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The Contribution of the Prefrontal Cortex in Fear Memory Specificity

Author

Vieira, Philip Andrew

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The Contribution of the Prefrontal Cortex in Fear Memory Specificity

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Philip Andrew Vieira

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Dissertation Committee:
    Dr. Edward Korzus, Chairperson
    Dr. Peter Hickmott
    Dr. Glenn Stanley

The Dissertation of Philip Andrew Vieira is approved:

Committee Chairperson

University of California, Riverside

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Dedication

This work, the direct culmination of half a decade graduate education, built upon a foundation of elementary, secondary, and baccalaureate education, is dedicated to my parents, Maria Fatima Garcia Vieira and José de Freitas Lourenço Vieira, and to my daughter, Ronia Lora Bunckenburg.

ABSTRACT OF THE DISSERTATION

The Contribution of the Prefrontal Cortex in Fear Memory Specificity

by

Philip Andrew Vieira

Doctor of Philosophy, Graduate Program in Psychology
University of California, Riverside, December 2014
Dr. Edward Korzus, Chairperson

Several mental disorders present with symptoms related to inaccurate fear memory discrimination, including post-traumatic stress disorder (PTSD), in which a non-aversive stimulus resembling a previously experienced aversive situation triggers a fear response. The neural mechanisms underlying the attainment of fear memory accuracy for appropriate discriminative responses to aversive and non-aversive stimuli are unclear. While much research has involved the hippocampus in these processes, more recent studies implicate the medial prefrontal cortex (mPFC). By using a combination of novel behavioral paradigms and viral-mediated genetic manipulation, we were able to disrupt specific processes within the mPFC and measure their effect on fear memory accuracy. We found that several elements important to the cellular mechanisms underlying memory consolidation within the mPFC are required for appropriate discrimination of aversive and non-aversive stimuli with overlapping qualities of similarity. These include cyclic

AMP response element binding protein (CREB), CREB-binding protein (CBP) histone acetyltransferase (HAT) activity, and *N*-methyl-D-aspartate receptors (NMDARs). This work contributes to the growing body of literature on prefrontal contributions to memory systems at the basic level, and can be applied to our understanding of deficient memory specificity found in disorders like PTSD.

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Introduction

Memory specificity, or the ability to recognize and discriminate multiple distinctive stimuli that share common features, is an important adaptive process that allows animals to respond appropriately to an ever-changing environment. Likewise, memory generalization enables animals to approach novel experiences by referring to previously stored instances that resemble the new situation. There exists a normal balance between memory specificity and memory generalization, but when this balance becomes disrupted, it can lead to inappropriate responses to the environmental stimuli. This is seen in the overgeneralization characteristic of posttraumatic stress disorder (PTSD) and major depression, in which a safe environmental cue triggers a past traumatic event (Gotlib and Joormann 2010, Mahan and Ressler 2012), and in the overspecificity seen in autism spectrum disorder (ASD), obsessive compulsive personality disorder (OCPD) and attention deficit/hyperactivity disorder (ADHD), in which common environmental cues become sources of focus and distraction (Goldberg, Mostofsky et al. 2005, Sahay, Wilson et al. 2011). Understanding the delicate balance between memory specificity and generalization is important for the diagnosis and treatment of these disorders, and has led to numerous theories about the underlying neurobiological processes involved in memory discrimination.

As with most memory-related processes, early research focused on the hippocampus as a site for memory specificity and generalization (Freeman, Kramarcy et al. 1973, Frankland, Cestari et al. 1998). More recently, evidence suggests that the medial prefrontal cortex (mPFC) is involved with memory generalization (Xu, Morishita et al.

2012) and memory specificity via a functionally connected circuit between the nucleus reuniens (NR), the hippocampus (HPC), and the mPFC (Xu and Sudhof 2013). In the latter paper, researchers used a combination of viral-mediated synaptic silencing via tetanus-toxin and optogenetic probes to stimulate specific populations of neurons within this network, showing that the mPFC controls memory specificity through a direct connection to the NR, which has not only reciprocal connectivity with the mPFC, but also a functional connection to the HPC, which in turn connects directly and indirectly to the mPFC. This effect was shown to be specific during the acquisition stage of memory, which is consistent with the literature supporting a role of the mPFC as an executive control for working memory (Fuster 2001).

Less research has focused on the mPFC as a site for encoding of discrimination memory for long-term storage. Eichenbaum demonstrated that disruption of the mPFC with ibotenic acid infusions (resulting in significant cell death) impairs relational memory in an olfactory discrimination task with overlapping odors for both acquisition and retrieval (DeVito, Lykken et al. 2010). These results are confounded by the severity of ibotenic acid treatment (damaging up to 81% of the mPFC) and the presence of mPFC lesion throughout all stages of the olfactory discrimination task.

Decades of research on the cellular factors underlying memory consolidation offer several candidate mechanisms to investigate. Pharmacological studies implicated *N*-methyl-D-aspartate receptors (NMDARs) in long-term plasticity and memory (Morris, Anderson et al. 1986), which was later confirmed by selective genetic deletion of the *NR1* gene encoding an obligatory subunit for NMDAR, resulting in impaired memory and deficient long-term plasticity (Tsien, Huerta et al. 1996). Persistent synaptic activation opens NMDARs, which flux calcium to bind to calmodulin, which in turn activates adenylyl cyclase, producing cyclic AMP (cAMP), which activates protein kinase A (PKA), triggering eventual phosphorylation of cAMP response element binding protein (CREB) (reviewed in Klann 2002), a protein that has been shown to be required for memory consolidation (Dash, Hochner et al. 1990, Bourtchuladze, Frenguelli et al. 1994, Yin, Wallach et al. 1994, Josselyn, Shi et al. 2001, Kida, Josselyn et al. 2002, Pittenger, Huang et al. 2002). Phosphorylation of CREB at serine 133 is required for interaction with the nuclear protein CREB-binding protein (CBP) (Gonzalez, Yamamoto et al. 1989, Chrivia, Kwok et al. 1993), which promotes gene transcription by providing a scaffold for transcription factor integration, bridging DNA-binding transcription factors and transcriptional machinery, and by modifying transcription factors and chromatin through its intrinsic histone acetyltransferase (HAT) activity (Bannister and Kouzarides 1996, Korzus, Torchia et al. 1998, Janknecht 2002). CBP HAT activity has been recently studied through genetic mutation, in which the acetyl-CoA binding site (amino acids Y1540/F1541) was substituted with alanine, generating a CBP mutant lacking HAT activity (Bannister and Kouzarides 1996, Korzus, Torchia et al. 1998, Korzus, Rosenfeld et al. 2004). This mutation maintains all other protein binding domains on CBP, allowing it to carry out other functions except histone acetyltransferase, an essential epigenetic mechanism that transfers a acetyl group to the histone tails, causing the chromatin the relax to allow subsequent gene transcription. By driving this mutation to excitatory forebrain neurons under conditional control of doxycycline, this group found that CBP

HAT activity is required for memory consolidation(Korzus, Rosenfeld et al. 2004). Requirement for CBP in memory consolidation has been confirmed in other studies (Alarcon, Malleret et al. 2004, Korzus, Rosenfeld et al. 2004, Wood, Kaplan et al. 2005, Chen, Zou et al. 2010, Barrett, Malvaez et al. 2011, Valor, Pulopulos et al. 2011, Peixoto and Abel 2012, Maddox, Watts et al. 2013, Valor, Viosca et al. 2013). Taken together, NMDAR, CREB, and CBP HAT form a hierarchical system of long-term synaptic changes thought to underlie memory consolidation.

We therefore sought to characterize the role of the mPFC in the discrimination of aversive and non-aversive stimuli reliant on memory specificity. By implementing a contextual fear discrimination task and a novel auditory fear discrimination task, we were able to track fear memory acquisition and retrieval, generalization, and eventual discrimination across time. We also took advantage of viral and genetic mediated manipulation of specific neural populations in discrete brain regions to tease apart several steps along the pathway toward long-term synaptic plasticity, including NMDA receptor (NMDAR) knockdown, inhibition of cAMP response element binding protein (CREB), and specific manipulation of CREB-binding protein (CBP) histone acetyltransferase (HAT) activity. These three elements are critical to long-term memory formation and consolidation. NMDAR has been extensively researched and shown to be a requirement for long-term synaptic plasticity and memory (Collingridge and Bliss 1995), especially fear memory consolidation (Shimizu, Tang et al. 2000), fear memory extinction (Milad and Quirk 2002), and discrimination memory (McHugh, Jones et al. 2007). CREB has been long shown to be a requirement for cellular memory consolidation, as demonstrated in Drosophila, Aplysia, and mice (Dash, Hochner et al. 1990, Bourtchuladze, Frenguelli et al. 1994, Yin, Wallach et al. 1994, Josselyn, Shi et al. 2001, Kida, Josselyn et al. 2002, Pittenger, Huang et al. 2002). CBP HAT activity has also been implicated in memory consolidation as well as long-term synaptic plasticity (Alarcon, Malleret et al. 2004, Korzus, Rosenfeld et al. 2004). We found evidence to support the role of the mPFC in memory specificity due to long-lasting changes within circumscribed populations of neurons. Specifically, we observed a consistent deficit in discrimination memory when inhibiting CBP HAT activity, CREB activity, and after deletion of the NR1 obligatory subunit of the NMDA receptor within the mPFC. These observations suggest a critical role for long-term plasticity dependent-memory consolidation within the mPFC in balancing memory specificity and generalization.

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Chapter I:

Prefrontal consolidation supports the attainment of fear memory accuracy

Abstract

Considerable evidence indicates that coactivator of transcription and histone acetyltransferase cAMP response element binding protein (CREB) binding protein (CBP) is critically required for normal neural function. CBP hypofunction leads to severe psychopathological symptoms in human and cognitive abnormalities in genetic mutant mice with severity dependent on the neural locus and developmental time of the gene inactivation. Here, we showed that an acute hypofunction of CBP in the medial prefrontal cortex (mPFC) results in a disruption of fear memory accuracy in mice. In addition, interruption of CREB function in the mPFC also leads to a deficit in auditory discrimination of fearful stimuli. While mice with deficient CBP/CREB signaling in the mPFC maintain normal responses to aversive stimuli, they exhibit abnormal responses to similar but non-relevant stimuli when compared to control animals. These data indicate that improvement of fear memory accuracy involves mPFC-dependent suppression of fear responses to non-relevant stimuli. Evidence from a context discriminatory task and a newly developed task that depends on the ability to distinguish discrete auditory cues indicated that CBP-dependent neural signaling within the mPFC circuitry is an important component of the mechanism for disambiguating the meaning of fear signals with two opposing values: aversive and non-aversive.

Introduction

The ability to discriminate between similar, yet different, stimuli is critical for cognitive functioning (O'Reilly and McClelland 1994) and is referred to as memory specificity or memory accuracy. Failure to discriminate between aversive and non-aversive stimuli during recall may indicate decreased memory resolution (i.e. reduced access to memory details) or generalized fear or both, and may lead to inappropriate stimulus generalization. Generalization is not always inappropriate and this type of reduced fear memory accuracy is observed when one responds the same to two stimuli that are not identical. After initial generalization, fear memory accuracy can be increased through additional experiences with reinforced aversive stimulus and non-reinforced non-aversive stimulus. Conversely, overgeneralized fear is a typical symptom of anxiety disorders including phobias and posttraumatic stress disorder (PTSD), which are triggered by cues resembling traumatic experience in a secure environment (Mahan and Ressler 2012). Studies of neural substrates and mechanisms underlying memory resolution are focused on the hippocampal circuit (Leutgeb et al. 2007; Sahay et al. 2011). Recent studies also implicate prefrontal circuitry in the contextual fear memory specificity and generalization (Xu et al. 2012; Xu and Sudhof 2013) or discrimination of more discrete multiple odor stimuli (DeVito et al. 2010).

