioning is a suitable model for studying the underlying dysregulation of fear seen in posttraumatic stress disorder. We show that *homer1a* (a gene previously associated with various forms of psychiatric disease) is rapidly transcribed with Pavlovian fear conditioning and is also regulated through BDNF-trkB signaling mechanisms. Furthermore, we show that fear-induced and BDNF-induced upregulation of *homer1a* is preceded by differential posttranslational modifications of histone tails in both the amygdala and the hippocampus. We next show that inhibiting histone deacetylase enzymes can effect these histone modifications, as well as corresponding changes in mRNA levels and behavioral expression of fear memories. We also find that fear induced plasticity results in an increase in CREB and CBP binding around the Homer1 promoter. Finally, we demonstrate that inhibiting CREB expression can impair BDNF's ability to recruit CBP binding to the Homer1 promoter, histone modifications around the Homer1 promoter, and *homer1a* mRNA upregulation in primary cell culture. This body of work significantly advances our understanding of the mechanisms of gene transcription that may underlie synaptic plasticity and Pavlovian fear conditioning. Given the association of Homer1 with psychiatric disease, these findings may help us to better understand how genetic differences could lead to greater susceptibility to mental illness.

5.2 Limitations of Findings

Here we show that both BDNF-induced plasticity and Pavlovian fear – induced plasticity can result in upregulation in *homer1a*. There is substantial evidence suggesting

that fear conditioning requires BDNF signaling in the amygdala and hippocampus (Rattiner et al., 2004b; Rattiner et al., 2004a; Chhatwal et al., 2006; Heldt et al., 2007; Musumeci et al., 2009), however we never directly prove that BDNF is necessary and sufficient to produce an upregulation of *homer1a* during Pavlovian fear conditioning. While we know that BDNF can increase *homer1a* expression there are a variety of other neurotransmitter, receptor-ligand systems involved in Pavlovian fear conditioning that could potentially also upregulate *homer1a* during the consolidation of fear.

For instance, NMDA activation has been shown to upregulate *homer1a* in primary cerebellar neuronal culture (Ango et al., 2000; Sato et al., 2001) and in the suprachiasmatic nucleus (Nielsen et al., 2002). Also the PACAP receptor, which is a robust activator of the cAMP-CREB pathway was recently implicated in PTSD and fear conditioning (Ressler et al., 2011). PACAP has also been shown to upregulate *homer1a* expression in the suprachiasmatic nucleus (Nielsen et al., 2002) in sympathetic neurons (Kammermeier, 2008) and in both central and peripheral neurons (Girard et al., 2004). Both NMDA and PACAP have all been implicated in Pavlovian fear conditioning (Ressler et al., 2011). Therefore, while it is acceptable to conclude that BDNF-trkB signaling is one mechanism by which Pavlovian fear conditioning may upregulate *homer1a*, we cannot rule out the possibility that these other receptor-ligand systems also play a role in Pavlovian fear induced upregulation of *homer1a* mRNA.

The use of primary amygdala and hippocampal cell culture is limited in face validity in that cells in culture lack the functional connectivity that amygdala and hippocampal neurons have *in vivo*. Primary neuronal culture might also lack glial and extracellular factors that might also influence neuronal signaling and plasticity. This

could potentially limit the interpretations we could make from the manipulations of the TrkB and CREB signaling pathways that were conducted *in vitro* with BDNF induced plasticity.

However, we saw many parallels in changes in mRNA levels as well as histone modifications *in vivo* and *in vitro*. In addition, differences seen in amygdala and hippocampal tissues after fear-induced plasticity persisted when examined in cell culture using BDNF induced plasticity. This suggests that distinct epigenetic regulation of amygdala and hippocampal expression of *homer1a* may be due to unique molecular properties of these neurons separate from their functional connectivity in the brain. Therefore, our *in vitro* data appears to make very interesting advances in the understanding of the mechanisms of *homer1a* mRNA expression and epigenetic regulation. While the role of CREB and trkB signaling in Pavlovian fear conditioning have been extensively demonstrated, future studies looking at the role of these signal pathways in homer1a expression during Pavlovian fear conditioning would provide more insight into the mechanism.