Regulatory mechanisms direct cAMP response element binding protein (CREB)-dependent transcription subsequent to learning-induced molecular changes in which neurons play a pivotal role in the conversion of short-term to long-term memory across species (Dash et al. 1990; Bourtchuladze et al. 1994; Yin et al. 1994; Josselyn et al. 2001;

Kida et al. 2002; Pittenger et al. 2002). Phosphorylation of CREB at serine 133 is required for the recruitment of the chromatin-remodeling factor with intrinsic acetyltransferase activity CREB binding protein (CBP), both events critical for CREB-dependent transcription (Gonzalez et al. 1989; Chrivia et al. 1993). CBP integrates multiple signaling pathways via direct interactions with independently regulated multiple transcriptional factors and components of transcriptional machinery. In addition, CBP comprises enzymatic activity referred to as HAT (histone acetyltransferase), which enables acetylation of conserved lysine amino acids on proteins by catalyzing a transfer of an acetyl group of acetyl CoA to form ε-*N*-acetyl-lysine (Bannister and Kouzarides 1996; Korzus et al. 1998). Initially histones were considered as primary natural substrates for CBP enzymatic activity. However histones are not the only targets for CBP's HAT activity and a number of non-histone potential targets for CBP's HAT activity have been found, including proteins regulating chromatin remodeling and gene expression such as p53 (Gu and Roeder 1997), CREB (Lu et al. 2003) and many others (Kouzarides 2000; Sterner and Berger 2000; Yang 2004; Glozak et al. 2005; Kimura et al. 2005). Impact of histone and non-histone protein acetylation by CBP is not fully understood. Despite uncertainty in respect to how CBP controls neuronal function via its interaction with multiple regulatory proteins and acetyltransferase activity, considerable evidence indicates that CBP is a critical component of the neural signaling underlying cognitive functioning (Alarcon et al. 2004; Korzus et al. 2004; Wood et al. 2005; Chen et al. 2010; Barrett et al. 2011; Valor et al. 2011; Peixoto and Abel 2012; Maddox et al. 2013; Valor et al. 2013). However it is difficult to separate developmental defects, compensatory developmental effects and acute function in the adult brain of a gene with pronounced developmental functions. To avoid developmental confounds, four independent manipulations to downregulate CBP acetyltransferase activity specifically in the adult living brain have been reported to date. Acute CBP hypofunction targeted specifically in adult mice by means of Tet-regulatable expression of CBPΔHAT targeted to excitatory forebrain neurons (Korzus et al. 2004) or hippocampal focal knockout of CBP (Barrett et al. 2011) or intra lateral amygdala infusion of c646, a selective pharmacological inhibitor of p300/CBP activity, shortly following fear conditioning (Maddox et al. 2013) resulted in selective impairment of long-term potentiation (Barrett et al. 2011; Maddox et al. 2013) and long-term memory (Korzus et al. 2004; Barrett et al. 2011; Maddox et al. 2013). In addition, ablation of CBP in adult brain resulted in impaired environmental enrichment-induced neurogenesis (Lopez-Atalaya et al. 2011), which suggest additional role of CBP in adult neurogenesis-dependent enhancement of adaptability toward novel experiences (Aimone et al. 2011; Sahay et al. 2011). These data strongly implicate CBP acetyltransferase activity in neural epigenetic signaling underlying long-term memory consolidation.

Although there has been extensive research into the function of the PFC during information acquisition and retrieval, a fundamental question that has escaped resolution is whether CBP-dependent signaling within the prefrontal cortex supports mechanisms in which fear memories are encoded and retrieved without confusion. Using mutant mice expressing dominant negative CBP with eliminated acetyltransferase activity, we have tested the impact of CBP-dependent mechanisms in the mPFC on fear memory accuracy.

Evidence from context and auditory discriminatory tasks indicated that the mPFC circuitry is critical for the acquisition of fear memory accuracy necessary for the recognition of subtle differences between aversive and non-aversive stimuli. These data indicate that CBP-dependent signaling in the mPFC is critical for the suppression of fear responses to non-relevant stimuli, which is a necessary process towards improvement of fear memory accuracy.

Results

Impairment of contextual fear memory specificity in CBPΔHAT^{PFC} mice

The CBPΔHAT mutant, a dominant-negative inhibitor of CBP-dependent lysine acetylation, harbors a substitution mutation of two conserved residues (Tyr^{1540}/Phe^{1541} to Ala^{1540}/Ala^{1541}) in the acetyl CoA binding domain (Korzus et al. 1998; Korzus et al. 2004). This mutant has no intrinsic acetyltransferase activity due to its inability to interact with a donor of acetyl group, acetyl-CoA but retains all protein-protein interaction domains (Korzus et al. 1998). When expressed acutely in adult excitatory neurons, CBPΔHAT functions as a specific blocker of long-term memory consolidation without affecting information acquisition or short-term memory (Korzus et al. 2004). To test the impact of CBP-dependent signaling in the medial prefrontal cortex (mPFC) on fear memory specificity, we generated mice expressing CBPΔHAT and eGFP in the mPFC using virus-mediated gene transfer (referred to as CBPΔHAT^{PFC} mice) (Fig. 1.1A). For control mice, we injected virus-expressing eGFP only in the mPFC. Cytohistological analysis of brain tissue isolated from CBPΔHAT^{PFC} and control animals revealed that the majority of cells expressing mutant protein in the mPFC were neurons (Ctrl: 93.85 ± 0.006%, n = 3; CBPΔHAT^{PFC}: 92.06 ± 0.012 %, n = 3; $t_{(2)}$ = -0.03, p = 0.511, r = 0.013, data not shown). Conditioned CBPΔHAT^{PFC} mice display decreased level of acetylated histone H3 (t-test: t(10) = 2.38 , p = 0.0382, r = 0.6013; Ctrl: 1 ± 0.06, n = 5, CBPΔHAT^{PFC}: 0.74 ± 0.06, n = 7) and acetylated histone H4 (Ac-H4; left panel; t-test: t(10) = 2.9718 , p = 0.0140, r = 0.6848; Ctrl: 1 ± 0.04, n = 6, CBPΔHAT^{PFC}: 0.67 ± 0.10, n = 6) in cells expressing GFP when compared to conditioned control animals (Fig. 1.1B). These data are consistent with previous studies reporting decreased levels of acetylated histones in CBP mutant mice (Alarcon et al. 2004; Wood et al. 2005; Chen et al. 2010; Barrett et al. 2011; Valor et al. 2011; Peixoto and Abel 2012).

We examined CBPΔHAT^{PFC} mice using the fear-conditioning paradigm (Fig. 1.1C). CBPΔHAT^{PFC} mice performed similar to controls in the contextual version of the fear conditioning task after a 24 h delay (Fig. 1.1C; Ctrl: 25.78 %, n = 10; CBPΔHAT^{PFC}: 22.14 %, n = 10; $t_{(18)}$ = 1.28, p = 0.108). To determine whether the mPFC supports fear memory accuracy, we examined CBPΔHAT^{PFC} mice using the context fear discrimination task (Lovelace et al. 2014) (Fig. 1.1D). First, we tested CBPΔHAT^{PFC} and control mice on a generalization task, in which we examined the freezing responses to novel context B after training on the fear conditioning task to context A. Context B was similar yet not identical to the training context A (see Methods). We found no difference in freezing responses to context B or A in CBPΔHAT^{PFC} and control mice (Fig. 1.1E. Context A vs. B t-test: Ctrl, n = 9, p = 0.805; CBPΔHAT^{PFC}, n = 11, p = 0.851). Thus, CBPΔHAT^{PFC} mice did not demonstrate any obvious abnormalities in fear memory generality during the initial presentation of novel context B. Next, CBPΔHAT^{PFC} mice and control littermates were trained to distinguish between the conditioned context A, which was paired with a footshock (CS$^+$) and an unconditioned context B, which was not paired with any reinforcement (CS$^-$) over multiple training sessions (Fig. 1.1D). This task requires temporal integration because animals learn subtle differences between context A and B over many days with a single exposure to each context only once per day. Initially, the control and CBPΔHAT^{PFC} mice generalized their conditioned responses and exhibited similar freezing levels to both the CS$^+$ and CS$^-$ contexts (block trials 1-4). However, the control animals began to freeze significantly less in response to context B compared to context A after 4 block trials of training, demonstrating the ability to consistently distinguish between similar yet different contexts (block trials 5-6) (Fig. 1.1F; RM-ANOVA of trial block and context: Context: $F_{(1,8)} = 9.423$, $p = 0.015$; Trial block, $F_{(5,40)} = 3.24$, $p = 0.015$; Trial block x Context: $F_{(5,40)} = 6.58$, $p = 0.0001$; $n = 9$). *Post hoc* analysis using Bonferroni correction for multiple comparisons indicated that differences were present during trial blocks 5 ($p = 0.003$) and 6 ($p = 0.005$). In contrast to the control animals, CBPΔHAT^{PFC} mice failed to distinguish between context A and B and continued to generalize their conditioned responses throughout all 12 days of training (Fig. 1.1G, RM-ANOVA of trial block and context: Context: $F_{(1,10)} = 5.42$, $p = 0.04$; Trial block: $F_{(2,15)} = 11.09$, $p = 0.002$; Trial Block x Context: $F_{(3,27)} = 1.62$, $p = 0.21$; $n = 11$). These data demonstrated that CBPΔHAT expressed in the mPFC resulted in imbalanced neural processes underlying fear memory specificity and generalization. Analysis of the context discrimination ratio confirmed that at the end of the training, the control animals performed better on the context discrimination task compared to the CBPΔHAT^{PFC} mice. Figure 1.1H shows no difference in performance between control and CBPΔHAT^{PFC} animals on trial block 1 (t-test: $t_{(18)} = 0.02$, $p = 0.99$, $r = 0.005$), but a marked difference on trial block 6 (t-test: $t_{(18)} = 2.60$, $p = 0.018$, $r = 0.52$). These findings demonstrate that CBPΔHAT^{PFC} mice have a strong deficit in context discrimination.

Hypothetically, learning of appropriate responses to fearful and similar but not relevant stimuli may involve changes in response to aversive stimuli or non-aversive or both across the entire training. Therefore we analyzed fear responses to Context A (CS+) and, separately, to Context B (CS-) in CBPΔHAT^{PFC} and control mice. There was no difference in responses to conditioned stimuli CS+ between CBPΔHAT^{PFC} and control mice across the entire context discrimination training (Fig. 1.1F-G; RM-ANOVA of trial blocks 1-5 and group: Trial Block X Group: $F_{(2.7,\ 47.9)} = 1.782$, $p = 0.169$). However, CBPΔHAT^{PFC} and control mice responded differently to non-relevant stimuli CS- across training on the context discriminatory task (Fig. 1.1F-G; RM-ANOVA of trial blocks 1-5 and group: Trial Block x Group: $F_{(2.9,\ 51.6)} = 4.919$, $p = 0.005$). Change in freezing to CS- across the training (freezing delta) was significantly higher in CBPΔHAT^{PFC} when compared to control mice (Figure 1.1I; t-test: $t_{(18)} = -2.235$, $p = 0.038$). However, calculations of freezing delta consider only performance on trial blocks 1 and 6. In order to include performance of tested animals on each day across the entire training on the contextual discriminatory task (Fig. 1.1F-G; Trial Blocks 1-6), we compared average slopes (α) of fitted learning curves (Fig. 1.1J). The learning of appropriate responses to CS+ shows a positive slope in both control ($\alpha = 4.76 \pm 1.07$; where $\alpha =$ slope) and

$\mathrm{CBP\Delta HAT^{PFC}}$ (α= 6.35±1.61) mice and there is no difference between groups (t-test; $t_{(18)} = -0.778$, p = 0.446). The learning of appropriate response to CS- shows a negative slope in the control group (α= -0.88±1.34), which significantly improved fear memory accuracy at the end of training (Fig. 1.1F). In contrast, the $\mathrm{CBP\Delta HAT^{PFC}}$ group, which failed to improve fear memory accuracy across training (Figure 1.1G), showed a positive slope for CS- (α= 4.26±1.4), a marked difference from control responses to the CS- (CS-/Ctrl: α= -0.88±1.34; CS-/$\mathrm{CBP\Delta HAT^{PFC}}$: α= 4.26±1.4); CS- slope/Ctrl vs $\mathrm{CBP\Delta HAT^{PFC}}$ t-test; $t_{(18)} = -2.614$, p = 0.018). In summary, analysis of patterns of responses to Context A (CS+) and Context B (CS-) in control animals revealed that the improvement of contextual fear memory accuracy was due to increased freezing behavior to the CS+ and a decrease in freezing to CS-. CBP hypofunction in the mPFC altered the ability to learn discriminatory responses to CS+ versus CS- by disrupting the pattern of the learning curve for CS- only. These data suggest that the mPFC supports the improvement of contextual fear memory accuracy by controlling acquisition of appropriate responses to non-relevant stimuli.