Another limitation to the interpretation of our results comes from the choice of sodium butyrate (NaB) to inhibit histone deacetylase. NaB was an advantageous choice in that a single intraperitoneal injection has been previously shown to enhance fear memories. In our studies, we further showed that NaB also enhanced hippocampal *homer1a* mRNA levels as well as increased H3 acetylation around the Homer1 promoter. As a histone deacetylase inhibitor, NaB is inefficient due to its lack of stability and low retention rate (Monneret, 2005). In addition, behaviorally, mice injected with sodium butyrate had acute motor deficits after injection. They were not injected until after

acquisition/training so these effects could not affect the animal's abilities to perceive the auditory or footshock stimulus. However, these deficits might still have affected consolidation in some other indirect way not due to the drug's histone deacetylase activity.

While we could have chosen a more specific histone deacetylase inhibitor, the more specific agents with greater stability and fewer side effects rarely cross the blood brain barrier. Though systemic injections don't provide localized manipulation of histone modifications during Pavlovian fear conditioning, they do allow for one to examine the effects of HDAC inhibition of both the amygdala and hippocampus as well as being more translationally relevant towards developing novel pharmacotherapies for PTSD. Furthermore, NaB has been used previously in the literature to show an enhancement of contextual fear conditioning (Levenson et al., 2004) and extinction (Lattal et al., 2007). This allowed us to have a positive control, contextual fear memories, to confirm that our pharmacological manipulation was effective. NaB is the only HDAC inhibitor demonstrated to enhance contextual fear memories when injected systemically (Levenson et al., 2004).

6.3 Epigenetics and Pavlovian Fear Conditioning

Histone deacetylase (HDAC) inhibitors were originally designed as drugs to inhibit cancerous tumors (Zhu and Otterson, 2003). However, it was discovered that sodium butyrate might also have implications for memory related disorders. Evidence suggests that sodium butyrate can enhance memory in an animal model for Alzheimer's disease (Govindarajan et al., 2011), in animal models of traumatic brain injury (Dash et al., 2009) as well as in consolidation of contextual fear conditioning (Levenson et al.,

2004; Federman et al., 2009) and extinction of contextual fear conditioning (Lattal et al., 2007; Kaplan and Moore, 2011). While our data suggest that a systemic injection of sodium butyrate does not enhance auditory cued fear conditioning, others have shown that injecting trichostatin-A (TSA), another HDAC inhibitor, directly into the lateral amygdala (LA) can enhance auditory fear conditioning (Monsey et al., 2011). Our data did not show an enhancement in auditory fear conditioning which may be due to the difference in drug (NaB or TSA) or difference in type of infusion (systemically or locally). While a local injection into the LA would have given us a more mechanistic data, systemic injections gave us a more translational-relevant model as well as allowing us to look at the effects of hippocampal and amygdala HDAC inhibition in the same animal. TSA does not cross the blood-brain barrier and would not have been useful for an examination of systemic inhibition of HDAC.

We show that despite an upregulation of *homer1a* in both the hippocampus and amygdala during Pavlovian fear conditioning, epigenetic changes that regulate dynamic expression of *homer1a* are different in these two distinct brain regions. In the hippocampus *homer1a* expression was dependent upon H3 acetylation, whereas in the amygdala it was H3K9 methylation dependent. If this result replicates for other genes as well, then manipulation of the enzymes that specifically methylate H3K9 residues might be a methodological way of only targeting amygdala dependent expression of *homer1a*.