We also found that $\mathrm{CBP\Delta HAT^{PFC}}$ mice performed similar to controls in the cued version of the fear conditioning task during acquisition (data not shown: $F_{(5,90)}=1.49$, p = 0.201) and after a 24 h delay (Fig. 1.2A. Ctrl: 47.17 ± 5.82 %, n = 10; $\mathrm{CBP\Delta HAT^{PFC}}$: 57.27 ± 7.21 %, n = 10; $t_{(18)} = -1.042$, p = 0.324, r = -0.096). These data indicate that information acquisition and long-term memory examined with a 24 hr delay on contextual (Fig. 1.1C) and cued fear-conditioning (Fig. 1.2A, 1.4A-B) were normal in $\mathrm{CBP\Delta HAT^{PFC}}$ mice. The normal performance of $\mathrm{CBP\Delta HAT^{PFC}}$ on these fear-conditioning tasks (Fig. 1.1C, 1.2A, 1.4A-B) indicates that CBPΔHAT^{PFC} mice have functioning circuitry underlying Pavlovian conditioning.

CBPΔHAT^{PFC} mice showed normal levels of locomotor activity (Fig. 1.2B-D. Total Distance Traveled: Ctrl, 46159.94 ± 1335 mm, n = 12; CBPΔHAT^{PFC}, 43563.67 ± 4730.60 mm, n = 16; $t_{(11)} = -0.43$, $p = 0.6627$, $r = 0.1289$. Average Velocity: Ctrl: 51.52 ± 1.50 mm/s, n = 12; CBPΔHAT^{PFC}: 48.43 ± 5.23 mm/s, n = 16; $t_{(11)} = -0.367$, $p = 0.6399$, $r = 0.1101$) and normal anxiety-related responses (Fig. 1.2E. Thigmotaxis: Ctrl: 58.58 ± 4.12 %, n = 12; CBPΔHAT^{PFC}: 66.37 ± 6.14 %, n =16; $t_{(11)} = 0.34$, $p = 0.3689$, $r = 0.1030$).

Impairment of auditory memory specificity in CBPΔHAT^{PFC} mice

To evaluate if the deficient discrimination of aversive and non-aversive external stimuli was sensory input-specific, we examined CBPΔHAT^{PFC} mice using a novel auditory discrimination task, which tests the ability of subjects to recognize the direction of frequency modulated (FM)-sweeps (trains of upward and downward FM-sweeps) (Fig.1.3). This assay includes 3 days of acquisition (single CS^+ footshock pairing) followed by a 24hr test on day 4 and a generalization test on day 4-5. Discrimination training takes place on days 7-12 in which animals are run through 3 sessions: first, they are tested for freezing to CS^+ and CS^- (in context C); second, they are exposed to CS^+ (or CS^-); third, they are exposed to CS^- (or CS^+).

In parallel experiments, we also microinjected into the mPFC an HSV virus encoding a mutant form of CREB (mCREB) and tested these mice (mCREB^{PFC}) in the auditory discrimination task. CREB is implicated in memory consolidation across variety of species (Dash et al. 1990; Bourtchuladze et al. 1994; Yin et al. 1994; Josselyn et al. 2001; Kida et al. 2002; Pittenger et al. 2002) and functions immediately upstream of CBP. mCREB (CREB^{S133A} mutation) cannot be phosphorylated at the key serine 133 residue and, therefore, cannot recruit CBP and activate transcription (Gonzalez et al. 1989; Chrivia et al. 1993). Thus we have tested a possible involvement of this well-recognized mediator of memory consolidation in auditory fear discrimination in parallel experiments to those performed in CBPΔHAT^{PFC} mice.

We first examined FM-sweep fear conditioning acquisition in CBPΔHAT^{PFC} and mCREB^{PFC} mice. All three groups: the CBPΔHAT^{PFC}, mCREB^{PFC} and control mice similarly acquired this form of Pavlovian conditioning (Fig. 1.4A; RM-ANOVA of Day and Group: $F_{(4,82)} = 0.975$, $p = 0.426$) and showed the same performance on the 24-hr memory test (Fig. 1.4B; two way ANOVA of Group and Baseline/24 h-Test; Group: $F_{(2,82)} = 0.777$, $p = 0.463$; Baseline/24 h-Test: $F_{(1,82)} = 688.3$, $p = 1.2\text{x}10^{-41}$; Group x Baseline/24 h-Test: $F_{(2,82)} = 0.205$, $p = 0.815$). These data demonstrate that information acquisition and long-term memory tested after a 24-hr delay on FM-sweep fear conditioning was normal in CBPΔHAT^{PFC} and mCREB^{PFC} mice. We also tested CBPΔHAT^{PFC}, mCREB^{PFC} and control mice on generalization tasks, in which we examined their freezing responses to novel downward FM sweep (CS-) after training on the upward FM-sweep (CS+) fear conditioning task. The generalization test revealed that there was no difference in the freezing responses to the CS- or CS+ between CBPΔHAT^{PFC}, mCREB^{PFC} and control mice (Fig. 1.4C; ANOVA of FM-sweep direction and group during day 4 and 5: Group: $F_{(2,82)} = 0.37$, $p = 0.692$; ANOVA of FM-sweep direction: $F_{(1,82)} = 3.458$, $p = 0.067$; Group x FM-Sweep Direction: $F_{(2,82)} = 0.090$, $p = 0.914$). These data indicate that strong generalization was observed during days 4 and 5 in all three tested groups.

Next, the animals underwent auditory discrimination training (Fig. 1.4D-F). Initially, the control, CBPΔHAT^{PFC} and mCREB^{PFC} mice generalized their conditioned responses and exhibited similar levels of freezing responses to both CS^+ and CS^- (days 1-2). However, after 2 days of training, the control animals exhibited a higher number of freezing responses to CS^+ and significantly fewer freezing responses to CS^- compared to CS^+, demonstrating the ability to consistently distinguish between similar yet different auditory patterns (days 9-12) (Fig. 1.4D; RM-ANOVA of Day and FM-sweep direction: Day x FM-sweep direction: $F_{(2.2,33.5)} = 10.776$, $p = 0.0002$, $n = 16$). *Post hoc* analysis using Bonferroni correction (alpha = 0.0083) for multiple comparisons indicated that differences were present during days 9 (CS^+ vs CS^- t-test: $t_{(30)} = 3.632$, $p = 0.001$, $r = 0.55$), 10 ($t_{(30)} = 5.227$, $p = 0.00001$, $r = 0.69$), 11 ($t_{(30)} = 7.540$, $p = 2.1 \times 10^{-08}$, $r = 0.81$) and 12 ($t_{(30)} = 9.253$, $p = 2.7 \times 10^{-10}$, $r = 0.86$) only.

CBPΔHAT^{PFC} mice demonstrated weak ability to discriminate between CS^+ and CS^-, and only during the last two days of training (Fig. 1.4E, RM-ANOVA of Day and FM-sweep direction: Day x FM-sweep direction: $F_{(5,70)} = 5.071$, $p = 0.001$, $n = 15$). *Post hoc* analysis using Bonferroni correction for multiple comparisons indicated that differences were present during days 11 (CS^+ vs CS^- t-test: $t_{(28)} = 3.149$, $p = 0.004$, $r = 0.51$) and 12 ($t_{(28)} = 3.325$, $p = 0.002$, $r = 0.53$) only. In contrast to the control animals, CBPΔHAT^{PFC} mice continued to generalize their conditioned responses after 2 days of training and failed to distinguish between context A and B during days 9 and 10 (Day 3: $p = 0.286$; Day 4: $p = 0.291$).

Clearly, CBPΔHAT^{PFC} mice demonstrated strong deficit in auditory memory specificity when compared to controls (Fig. 1.4D-E, RM-ANOVA of Group and FM-sweep direction and Day 7-12: Group x FM-sweep direction x Day: $F_{(2.8,81.4)} = 3.033$, $p = 0.037$; Group x FM-sweep direction: $F_{(1,29)} = 7.86$, $p = 0.009$; CBPΔHAT^{PFC}, n=15; Ctrl, n = 16). Furthermore, analysis of discrimination ratios shows difference in performance between control and CBPΔHAT^{PFC} animals on days 10-12 (Fig. 1.4G. Discrimination Index CBPΔHAT^{PFC} vs. Ctrl t-test: Day 10: $t_{(29)} = 2.813$, $p = 0.0087$, $r = 0.46$; Day 11: $t_{(29)} = 3.546$, $p = 0.001$, $r = 0.55$, Day 12: $t_{(29)} = 3.643$, $p = 0.001$, $r = 0.56$; CBPΔHAT^{PFC}, n=15; Ctrl, n = 16) but not during the initial phase of training. Clearly, control mice show better performance then CBPΔHAT^{PFC} mice on auditory discrimination (Fig. 1.4D-E, G). Taken together, these data demonstrate that CBPΔHAT expressed in the mPFC resulted in abnormal auditory (FM-sweep direction) fear memory specificity.

Similarly to CBPΔHAT^{PFC} animals, mCREB^{PFC} mice demonstrated a strong deficit in memory specificity during the discrimination phase when compared to controls on the auditory discrimination task (Fig. 1.3F; RM-ANOVA, Group x FM-sweep direction x Day: $F_{(2.8,79.6)} = 4.644$, $p = 0.006$; mCREB^{PFC}, n=14; Ctrl, n = 16). These data demonstrated that mCREB^{PFC} expressed in the mPFC prevented an improvement of auditory memory accuracy across the training as observed in control mice (Fig. 1.4D). Analysis of the auditory discrimination ratio confirmed that at the end of the training, the control animals performed better on the auditory discrimination task compared to the mCREB^{PFC} mice (Fig. 1.4H, RM-ANOVA of Day and Group: Day x Group: $F_{(2.5,69.0)}$ = 5.149, p = 0.005; mCREB^{PFC}, n=14; Ctrl, n = 16). Furthermore, analysis of discrimination ratios showed a strong difference in performance between control and mCREB^{PFC} animals on days 10-12 (t-test; day 10: $t_{(28)}$ = 2.232, p = 0.034, r = 0.39; day 11: $t_{(28)}$ = 4.130, p = 0.0003, r = 0.62; day 12: $t_{(28)}$ = 4.313, p = 0.0002, r = 0.63; mCREB^{PFC}, n=14; Ctrl, n = 16).

Next, we performed an analysis of fear responses to upsweep (CS+) and, separately, to downsweep (CS-) in control, CBPΔHAT^{PFC} and mCREB^{PFC} mice tested on FM-sweep direction fear discriminatory task (Fig. 1.4). There was no difference in responses to conditioned stimuli CS+ between CBPΔHAT^{PFC} and control mice across the entire FM-sweep direction discrimination training (Fig. 1.4D-E; CS+/CBPΔHAT^{PFC} vs Ctrl; RM-ANOVA of days 7-12 and group: Day X Group, $F_{(2.8, 81.6)}$ = 0.756, p = 0.514). Similarly, there was no difference in responses to conditioned stimuli CS+ between mCREB^{PFC} and control mice across entire FM-sweep direction discrimination training (Fig. 1.4D, F; CS+/mCREB^{PFC} vs Ctrl; RM-ANOVA of days 7-12 and group: Day X Group: $F(_{2.8, 79.5)}$ = 1.808, p = 0.155). An analysis of learning curves (Fig. 1.4J) showed a positive slope to CS+ in control (α= 2.366±0.82) and CBPΔHAT^{PFC} (α=2.384±0.894) mice or no change in freezing responses to CS+ in mCREB^{PFC} mice (α=-0.278±1.15) across the entire FM-sweep direction fear discriminatory task. In fact, there was no difference in the learning (slopes) of appropriate responses to CS+ between CBPΔHAT^{PFC} and control groups (Fig. 1.4J; CS+ slope/Ctrl vs CBPΔHAT^{PFC} t-test; $t_{(29)}$ = -0.015, p = 0.988) or mCREB^{PFC} and control mice (Fig. 1.4J; CS+ slope/Ctrl vs mCREB^{PFC} t-test; $t_{(28)}$ = 1.906, p = 0.067).

However, CBPΔHAT^{PFC} and mCREB^{PFC} mice responded differently to non-relevant stimuli CS- across training on the auditory discriminatory task when compared to normal mice (Fig. 1.4D-E; CS-/CBPΔHAT^{PFC} vs Ctrl; RM-ANOVA of days 1-5 and group: Day X Group, $F_{(3.8,\ 111,4)} = 6.151$, $p = 0.0002$; Fig. 1.4D,F; CS-/mCREB^{PFC} vs Ctrl; RM-ANOVA of days 1-5 and group: Day X Group: $F_{(3.7,\ 103.8)} = 5.685$, $p = 0.0005$). When compared to control mice, change in freezing (freezing delta) to CS- across the training was also significantly different in CBPΔHAT^{PFC} (Fig. 1.4I; t-test: $t_{(29)} = -2.798$, $p = 0.009$) and in mCREB^{PFC} mice (Fig. 1.4I; t-test: $t_{(28)} = -2.466$, $p = 0.02$). The marked improvement of discrimination observed on the FM-sweep direction fear discriminatory task in control mice (Fig. 1.4D, G, J) coincides with the significant negative slope of the learning curve for CS- (Fig. 1.4J; $\alpha = -6.176 \pm 1.22$). The CBPΔHAT^{PFC} group, which failed to improve fear memory accuracy across training (Fig. 1.4E, G), shows only a slight negative slope for CS- across the training (Fig. 1.4J; $\alpha = -1.22 \pm 0.78$) and a marked difference when compared to the CS- slope observed in control animals (Fig. 1.4J; CS- slope/Ctrl vs. CBPΔHAT^{PFC} t-test; $t_{(29)} = -3.368$, $p = 0.002$). The mCREB^{PFC} group, which did not improve performance on auditory discrimination task as well (Fig. 1.4F, H), exhibited similar patterns of learning to the CBPΔHAT^{PFC} mice. While responses to CS+ do not vary from those observed for control mice (Fig. 1.4I, J), the CS- learning curve is significantly different in mCREB^{PFC} mice compared to control mice (Fig. 1.4J; CS-/Ctrl: $\alpha = -6.176 \pm 1.22$; CS-/mCREB^{PFC}: $\alpha = -0.746 \pm 1.03$; CS- slope/Ctrl vs mCREB^{PFC} t-test: $t_{(28)} = -3.347$, $p = 0.002$).

In summary, analysis of patterns of responses to CS+ and CS- in control animals tested on the FM-sweep direction fear discriminatory task revealed that the improvement of auditory fear memory accuracy was due to only slight incline in freezing to CS+ and rapid decline in freezing to CS-. CBP hypofunction or CREB hypofunction in the mPFC altered the ability to learn auditory discriminatory responses to CS+ versus CS- by disrupting the pattern of learning for CS- only, while responses to CS+ remained similar to control mice. Consistent with conclusions regarding contextual fear memory specificity, these data demonstrate that the mPFC supports the improvement of auditory fear memory accuracy by controlling acquisition of appropriate responses to non-relevant stimuli.

Discussion

The present findings are the first evidence of the critical role that the mPFC plays in the attainment of fear memory accuracy for appropriate discriminative responses to aversive and non-aversive stimuli. They add substantially to the understanding of the circuitry and molecular mechanisms underlying fear memory specificity and generalization. We demonstrated that CBP-dependent signaling in the mPFC is required for fear memory accuracy. In addition, fear memory accuracy was also abnormal in mutant mice with disrupted CREB function, which is one of the most widely studied mediators of cellular memory consolidation in Drosophila, Aplysia, and mice (Dash et al. 1990; Bourtchuladze et al. 1994; Yin et al. 1994; Josselyn et al. 2001; Kida et al. 2002; Pittenger et al. 2002). The requirement of CBP acetyltransferase activity for memory consolidation has been demonstrated before including acetylatation/deacetylation-targeted pharmacological rescue of memory consolidation in CBPΔHAT mutant mice (Alarcon et al. 2004; Korzus et al. 2004) or late-phase LTP in CBP deficient mutant mice (Alarcon et al. 2004), and also in Aplysia (Guan et al. 2002).

It is important to note that Pavlovian auditory and contextual fear conditioning were intact in CBPΔHAT^{PFC} and mCREB^{PFC} mice. Memory generalization measured immediately after initial fear conditioning was also unchanged in CBPΔHAT^{PFC} and mCREB^{PFC} mice. In addition, there was no difference between tested groups in responses to CS+ across the entire contextual or auditory discriminatory tasks. The abnormal performance of mutant mice in contextual and auditory discriminatory tasks was specific to deficits in responsiveness to CS- only and during later phases of the tasks. These data suggest that prefrontal circuit is critically involved in learning appropriate responses to non-relevant stimuli that are similar yet not identical to aversive stimuli. These data are consistent with the previously described function of the PFC in fear memory extinction. Increasing evidence from human(Kesner and Rogers 2004; Blumenfeld and Ranganath 2007) and animal(Hirsch and Crepel 1992; Morris et al. 1999; Takita et al. 1999; Quirk et al. 2000; Izaki et al. 2002; Maroun and Richter-Levin 2003; Santini et al. 2004; Kawashima et al. 2006; Richter-Levin and Maroun 2010) studies implicate the PFC in extinction of conditioned fear(Sotres-Bayon et al. 2006; Quirk and Mueller 2008) and conditioned taste aversion(Akirav et al. 2006).

There is converging evidence that links fear memory specificity and generality with information processing in the hippocampus-thalamus-PFC-amygdala circuit (Marr 1971; O'Reilly and McClelland 1994; Leutgeb et al. 2007; McHugh et al. 2007; Kumaran and

McClelland 2012; Nakashiba et al. 2012; Xu et al. 2012; Navawongse and Eichenbaum 2013; Xu and Sudhof 2013). Involvement of the PFC in context or odor discrimination during information acquisition has been previously studied (DeVito et al. 2010; Xu et al. 2012; Xu and Sudhof 2013); however, the contribution of the PFC in the discrimination of auditory patterns, such as FM-sweep direction, has not been previously explored. FM-sweep direction discrimination is important in speech recognition (Zeng et al. 2005) but its underlying neural mechanism is unknown. Auditory fear conditioning has been extensively studied and depends on synaptic plasticity within the amygdala (Fanselow and LeDoux 1999; LeDoux 2000) but neural substrates for auditory fear discrimination is less well studied in mice.. Recently, it was suggested that stimulus convergence in the auditory cortex is necessary for the associative fear learning of frequency-modulated sweeps (Letzkus et al. 2011). A reduced reliance on FM-sweep direction stimuli in CBPΔHAT^{PFC} and mCREB^{PFC} mice indicates that the mPFC supports directly auditory fear memory specificity.

There is a general difference in the patterns of freezing responses to CS+/CS- between auditory and context discrimination in control animals. While the direction of learning curves (upwards/downwards) remains the same, their steepness varies. In the context discrimination assay (Fig. 1.1J), the learning of appropriate responses to CS+ showed a significantly positive slope (Fig. 1.1J; CS+/Control, $\alpha= 4.76\pm1.07$; where $\alpha=$ slope), while the learning of appropriate response to CS- showed a slight negative slope (Fig. 1.1J; CS-/Control, $\alpha= -0.88\pm1.34$). The marked improvement of discrimination observed on the FM-sweep direction fear discriminatory task in control mice (Fig. 1.4D) coincides with the slight positive slope of the learning curve for CS+ (Fig. 1.4J; CS+/Control, α= 2.366±0.82) and the significant negative slope of the learning curve for CS- (Fig. 1.4J; CS-/Control, α= -6.176±1.22). Two possible factors may have an effect on the steepness of learning curves for acquired responses to CS+/CS- in these discriminatory tasks. First, it is possible that a "floor" effect on CS- curve in the contextual discriminatory task and a "ceiling" effect on CS+ curve in the auditory discriminatory task may account for these differences. Initial level of freezing is substantially lower in the contextual discriminatory task (Fig 1.1F-G; ~25% of initial freezing) when compared to the auditory discrimination task (Fig. 1.4D-F; above 75% of initial freezing). Second, it may be more difficult to extinguish responses to non-relevant stimuli (Context B) because of high complexity of contextual stimuli (multi-modality, more details). Conversely, the rapid decline of responses to downsweep (CS-) may result from the lower complexity (single modality) of the auditory stimuli and, subsequently, more effective discrimination training.

Recently, it has been proposed that disruption of the PFC circuit during information acquisition may result in over-generality. Inactivation of prefrontal inputs to the nucleus reuniens resulted in an increased fear generalization to novel contextual stimuli (Xu et al. 2012). Our manipulation of the mPFC differed and targeted CBP-dependent nuclear processes, which may not produce immediate global effects on firing properties of the mPFC neurons during information acquisition, but rather have effects on the properties of the neural circuits relevant to long-term memory consolidation. However, it is unclear whether the abnormality in fear memory accuracy found in CBPΔHAT^{PFC} mice resulted from fear driven over-generalization or a deficit to access memory details (i.e. memory resolution).