One of the major limitations of the epigenetic field is the lack of tools to manipulate histone modifications at specific genes. While the development of *in vivo* chromatin immunoprecipitation methods have allowed us to look at posttranslational modifications of histones at specific gene promoters, our ability to inhibit or manipulate

enzymes that catalyze the modifications are restricted to global genome wide manipulations. Thus, we were not able to manipulate methylation or acetylation of histones, specifically around the Homer1 promoter. Pharmacologically, there are no HDAC inhibitors that regulate specific genes. Molecularly, studies have used lentiviral inhibition of histone methylation enzymes (Maze et al., 2010), but again these methods inhibit methylation at the global gene level. One idea would be to design genetically engineered proteins with leucine zipper motifs that recognize specific sequences of DNA, which are fused to histone modifiying enzymes or transcription factors. There has been some work in yeast models and at the *in vitro* level (Lohmer et al., 1991; Fan et al., 2011), but this methodology has not yet been designed or tested in animal models of behavioral psychiatry.

6.4 Homer1a and Pavlovian Fear conditioning

There is a small, but solid body of evidence that suggests that homer1a plays a role in Pavlovian fear conditioning. *Homer1a* mRNA is rapidly upregulated in the nucleus of neurons in the hippocampus after Pavlovian fear conditioning (Hashikawa et al., 2011). Homer1a knockout mice show impaired consolidation and retention of contextual fear memories (Inoue et al., 2009). Finally, over-expressing homer1a in the hippocampus enhances contextual memory (Tronson et al., 2010). The studies described above all examine contextual fear memories, but fail to look at amygdala dependent cued fear conditioning. Given our data, it would be valuable to examine these homer1a KO mice and *homer1a* over-expressing viral vectors, in auditory cued fear conditioning and amygdalar homer1a signaling. In addition, the Inoue study could be further added to by

accounting for pain sensitivity and perhaps trying to rescue behavioral deficits with a virus expressing the *homer1a* gene.

While there are many future experiments that can be done to clarify how important *homer1a* expression and signaling is for this behavior, it is clear that *homer1a* is involved in the consolidation of fear memories. However, a major outstanding question that remains is: (as described in chapter 2) how does a molecule that is involved in homeostatic plasticity play a role in the consolidation of fear memories? Evidence suggests that auditory cued fear conditioning involves LTP (Rogan et al., 1997; Maren, 2005), an increase in NMDA functioning (Nedelescu et al., 2010), and an increase in surface expression of AMPA receptors (Rumpel et al., 2005; Mokin et al., 2007; Brigman et al., 2010; Liu et al., 2010; Nedelescu et al., 2010). In contrast, *homer1a* expression, at least in primary cell cultures, appears to decrease AMPA receptor surface expression (Hu et al., 2010) and inhibit NMDA functioning (Bertaso et al., 2010).

These findings are paradoxical in nature and understanding how this homeostatic plasticity plays a role in fear conditioning will be important in future studies. This paradox could be explained by: 1) an increase in AMPA receptor insertion/NMDA function may occur in certain cell populations but with a decrease within distinct cell populations of the same brain structures, 2) Changes in AMPA receptor expression/NMDA function may occur in opposite directions at different synapses within the same population of cells, or 3) There may be differential timing in AMPA insertion/NMDA function; for example, an initial decrease in AMPA receptor insertion/NMDA function may be followed by an increase in AMPA receptor

insertion/NMDA function. Clearly, further research is needed to understand how homeostatic plasticity plays a role in consolidation of fear.

6.5 Homer1a in PTSD

While we only have preliminary evidence for a single nucleotide polymorphism being associated with development of PTSD, based on the published evidence from genome wide association studies in other psychiatric disorders (Dahl et al., 2005; De Luca et al., 2009; Rietschel et al., 2010) and animal models of PTSD (Szumlinski et al., 2006), it is likely that polymorphisms within this gene could account for resiliency in certain populations of trauma victims. The Homer1 gene family has been associated both through genome wide association studies in humans and molecular/pharmacological studies in animal models (Szumlinski et al., 2006) in particular with fear conditioning (Inoue et al., 2009), depression and drug addiction. Molecularly the Homer1 gene family is a crucial mediator of signal transduction during synaptic plasticity (Bertaso et al., 2010; Hu et al., 2010). Future research is necessary to determine specifically the potential role of homer1a and disordered synaptic plasticity in the development of posttraumatic stress disorder.