The difficulties with studying CBP function in cognition is confounded by the high complexity of the CBP protein, which can integrate or antagonize multiple signaling pathways and by its distinctive roles in developing and mature circuits. Haploid insufficiency mutations in CBP (Chrivia et al. 1993) or its homolog p300 (Eckner et al. 1994) results in Rubinstein-Taybe syndrome (RTS) (Rubinstein and Taybi 1963; Petrij et al. 1995), which is developmental disorder characterized by severe mental retardation. CBP and p300 both share a very similar molecular structure(Arany et al. 1994) including intrinsic acetyltransferase activity (Ogryzko et al. 1996) and are capable to mediate similar cellular functions including CREB-dependent transcriptional activation. The functional differences between these two redundant genes are due to their highly overlapping but different patterns of expression and not yet understood functional specificity. Prenatal lethality in CBP knockout mice demonstrates an essential role of this gene in embryogenesis (Yao et al. 1998). CBP hemizygote or CBP mutations targeted to excitatory forebrain neurons using CamKIIα promoter driven expression such as conditional knockout or transgenic mice expressing dominant negative variants display specific deficits in long-term memory but not in short-term memory suggesting that CBP function may support long-term memory encoding. However these results are not consistent across all CBP mutant strains. In one study, CamKIIα-dependent conditional knockout of CBP targeted to excitatory neurons during postnatal brain development resulted in deficient short-term memory(Chen et al. 2010). Although, CamKIIα gene product levels are low during early phases of brain development, a large increase in the expression is usually observed between postnatal days 10 to 30 (Sugiura and Yamauchi 1992; Kojima et al. 1997) coinciding with postnatal brain development. Since the developmental time of CBP conditional deletion was not reported in this study, one cannot eliminate developmental confounds underlying the behavioral phenotype. Thus, it is difficult to dissociate between developmental defects, developmental compensatory effects and acute deficits in mutant mice with CBP hypofunction during critical periods of postnatal brain development. However, when manipulation of CBP activity is performed in the adult brain, data consistently implicate CBP acetyltransferase function in neural epigenetic signaling underlying long-term synaptic plasticity and long-term memory consolidation (Korzus et al. 2004; Barrett et al. 2011; Maddox et al. 2013). In addition, testing of CamKIIα positive cells-restricted and adult mice induced CBP knockout mice indicated that environment-induced adult neurogenesis is extrinsically regulated by CBP function in mature hippocampal granule cells (Lopez-Atalaya et al. 2011). Considering that adult neurogenesis in the hippocampus constitutes an adaptive mechanism to optimally encode contextual information important for memory resolution (Aimone et al. 2011; Sahay et al. 2011) and CBP mutant demonstrates deficiency in spatial discrimination (Lopez-Atalaya et al. 2011) it is likely that CBP is also involved is adult neurogenesis-dependent long term encoding of contextual information. However in $\mathrm{CBP\Delta HAT^{PFC}}$ or $\mathrm{mCREB^{PFC}}$ mice hypofunction was targeted to the mPFC and it is unlikely that this manipulation would have an effect on adult neurogenesis in the hippocampus.

How can CBP enzymatic activity regulate neural function? The regulation of gene expression requires not only an activation of transcription factors but also the recruitment of multifunctional coactivators that are independently regulated and directly involved in the chromatin remodeling underlying epigenetic regulatory mechanisms (Rosenfeld and Glass 2001). For example, recent work demonstrated the importance of chromatin remodeling factors like the SWI/SNF complex in neuronal function underlying memory (Vogel-Ciernia et al. 2013). While CBP's function as a platform to recruit other required coactivators appears to be indispensable for CREB-dependent transcription, the recruitment for lysine acetyltransferase activity is transcription unit specific and may depend on the structure of chromatin at a specific locus and/or a specific cell type (Puri et al. 1997; Korzus et al. 1998). Changes in histone acetylation are predictive for gene expression (Allfrey et al. 1964; Pogo et al. 1966). The concordance between the histone acetylation and transcription levels increases over time and the positive correlation between both has been confirmed in genome-wide studies (Kurdistani and Grunstein 2003; Karlic et al. 2010; Markowetz et al. 2010). It is important to emphasize that these are correlations only and that causal relationships between histone modification and gene expression in the brain in vivo will require additional investigation. In addition, a number of non-histone proteins have been identified as substrates for CBP (Kouzarides 2000; Sterner and Berger 2000; Yang 2004; Glozak et al. 2005; Kimura et al. 2005) including CREB (Lu et al. 2003). Regardless of the uncertainty of the CBP's acetyltransferase critical target(s), genetic and pharmacological studies have indicated that hypofunction of CBP's acetyltransferase activity interferes with mechanisms that support memory consolidation and reconsolidation in brain neural networks (Korzus et al. 2004; Maddox et al. 2013). Current data indicate that the acquisition fear memory accuracy involves CBP-dependent mechanism within mPFC circuitry.

Thus, locomotor activity, anxiety-related responses, and fear conditioning were normal in $CBP\Delta HAT^{PFC}$ mice, yet these mutant mice showed a strong deficit in fear memory accuracy in both contextual and auditory discrimination assays. Both context and auditory fear discrimination tasks required temporal integration because the animals learned subtle differences between relevant and non-relevant stimuli over many days with a single exposure to either CS+ and CS- per day. Inhibition of a component of neural signaling immediately upstream of CBP by a direct blockade of CREB ability to recruit CBP to the target promoter in the mPFC produced identical effects as CBPΔHAT on the capability of mice to learn the distinction between auditory stimuli. Thus, impairment of either component of CREB/CBP-dependent signaling (CREB phosphorylation or CBP's acetyltransferase activity) within the mPFC circuitry resulted in a deficit in auditory fear memory specificity indicating that the mPFC circuitry supports the disambiguation of auditory fear signals.

How CBP and CREB control memory accuracy in the mPFC is unclear. Both CBP and especially CREB have been implicated in long-term plasticity and memory consolidation in Aplysia, Drosophila and mice. Thus it is possible that long term coding within mPFC network involving LTP-mediated modification of prefrontal circuits is critical during contextual and auditory fear discrimination. This type of plasticity in the mPFC might be required to extinguish CS- responses, which would be consistent with the recognized role of the mPFC in fear memory extinction. In addition, CREB has been strongly implicated in adaptive alteration of neuronal excitability and memory allocation (Rogerson et al. 2014) and it is possible that CBP may mediate CREB-dependent changes in neuronal excitability.

There is converging evidence that links contextual fear memory specificity and generality with information processing in the hippocampus-thalamus-PFC-amygdala circuit (Marr 1971; O'Reilly and McClelland 1994; Leutgeb et al. 2007; McHugh et al. 2007; Kumaran and McClelland 2012; Nakashiba et al. 2012; Xu et al. 2012; Navawongse and Eichenbaum 2013; Xu and Sudhof 2013). Our findings are consistent with the conclusions reported by DeVito et al., who suggested that the mPFC circuit was critical for the acquisition of overlapping odor discrimination problems (DeVito et al. 2010). Thus, the present findings of the critical role of the mPFC in auditory and context discrimination provides further evidence for the high integration-dependent disambiguation function of the mPFC because similar contexts (or up/down FM-sweeps) were both presented during multiple day training consisting of discontinuous episodes before the animals acquired the ability to properly respond to these signals. These data indicate that certain types of prefrontal dysfunction are likely to contribute to overgeneralized fear, a clinical condition present in anxiety related disorders such as PTSD.

Materials and Methods:

Subjects

C57BL/6J mice were used for all experiments. Prior to any procedure, the mice are weaned at postnatal day 21, housed 4 animals to a cage with same sex littermates, maintained on a 12 hr light/dark cycle, and had *ad libitum* access to food and water. Autoclaved bedding was changed every week. All procedures were approved by the UC Riverside Institutional Animal Care and Use Committee in accordance with the NIH guidelines for the care and use of laboratory mice.

Surgery

The injection protocol is modified from previously described work (Cetin et al. 2006). In this study, 2-4-month-old mice were individually housed and weighed to determine the appropriate drug ratios to use. Atropine was injected to help with breathing [.02 mg/kg body weight]. The mice were then placed into an isoflurane chamber to induce anesthesia, mounted in a heated stereotaxic apparatus and supplied with a constant flow of isoflurane/oxygen mix. The scalp was shaved and sanitized with 70% ethanol. The ear bars, bite bar, and nose clamp were adjusted to firmly hold the head in place. A midline incision was made on the scalp, and surgical hooks were placed to keep the skull exposed. Sterile PBS was added as needed to prevent the skull from drying. The head was leveled by comparing bregma and lambda coordinates until they were equivalent. Injection sites were calculated based on bregma coordinates, and a dental drill was used to thin the skull over the injection site. A 27G needle was then used to remove the thinned bone. A 5-μl calibrated glass micropipette [8 mm taper, 8 μm internal tip diameter] was fitted with a plastic tube connected to a 10-ml syringe and lowered onto a square of Parafilm containing a 4-µl drop of virus. The syringe was aspirated to fill the micropipette with solution before moving it to the injection site. The micropipette was slowly lowered to the proper stereotaxic coordinates and pressure was applied to the syringe to inject 1 µl of solution at a rate of 50 nl/min. After the total volume was injected, the micropipette was withdrawn slowly to avoid backflow, and the injection site was cleaned with sterile cotton swabs. The skin was sutured, and antibiotic was applied to the scalp. Lidocaine was subcutaneously injected near the site followed by an intraperitoneal injection of sterile PBS [30 ml/kg body weight] to prevent dehydration. The mouse was kept warm by placing its cage on a heated plate and injected with buprenorphine [.05 mg/kg] for pain relief. On post-surgical days 1 and 2, the mouse received subcutaneous injections of meloxicam [1 mg/kg] to relieve pain. Animals were monitored for any signs of distress or inflammation for 3 days after surgery. Behavioral experiments were initiated 3 days after surgery. The infralimbic and prelimbic cortices were targeted at the following stereotaxic coordinates: Bregma; AP 1.8, ML±0.4, DV 1.4.

Viruses

Surgical procedures were standardized to minimize the variability of HSV virus injections, using the same stereotaxic coordinates for the mPFC and the same amount of HSV injected into the mPFC for all mice. CBPΔHAT or mCREB and/or EGFP were cloned into the HSV amplicon and packaged using a replication-defective helper virus as previously described (Lim and Neve 2001; Neve and Lim 2001). The viruses (HSV/CMV-CBPΔHAT-IRES2-EGFP, HSV/CMV-EGFP and HSV/mCREB-EGFP) were prepared by Dr. Rachael Neve (MIT, Viral Core Facility). The average titer of the recombinant virus stocks was typically 4.0 x 10^7 infectious units/ml. HSV viruses are effectively expressed in neurons in the PFC. The CBPΔHAT mutant, a dominant-negative inhibitor of CBP-dependent histone acetylation, harbors a substitution mutation of two conserved residues (Tyr^{1540}/Phe^{1541} to Ala^{1540}/Ala^{1541}) in the acetyl CoA binding domain (Korzus et al. 1998). It has been also demonstrated that CBPΔHAT lacks histone acetyltransferase activity (Korzus et al. 2004) and blocks c-fos expression in neurons (Korzus et al. 2004). The dominant negative CREB mutant (mCREB) carries substitution mutation Ser^{133} to Ala^{133}. Previous studies indicate that mCREB decreased CREB function and block neuronal CREB dependent gene expression (Gonzalez et al. 1989; Chrivia et al. 1993; Barrot et al. 2002; Olson et al. 2005).

Behavioral Assays

All behavioral experiments were performed under blind conditions.

Fear conditioning. Fear conditioning was performed as previously described (Korzus et al. 2004). Fear conditioning training was performed in the fear conditioning box from Coulburn Instruments Inc. After being handled, individual mice were exposed to context A. Context A was the unmodified fear conditioning box, which was placed inside of a sound attenuated chamber with the house light and house fan turned on. Performance was scored by measuring freezing behavior, the complete absence of movement (Fanselow 1980). Freezing was scored and analyzed automatically by a Video-based system (Freeze Frame software ActiMetrics Inc.). Video was recorded at 30 frames per s. The Freeze Frame software calculated a difference between consecutive frames by comparing gray scale value for each pixel in frame. Freezing was defined based on experimenter observations and set as sub-threshold activity for longer then 1 s. Freezing was expressed as a % Freezing, which was calculated as a percent of freezing time per total time spent in the testing chamber. The chamber was cleaned in between trials with Quatracide, 70% ethanol, and distilled water.

Contextual Fear Conditioning. Mice were trained in a standard Fear Conditioning Chamber Coulburn Instruments Inc.). The individual mice were exposed to context A for 180 s and received a 0.75 mA, 2 s foot shock (context A – foot shock pairing). The animals were then left for another 180 s inside the chamber. For the memory retention test, the mice are placed back into the training chamber for 180 s. Freezing was scored and analyzed automatically as described above.