6.6 Future Directions

The work described here has focused on regulation of a specific gene that is dynamically regulated during Pavlovian fear conditioning. However, it is likely that vast numbers of genes are being up-regulated or down-regulated during the course of consolidation of fear. Studies examining genome wide changes in histone acetylation and methylation during the consolidation of Pavlovian fear conditioning and extinction using

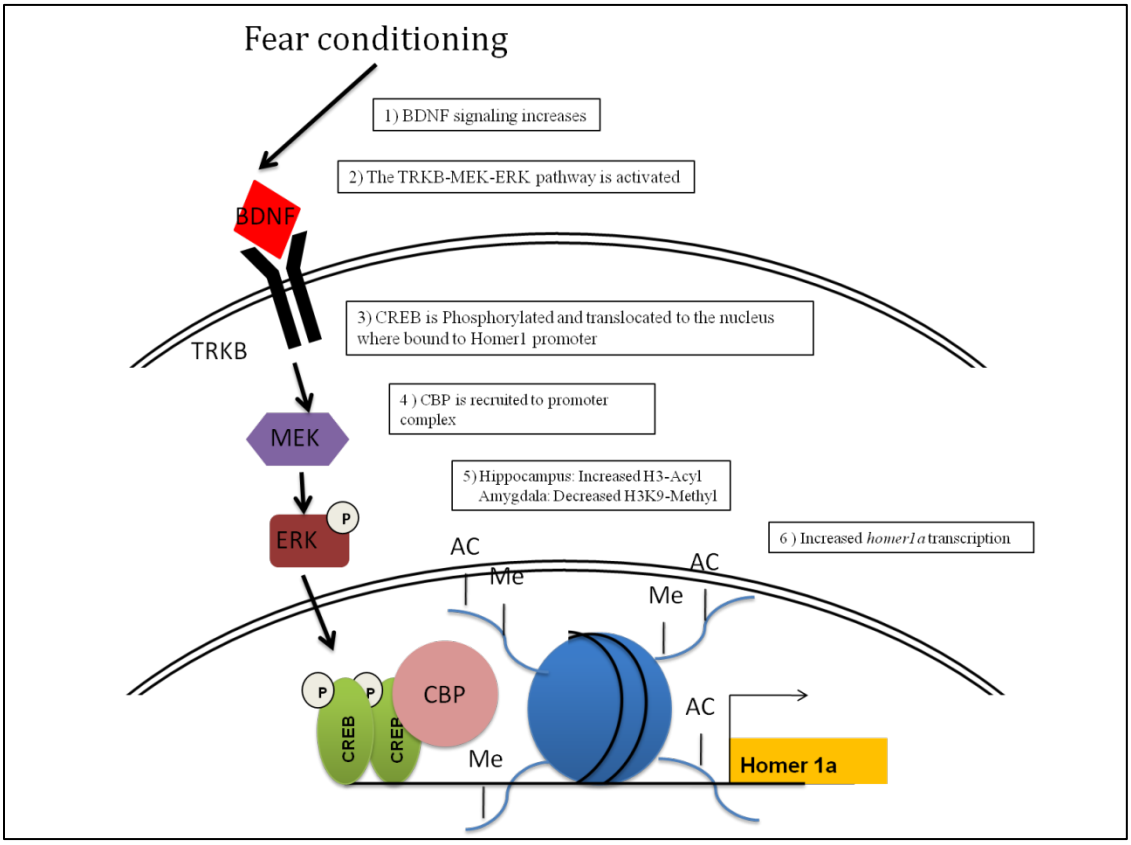
ChIP-seq or ChIP array will be useful for determining the role of epigenetic regulation of the Homer1 gene in the context of the whole genome.