Cued Fear Conditioning. Mice were trained in a standard Fear Conditioning Chamber Coulburn Instruments Inc.). After a three-minute baseline period, one, two, or three-20 second tones (2800 Hz, 75dB) were played and a shock (0.75 mA, 2 sec) was delivered during the final 2 sec of the tone. Twenty-four hours, mice were placed in a novel enclosure and after a three-minute baseline exposure, a series of three tones identical to that given in the training session was played. Freezing was scored and analyzed automatically as described above.

Context discrimination. The context discrimination assay was preformed similarly as previously described (Lovelace et al. 2014). After being handled, individual mice were exposed to context A one day before training. The protocol included 14 days of training, which was divided into three phases: initial training phase, generalization test and discrimination phase (Fig. 1.1D). During the initial training phase (day 1), mice were placed in the context A for 180 s followed by a single foot shock (arrow) and left for another 60 s inside the chamber. Context A (CS+) was the unmodified fear conditioning box (Coulburn Instruments Inc.), which was placed inside of a sound attenuated chamber with the house light and house fan on. The chamber was cleaned with Quatricide, 70% ethanol, and distilled water. For generalization test and during discrimination phase, the individual mice were exposed to Context A for 180 s and received a 0.75 mA, 2 s foot shock, and left for another 60 s inside the chamber. Four hours later, the mice were exposed to the similar Context B (CS-) for 242 s and received no footshock. Context A and B were similar but not the same. Context B was the modified fear conditioning chamber, with angular wall inserts, house fan off, and scented with Simple Green. Thus animals were exposed to CS^+ 13 times before the final test. The order of exposure to different contexts was counter balanced. Additionally, the context cues themselves were counter balanced within each group in order to isolate the effect of the CS+.

Auditory Discrimination. The auditory discrimination task is divided into three phases: initial training phase, generalization test and discrimination phase (Fig. 1.3). The conditioned stimuli (CS) for auditory fear conditioning were 20-s trains of frequency modulated (FM)-sweeps for a 400-ms duration, logarithmically modulated between 2 and 13 kHz (upsweep) or 13 and 2 kHz (downsweep) delivered at 1 Hz at 75 dB. After habituation, the CS+ was paired with a foot shock (2 s, 0.75 mA). The onset of the US coincided with the onset of the last sweep for the CS. For fear conditioning acquisition (days 1-3; initial training phase), the animals were presented with a single US-CS pairing per day. The FM-sweep Fear Retrieval (day 4) and Generalization (day 4-5) were tested (freezing to 3x CS^- for 30 s followed by 3x 30 s CS^+ without US; 3 min baseline and 3 min ITI) in context C, which significantly differed from the training chamber (context A). The discrimination phase of FM sweep direction discrimination training was performed over three sessions a day for 6 days (days 7-12): Session 1 was the performance test, Session 2 was the presentation to 1x UC-CS+ pairing after 3 min baseline, and Session 3 was the presentation to the US-CS- pairing after a 3 min baseline. The CS+ and CS- were counterbalance such that half of the CS+ group was upsweep and the other half CS+ was downsweep.

Open-field test. A 17” x 17” x 12” clear Plexiglas arena with a white acrylic floor was used for the open field test. The arena was placed in a sound attenuated chamber with a ceiling mounted camera and a dim light. After sanitizing the arena with Quatracide, 70% EtOH, and distilled water, the mice were individually placed inside the chamber and allowed to explore for 15 min before being returned to its home cage. Videos are analyzed offline using behavioral analysis software (CleverSys, Inc.) to quantify the level of anxiety and locomotion.

Histology

Mice were anesthetized using nembutal (200mg/kg, i.p. injection) and transcardially perfused first with PBS and then 4% PFA. The extracted brain was soaked in 4% PFA overnight and then transferred to PBS until histological sectioning. In this study, 100-µm-thick sections of the mPFC were obtained using a Compresstome VF-300 (Precisionary Instr., Greenville, NC) and placed in a 24-well plate for free-floating immunohistochemistry (IHC) according to a previously described protocol (Korzus, 2004). The sections are washed 3 times for 10 min in a wash buffer (PBS, 0.3% Triton x-100, 0.02% NaN_2) followed by a 1-hr incubation in blocking buffer (5% normal goat serum in washing buffer), followed by a 10-min incubation in the wash buffer. The sections were incubated overnight at 4C° with primary antibodies: anti-NeuN (Millipore 1:2000), chicken anti-GFP (Molecular Probes, 1:1000); anti-acetyl-Histone H3 (Millipore, 1:2000) or anti-acetyl-Histone H4 (Millipore, 1:2000). After three washes with the wash buffer, the sections were incubated with secondary antibodies (Alexa647-goat anti-mouse IgG; Alexa488 goat anti-chicken IgG; Alexa647-goat anti-rabbit IgG; Molecular Probes, 1:1000)), in blocking buffer for 4 hr at room temperature. The sections were washed again three times with the wash buffer before mounting for viewing. Negative control slices were performed for each row of the well plate, undergoing the same IHC procedure in addition to receiving primary antibodies. After immunostaining, the tissue was mounted directly onto glass slides, covered, and sealed with nail polish before imaging.

Imaging

The slides were placed on the stage of an Olympus FV1000 laser scanning confocal microscope controlled using the FluoView software. GFP, and Alexa-647 were imaged using 473-nm, and 647-nm lasers, respectively. The background fluorescence was measured and subtracted for each image. The fluorescence intensity was compared to the negative control slices, which did not receive any primary antibodies. Immunostained tissue was analyzed using a semi-automatic laser scanning confocal microscope

(Olympus FV1000) controlled by the FluoView software. Multiple brain sections were imaged using identical microscope settings. Eighty-micrometer z-stacks were obtained from the PL region in the mPFC, and ROI analysis was used for quantification. The background fluorescence was measured for each imaged and then subtracted. The intensity quantification was performed using the FluoView Olympus software and NIH Image J.

Histone acetylation assay

Individual mice were trained on a fear conditioning paradigm in which they were presented with a 20 sec auditory stimulus followed immediately by a 2 sec foot shock (0.75 mA intensity). The auditory stimulus is the same used for behavioral training in which logarithmically modulated upward (2 kHz-13 kHz) frequency-modulated sweeps are presented in 400ms bouts at a 1 Hz frequency for a total duration of 20 sec. 3 min after the foot shock, mice were placed in their home cage for 25 min undisturbed. Immunohistology and imaging were performed as described above. The region of interest (ROI) was a 5 μm circle placed on cells expressing GFP within cortical layer 2/3 in mPFC and fluorescence corresponding to acetylated histone H3 or H4 was measured from randomly selected 50-60 cell per hemisphere. The fluorescence intensity quantification was performed on original images by the use of Olympus Fluoview software.

Data analysis

The experimenters were blind to the group conditions. Data are expressed as the means ± SEM. N indicates number of animals unless stated otherwise. Statistical analysis was performed using Excel (Microsoft Inc.) or SPSS (IBM Inc.). The Student's *t*-test or ANOVA was used for statistical comparisons. Pearson's correlation (*r*) was used as an *effect size*. In cases where the repeated measures ANOVA (RM-ANOVA) was utilized and assumptions of sphericity were violated (via Mauchly's Test), the analysis was performed using the Greenhouse-Geisser correction. Where applicable, *post hoc* analysis with Bonferroni correction was performed for multiple comparisons, which allows for substantially conservative control of the error rate. A $p<0.05$ was considered statistically significant. The asterisks indicate statistical significance: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ and n.s. indicates not significant.

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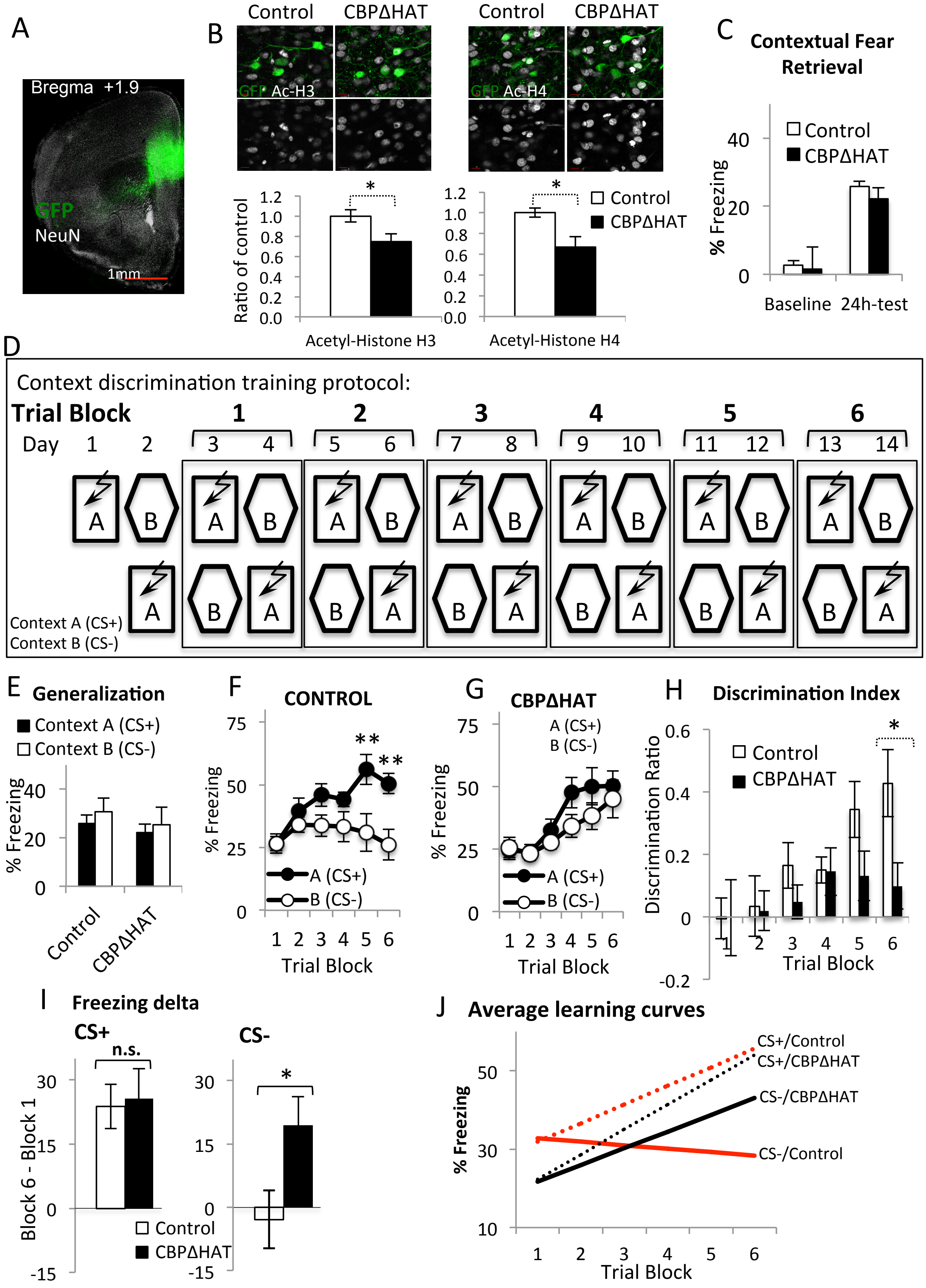
Figure 1.1