In addition, only four posttranslational modifications around *one* region of the Homer gene were analyzed. There are quite a few specific acetylation and methylation modifications that we did not examine, and the selection of the modifications we did look at was based on the limited currently available knowledge of histone regulation. It is known that histone modifications such as methylation and acetylation not only alter chromatin structure but also interact with other histone tails on the DNA up- and downstream along various promoters. For example, certain methylation modifications can induce acetylation at other histone tails. Understanding the entire histone/chromatin environment around the Homer1 promoter during Pavlovian fear conditioning and BDNF-induced plasticity will be interesting and important for future studies.

Finally, as mentioned above, it will be interesting see the functional role of homer1a during Pavlovian fear conditioning. It is currently known that homer1a increasingly binds to mGLUR receptors after fear conditioning, but it is unknown how *homer1a* expression affects NMDA functioning or AMPA receptor insertion after fear conditioning. Nor do we know how homer1a binding with the ryanodine, TRPC or IP3 receptors might play a role in fear consolidation. Understanding how homer1a interacts with these receptors at the synapse to effect plasticity, structural organization, and signal transduction will provide valuable insight into the mechanisms of Pavlovian fear conditioning, and regulation of fear during PTSD.

6.7 Figures

Figure 6.1 Summary of findings and working model of transcriptional regulation of *homer1a* during Pavlovian fear conditioning. Based on our findings and the literature, we suggest that during Pavlovian fear conditioning BDNF signaling is rapidly increased. This results in an activation of the TRKB pathway, activating MEK and ERK. ERK further phosphorylates CREB which is translocated to the nucleus where it binds to CRE sites in the promoter region of *homer1a*. CREB subsequently recruits CBP which induces specific histone changes in the amygdala (decreased methylation) and in the hippocampus (increased acetylation) both of which result in increased *homer1a* transcription.


Fear conditioning
1) BDNF signaling increases
2) The TRKB-MEK-ERK pathway is activated
BDNF
TRKB
3) CREB is Phosphorylated and translocated to the nucleus where bound to Homer1 promoter
MEK
4) CBP is recruited to promoter complex
ERK
P
5) Hippocampus: Increased H3-Acyl
Amygdala: Decreased H3K9-Methyl
6) Increased homer1a transcription
AC
Me
AC
Me
P
P
CBP
CREB
CREB
AC
Me
Homer 1a

6.8 References

Ango F, Pin JP, Tu JC, Xiao B, Worley PF, Bockaert J, Fagni L (2000) Dendritic and axonal targeting of type 5 metabotropic glutamate receptor is regulated by homer1 proteins and neuronal excitation. J Neurosci 20:8710-8716.

Bertaso F, Roussignol G, Worley P, Bockaert J, Fagni L, Ango F (2010) Homer1a-dependent crosstalk between NMDA and metabotropic glutamate receptors in mouse neurons. PLoS One 5:e9755.

Brigman JL, Wright T, Talani G, Prasad-Mulcare S, Jinde S, Seabold GK, Mathur P, Davis MI, Bock R, Gustin RM, Colbran RJ, Alvarez VA, Nakazawa K, Delpire E, Lovinger DM, Holmes A (2010) Loss of GluN2B-containing NMDA receptors in CA1 hippocampus and cortex impairs long-term depression, reduces dendritic spine density, and disrupts learning. J Neurosci 30:4590-4600.

Chhatwal JP, Stanek-Rattiner L, Davis M, Ressler KJ (2006) Amygdala BDNF signaling is required for consolidation but not encoding of extinction. Nat Neurosci 9:870-872.

Dahl JP, Kampman KM, Oslin DW, Weller AE, Lohoff FW, Ferraro TN, O'Brien CP, Berrettini WH (2005) Association of a polymorphism in the Homer1 gene with cocaine dependence in an African American population. Psychiatr Genet 15:277-283.

Dash PK, Orsi SA, Moore AN (2009) Histone deactylase inhibition combined with behavioral therapy enhances learning and memory following traumatic brain injury. Neuroscience 163:1-8.

De Luca V, Annesi G, De Marco EV, de Bartolomeis A, Nicoletti G, Pugliese P, Muscettola G, Barone P, Quattrone A (2009) HOMER1 promoter analysis in Parkinson's disease: association study with psychotic symptoms. Neuropsychobiology 59:239-245.

Fan HY, Morgan SA, Brechun KE, Chen YY, Jaikaran AS, Woolley GA (2011) Improving a designed photocontrolled DNA-binding protein. Biochemistry 50:1226-1237.

Federman N, Fustinana MS, Romano A (2009) Histone acetylation is recruited in consolidation as a molecular feature of stronger memories. Learn Mem 16:600-606.

Girard BM, Keller ET, Schutz KC, May V, Braas KM (2004) Pituitary adenylate cyclase activating polypeptide and PAC1 receptor signaling increase Homer 1a expression in central and peripheral neurons. Regul Pept 123:107-116.

Govindarajan N, Agis-Balboa RC, Walter J, Sananbenesi F, Fischer A (2011) Sodium Butyrate Improves Memory Function in an Alzheimer's Disease Mouse Model When Administered at an Advanced Stage of Disease Progression. J Alzheimers Dis.

Hashikawa K, Matsuki N, Nomura H (2011) Preferential Arc transcription at rest in the active ensemble during associative learning. Neurobiol Learn Mem 95:498-504.

Heldt SA, Stanek L, Chhatwal JP, Ressler KJ (2007) Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. Mol Psychiatry 12:656-670.

Hu JH, Park JM, Park S, Xiao B, Dehoff MH, Kim S, Hayashi T, Schwarz MK, Huganir RL, Seeburg PH, Linden DJ, Worley PF (2010) Homeostatic scaling requires group I mGluR activation mediated by Homer1a. Neuron 68:1128-1142.

Inoue N, Nakao H, Migishima R, Hino T, Matsui M, Hayashi F, Nakao K, Manabe T, Aiba A, Inokuchi K (2009) Requirement of the immediate early gene vesl-1S/homer-1a for fear memory formation. Mol Brain 2:7.

Kammermeier PJ (2008) Endogenous homer proteins regulate metabotropic glutamate receptor signaling in neurons. J Neurosci 28:8560-8567.

Kaplan GB, Moore KA (2011) The use of cognitive enhancers in animal models of fear extinction. Pharmacol Biochem Behav 99:217-228.

Lattal KM, Barrett RM, Wood MA (2007) Systemic or intrahippocampal delivery of histone deacetylase inhibitors facilitates fear extinction. Behav Neurosci 121:1125-1131.

Levenson JM, O'Riordan KJ, Brown KD, Trinh MA, Molfese DL, Sweatt JD (2004) Regulation of histone acetylation during memory formation in the hippocampus. J Biol Chem 279:40545-40559.

Liu Y, Formisano L, Savtchouk I, Takayasu Y, Szabo G, Zukin RS, Liu SJ (2010) A single fear-inducing stimulus induces a transcription-dependent switch in synaptic AMPAR phenotype. Nat Neurosci 13:223-231.

Lohmer S, Maddaloni M, Motto M, Di Fonzo N, Hartings H, Salamini F, Thompson RD (1991) The maize regulatory locus Opaque-2 encodes a DNA-binding protein which activates the transcription of the b-32 gene. EMBO J 10:617-624.

Maren S (2005) Synaptic mechanisms of associative memory in the amygdala. Neuron 47:783-786.