A
Bregma +1.9
GFP
NeuN
1mm
B
Control
CBPΔHAT
Control
CBPΔHAT
GFP Ac-H3
GFP Ac-H4
Ratio of control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
☐ Control
■ CBPΔHAT
Acetyl-Histone H3
Acetyl-Histone H4
C
Contextual Fear Retrieval
% Freezing
40
20
0
☐ Control
■ CBPΔHAT
Baseline
24h-test
D
Context discrimination training protocol:
Trial Block
1
2
3
4
5
6
Day
1
2
3
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5
6
7
8
9
10
11
12
13
14
A
B
Context A (CS+)
Context B (CS-)
E
Generalization
■ Context A (CS+)
☐ Context B (CS-)
% Freezing
40
20
0
Control
CBPΔHAT
F
CONTROL
% Freezing
75
50
25
0
**
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A (CS+)
B (CS-)
1 2 3 4 5 6
Trial Block
G
CBPΔHAT
A (CS+)
B (CS-)
% Freezing
75
50
25
0
A (CS+)
B (CS-)
1 2 3 4 5 6
Trial Block
H
Discrimination Index
Discrimination Ratio
0.6
0.4
0.2
0
-0.2
☐ Control
■ CBPΔHAT
*
1 2 3 4 5 6
Trial Block
I
Freezing delta
CS+
n.s.
CS-
*
Block 6 - Block 1
30
15
0
-15
☐ Control
■ CBPΔHAT
J
Average learning curves
% Freezing
50
30
10
CS+/Control
CS+/CBPΔHAT
CS-/CBPΔHAT
CS-/Control
1 2 3 4 5 6
Trial Block

Figure 1.1 Contextual Fear memory specificity was deficient in CBPΔHAT^{PFC} mice. (A) Viral-mediated delivery to the mPFC. Long-term expression HSV-1 viruses carrying CBPΔHAT (HSV/CBPΔHAT-IRES2-EGFP) or eGFP as the control (HSV/EGFP) were injected into the mouse mPFC. To determine the pattern of GFP-tagged virus expression, the imaged tissue was compared to the Paxinos and Franklin mouse atlas (Paxinos and Franklin 2001) and areas of maximal GFP expression were labeled as injection sites. A representative image of mPFC viral infection showed the precision of our viral-targeting procedures. The pattern of EGFP expression was similar 4 or 20 days after HSV virus injection into the mPFC. Green, GFP; white, NeuN neuronal marker. (B) CBPΔHAT blocks acetylation of histone H4 and H5 in the mPFC. To determine the effects of viral infection with CBPΔHAT on neural signaling, the levels of acetylated histones H3 and H4 were assessed in the brains of infected animals and compared to controls in a standard IHC analysis 25 min after auditory fear conditioning (see Methods). Cells expressing viral CBPΔHAT showed significantly lower levels of acetylated histone H3 and H4 when compared to control animals expressing GFP only. Representative images show GFP (in green) and acetylated histone H3 (Ac-H3, left panel; t-test: t(10) = 2.38 , p = 0.0382, effect size r = 0.6013) or acetylated histone H4 (Ac-H4; left panel; t-test: t(10) = 2.9718 , p = 0.0140, effect size r = 0.6848) CBPΔHAT^{PFC} mice. 3 animals were used per group; n indicates hemisphere. GFP, green; Ac-H3, white; Ac-H4, white; Red bar, 10 μm (C) Pavlovian contextual fear conditioning was normal in CBPΔHAT^{PFC} mice. CBPΔHAT^{PFC} and control (Ctrl) mice showed normal acquisition and retention of contextual fear conditioning. Contextual fear was tested in context A at 24 h after a single context A-foot shock pairing. (D) Experimental design for the context discrimination test. Context A and B were similar but not identical. The protocol included 14 days of training. The mice were placed in context A (CS+) for 180 s followed by a foot shock (arrow), and context B (CS-) lacked any reinforcement. (E) Generalization test. Freezing behavior to context A and a similar but not identical context B after conditioning to context A-foot shock pairing was not different, in both groups. Freezing in both tested groups were comparable in response to both contexts, indicating that context A was sufficiently similar to context B that generalization was occurring early in training. (F) After the initial generalization of fear conditioned responses, control mice exhibited robust fear memory specificity. (E) CBPΔHAT^{PFC} mice exhibited a deficit in context discrimination. (G) The context discrimination ratios (DI) were calculated using the freezing responses to CS+ and CS- according to the formula DI = ((Context A - Context B) / (Context A + Context B)). Analyses revealed differences in the performance during trial block 6 between CBPΔHAT^{PFC} and control mice, but not during trial blocks 1 – 5. CBPΔHAT^{PFC} mice, n=11. Control, n=9. (I) Change in freezing across training (freezing delta), calculated as the (freezing on Trial Block 6 – freezing on Trial Block 1). There was no difference in responses to conditioned stimuli CS+ between CBPΔHAT^{PFC} and control mice. Change in freezing to CS- across the training was significantly higher in CBPΔHAT^{PFC} when compared to control mice. (J) Average learning curves for learning of appropriate responses to CS+ and CS- were calculated based on the performance of control and CBPΔHAT^{PFC} group across the entire training (Fig. 1.1F-G; Block Trials 1 through 6) followed by fitting the regression line and t-test analysis on the mean of those slopes. The analysis of patterns of responses to CS+ and CS- in control animals tested on the context fear discriminatory task revealed that the improvement of fear memory accuracy was due to incline in freezing to CS+ and a slight decline in freezing to CS- (CS+/Ctrl:α= 4.76±1.07; CS-/Ctrl: α= -0.88±1.34). The learning of appropriate responses to CS+ shows a positive slope (α) in both control and CBPΔHAT^{PFC} mice and there is no difference between groups (CS+/Ctrl:α= 4.76±1.07; CS+/CBPΔHAT^{PFC}:α= 6.35±1.61; CS+ slope/Ctrl vs CBPΔHAT^{PFC} t-test; $t_{(18)} = -0.778$, $p = 0.446$). The CBPΔHAT^{PFC} group, which failed to improve fear memory accuracy, showed a positive slope for CS-, a marked difference from control responses to the CS- (CS-/Ctrl: α= -0.88±1.34; CS-/CBPΔHAT^{PFC}: α= 4.26±1.4); CS- slope/Ctrl vs CBPΔHAT^{PFC} t-test; $t_{(18)} = -2.614$, $p = 0.018$). The asterisks indicate statistical significance: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ and n.s. indicates not significant.

Figure 1.2

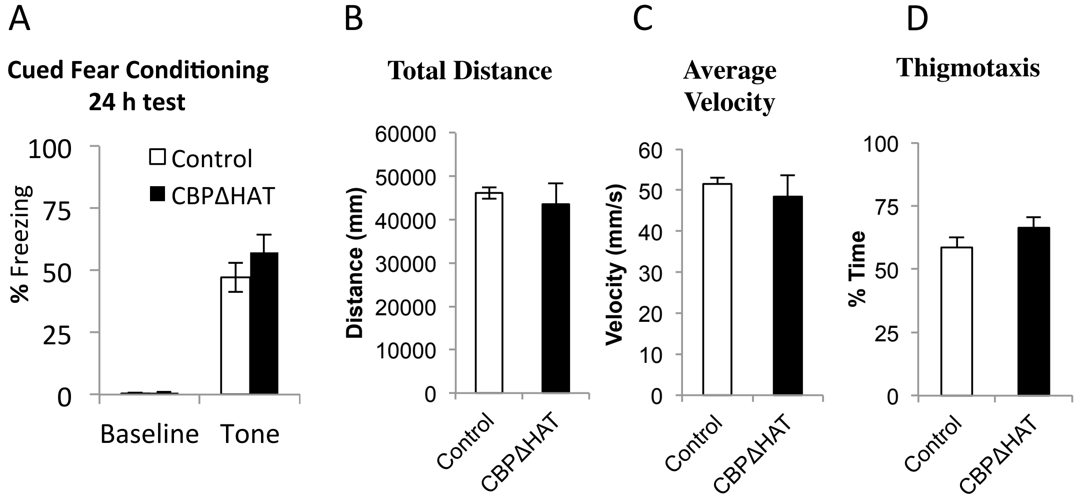


Figure 1.2 Pavlovian fear conditioning and locomotor activity are normal in CBPΔHAT^{PFC} mice. (A) Pavlovian cued fear conditioning was normal in CBPΔHAT^{PFC} mice. CBPΔHAT^{PFC} and control (Ctrl) mice showed normal acquisition and retention of contextual fear conditioning. Contextual fear was tested in context A at 24 h after the 5 CS-US pairing (CS, 2 s 2800 Hz tone; US -foot shock). CBPΔHAT^{PFC} mice, n=10. Control, n=10. (B-C) Non-induced locomotor activity and (D) anxiety-related responses were unaltered in CBPΔHAT^{PFC} mice.

Figure 1.3

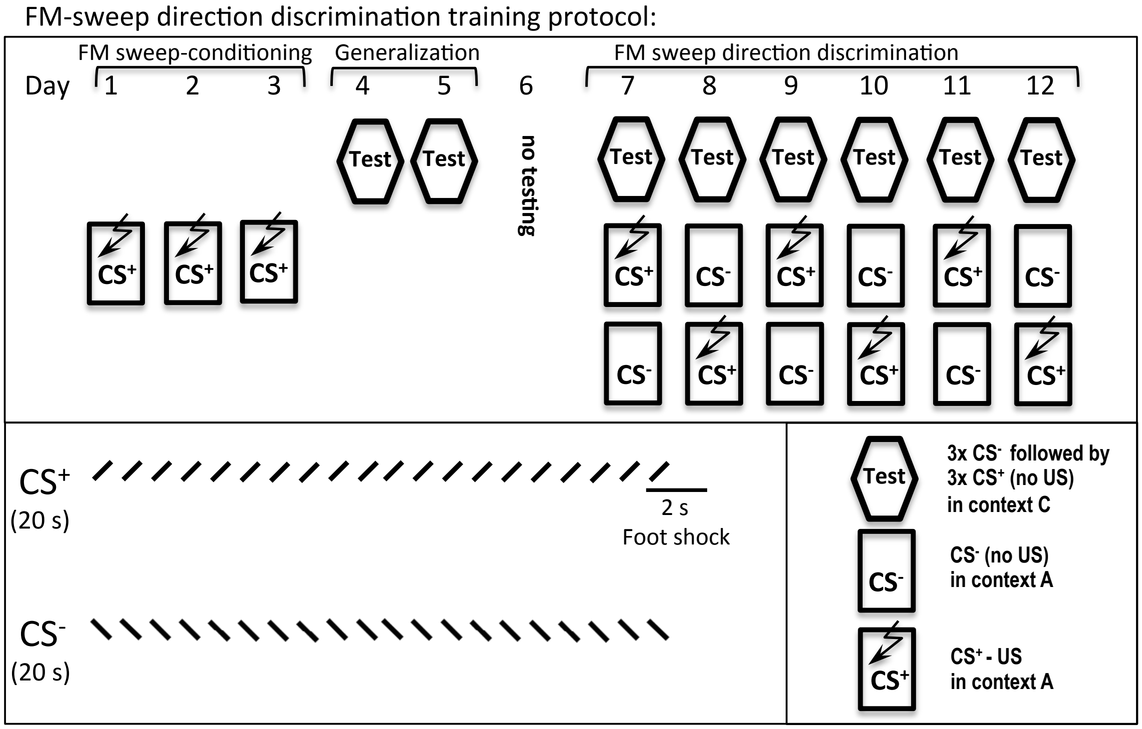
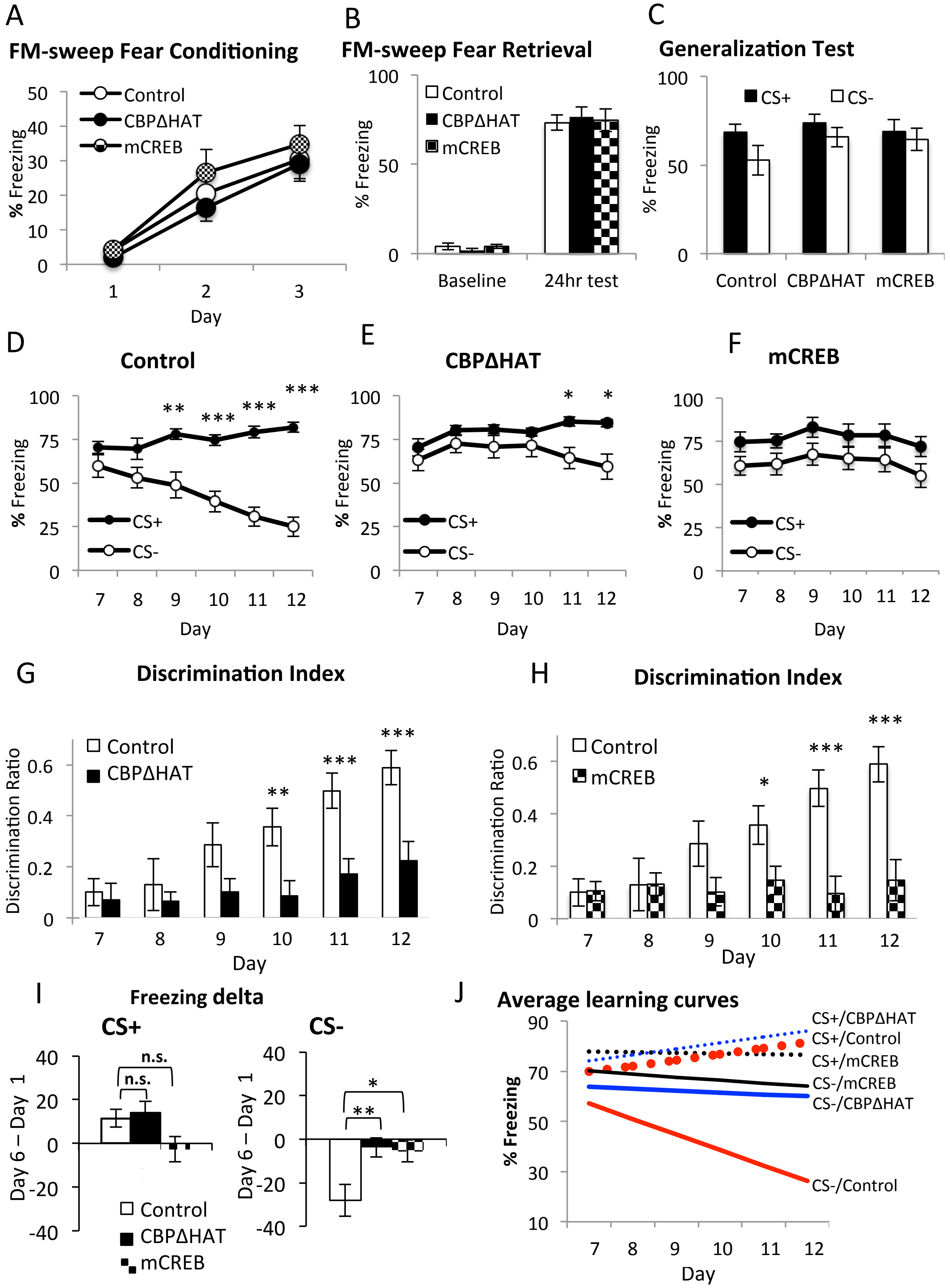