Maze I, Covington HE, 3rd, Dietz DM, LaPlant Q, Renthal W, Russo SJ, Mechanic M, Mouzon E, Neve RL, Haggarty SJ, Ren Y, Sampath SC, Hurd YL, Greengard P, Tarakhovsky A, Schaefer A, Nestler EJ (2010) Essential role of the histone methyltransferase G9a in cocaine-induced plasticity. Science 327:213-216.

Mokin M, Zheng Z, Keifer J (2007) Conversion of silent synapses into the active pool by selective GluR1-3 and GluR4 AMPAR trafficking during in vitro classical conditioning. J Neurophysiol 98:1278-1286.

Monneret C (2005) Histone deacetylase inhibitors. Eur J Med Chem 40:1-13.

Monsey MS, Ota KT, Akingbade IF, Hong ES, Schafe GE (2011) Epigenetic alterations are critical for fear memory consolidation and synaptic plasticity in the lateral amygdala. PLoS One 6:e19958.

Musumeci G, Sciarretta C, Rodriguez-Moreno A, Al Banchaabouchi M, Negrete-Diaz V, Costanzi M, Berno V, Egorov AV, von Bohlen Und Halbach O, Cestari V, Delgado-Garcia JM, Minichiello L (2009) TrkB modulates fear learning and amygdalar synaptic plasticity by specific docking sites. J Neurosci 29:10131-10143.

Nedelescu H, Kelso CM, Lazaro-Munoz G, Purpura M, Cain CK, Ledoux JE, Aoki C (2010) Endogenous GluR1-containing AMPA receptors translocate to asymmetric synapses in the lateral amygdala during the early phase of fear memory formation: an electron microscopic immunocytochemical study. J Comp Neurol 518:4723-4739.

Nielsen HS, Georg B, Hannibal J, Fahrenkrug J (2002) Homer-1 mRNA in the rat suprachiasmatic nucleus is regulated differentially by the retinohypothalamic tract transmitters pituitary adenylate cyclase activating polypeptide and glutamate at time points where light phase-shifts the endogenous rhythm. Brain Res Mol Brain Res 105:79-85.

Rattiner LM, Davis M, Ressler KJ (2004a) Differential regulation of brain-derived neurotrophic factor transcripts during the consolidation of fear learning. Learn Mem 11:727-731.

Rattiner LM, Davis M, French CT, Ressler KJ (2004b) Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. J Neurosci 24:4796-4806.

Ressler KJ, Mercer KB, Bradley B, Jovanovic T, Mahan A, Kerley K, Norrholm SD, Kilaru V, Smith AK, Myers AJ, Ramirez M, Engel A, Hammack SE, Toufexis D, Braas KM, Binder EB, May V (2011) Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. Nature 470:492-497.

Rietschel M et al. (2010) Genome-wide association-, replication-, and neuroimaging study implicates HOMER1 in the etiology of major depression. Biol Psychiatry 68:578-585.

Rogan MT, Staubli UV, LeDoux JE (1997) Fear conditioning induces associative long-term potentiation in the amygdala. Nature 390:604-607.

Rumpel S, LeDoux J, Zador A, Malinow R (2005) Postsynaptic receptor trafficking underlying a form of associative learning. Science 308:83-88.

Sato M, Suzuki K, Nakanishi S (2001) NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. J Neurosci 21:3797-3805.

Szumlinski KK, Kalivas PW, Worley PF (2006) Homer proteins: implications for neuropsychiatric disorders. Curr Opin Neurobiol 16:251-257.

Tronson NC, Guzman YF, Guedea AL, Huh KH, Gao C, Schwarz MK, Radulovic J (2010) Metabotropic glutamate receptor 5/Homer interactions underlie stress effects on fear. Biol Psychiatry 68:1007-1015.

Zhu WG, Otterson GA (2003) The interaction of histone deacetylase inhibitors and DNA methyltransferase inhibitors in the treatment of human cancer cells. Curr Med Chem Anticancer Agents 3:187-199.