Figure 1.3 Experimental design for the auditory discrimination test. The auditory discrimination task tests the ability of subjects to recognize a direction of FM-sweeps ((trains of upward and downward FM-sweeps). The conditioned stimuli (CS) for auditory fear conditioning were 20-s trains of FM-sweeps for a 400-ms duration, logarithmically modulated between 2 and 13 kHz (upsweep) or 13 and 2 kHz (downsweep) delivered at 1 Hz at 75 dB. As described in methods, these assay includes 3 phases: FM-sweep conditioning (day 1-3), generalization (day 4-5) and FM-sweep direction discrimination training (Day 6-12).

Figure 1.4


A
FM-sweep Fear Conditioning
% Freezing
50
40
30
20
10
0
Control
CBPΔHAT
mCREB
1
2
3
Day
B
FM-sweep Fear Retrieval
100
50
0
% Freezing
Control
CBPΔHAT
mCREB
Baseline
24hr test
C
Generalization Test
100
50
0
% Freezing
CS+
CS-
Control
CBPΔHAT
mCREB
D
Control
% Freezing
100
75
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25
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CS+
CS-
7
8
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CBPΔHAT
% Freezing
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7
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mCREB
% Freezing
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CS+
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Day
G
Discrimination Index
Discrimination Ratio
0.6
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Control
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Discrimination Index
Discrimination Ratio
0.6
0.4
0.2
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Control
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7
8
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11
12
Day
I
Freezing delta
CS+
Day 6 – Day 1
40
20
0
-20
-40
n.s.
n.s.
Control
CBPΔHAT
mCREB
CS-
Day 6 – Day 1
40
20
0
-20
-40
*
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J
Average learning curves
% Freezing
90
70
50
30
10
CS+/CBPΔHAT
CS+/Control
CS+/mCREB
CS-/mCREB
CS-/CBPΔHAT
CS-/Control
7
8
9
10
11
12
Day

Figure 1.4 FM-sweep direction fear memory specificity is deficient in CBPΔHAT^{PFC} mice. (**A-B**). Pavlovian FM-sweep fear conditioning was normal in CBPΔHAT^{PFC} and mCREB^{PFC} mice. CBPΔHAT^{PFC} and mCREB^{PFC} mice showed similar acquisition (**A**) and retention (**B**) of FM-sweep fear conditioning to control (Ctrl) mice. FM-sweep fear was tested in context C at 24 h after a three upsweeps-foot shock pairing. (**C**) All three groups (CBPΔHAT^{PFC} and mCREB^{PFC} and Ctrl) show no difference in the freezing responses to CS$^+$ and CS$^-$ ($p>0.05$) during day 4 and 5 of training, indicating that initially, the CBPΔHAT^{PFC} and mCREB^{PFC} mice generalized responses and did not discriminate between upsweep and downsweep. (**D**) After the initial generalization of fear conditioned responses, control mice exhibited robust fear memory specificity. (**E**) CBPΔHAT^{PFC} mice did not discriminate between upsweep and downsweep and exhibited a deficit in auditory fear memory specificity. CBPΔHAT^{PFC} mice demonstrated strong deficit in auditory memory specificity when compared to controls (Figure 1.1B-C, RM-ANOVA, Treatment x context x trial blocks 1-6: $F_{(2.806,\ 81.366)}=3.033$, $p=0.037$). (**F**) Similarly to CBPΔHAT^{PFC}, mCREB^{PFC} mice did not discriminate between upsweep and downsweep and exhibited a deficit in auditory fear memory specificity. (**G**) The FM-sweep direction discrimination ratios (DI) were calculated using the freezing responses to CS+ and CS- according to the formula DI = ((Upsweep - Downsweep) / (Upsweep + Downsweep)). Analyses revealed differences between CBPΔHAT^{PFC} and control mice in the performance during Days 11 - 12 between CBPΔHAT^{PFC} and control mice. CBPΔHAT^{PFC}, n=15; Ctrl, n = 16. (**H**) Analyses revealed differences between mCREB^{PFC} and control mice in the performance during Days 11 - 12. mCREB^{PFC}, n=14; Ctrl, n = 16).

(**I**) Change in freezing across training (freezing delta), calculated as the (freezing on Day 12 – freezing on Day 7). There was no difference in responses to conditioned stimuli CS+ between CBPΔHAT^{PFC}, mCREB^{PFC} and control mice. Change in freezing to CS- across the training was significantly higher in CBPΔHAT^{PFC} and mCREB^{PFC} when compared to control mice. (**J**) Average learning curves for learning of appropriate responses to CS+ and CS- were calculated based on the performance of control and CBPΔHAT^{PFC} group across the entire training (Fig. 1.4D-F; Days 7 to 14) followed by fitting the regression line and t-test analysis on the mean of those slopes (α). The analysis of patterns of responses to CS+ and CS- in control animals tested on the FM-sweep direction fear discriminatory task revealed that the improvement of auditory fear memory accuracy was due to slight incline in freezing to CS+ and rapid decline in freezing to CS- (CS+/Ctrl: α= 2.366±0.82; CS-/Ctrl: α= -6.176±1.22). There was no difference in the learning (slopes) of appropriate responses to CS+ between CBPΔHAT^{PFC} and control groups (CS+/Ctrl: α= 2.366±0.82; CS+/CBPΔHAT^{PFC}: α=2.384±0.894 ; CS+ slope/Ctrl vs CBPΔHAT^{PFC} t-test: $t_{(29)}$ = -0.015, p = 0.988) or mCREB^{PFC} and control mice (CS+/Ctrl: α= 2.366±0.82; CS+/mCREB^{PFC}: α=-0.278±1.15; CS+ slope/Ctrl vs mCREB^{PFC} t-test: $t_{(28)}$ = 1.906, p = 0.067). The CBPΔHAT^{PFC} group, which failed to improve fear memory accuracy, showed a positive slope for CS-, a marked difference from control responses to the CS- (CS-/Ctrl: α= -6.176±1.22; CS-/CBPΔHAT^{PFC}: α= -1.22±0.78; CS- slope/Ctrl vs CBPΔHAT^{PFC} t-test; $t_{(29)}$ = -3.368, p = 0.002). Similar to the CBPΔHAT^{PFC} group, the mCREB^{PFC} group did not improve performance on the auditory discrimination task and showed a positive slope for CS-, a marked difference from control responses to the CS-

(CS-/Ctrl: α= -6.176±1.22; CS-/mCREB^{PFC}: α= -0.746±1.03; CS- slope/Ctrl vs mCREB^{PFC} t-test: $t_{(28)} = -3.347$, $p = 0.002$). The asterisks indicate statistical significance: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ and n.s. indicates not significant.

Chapter II:

Prefrontal NMDA receptors gate discriminatory fear learning

Abstract

N-methyl-D-aspartate receptors (NMDAR) are critically involved in various learning mechanisms, brain development and brain disorders. In contrast to hippocampal NMDARs, less is known about the cognitive function of NMDARs in the medial prefrontal cortex (mPFC). The neural mechanisms governing discriminative fear learning for appropriate responses to aversive and safe stimuli are unclear. The objectives of the present study were to determine involvement of prefrontal NMDARs in discriminatory fear learning. To achieve this, we tested mice with locally deleted Grin 1 gene encoding the obligatory NR1 subunit of the NMDAR in the mPFC for their ability to distinguish frequency modulated (FM) tones in fear auditory conditioning. We demonstrated that NMDAR-dependent signaling in the mPFC is critical for discriminatory fear learning. While mice with deficient NMDAR signaling in the mPFC maintain normal responses to aversive stimuli, they exhibit abnormal responses to similar but safe stimuli. These data indicate that the improvement of fear memory accuracy involves mPFC-dependent extinction of fear responses to benign stimuli. These studies provide evidence that NMDAR-dependent neural signaling in the mPFC is a component of neural mechanism for disambiguating fear signals which supports discriminative fear learning by retaining proper gating information, *viz.* both aversive and benign cues.

Introduction

Fear memory accuracy is critical for survival, while fear generalization is effective for recalling and avoiding dangerous situations. However, failure to discriminate between aversive and non-aversive stimuli during recall is an indicator of reduced access to memory details, i.e., decreased memory resolution, generalized fear, or both. Overgeneralized fear is a typical symptom of anxiety disorders including generalized anxiety disorder (Gazendam, Kamphuis, and Kindt, 2013; Reinecke, Becker, Hoyer, and Rinck, 2010) and posttraumatic stress disorder (PTSD) (Jovanovic, Kazama, Bachevalier, and Davis, 2012), which are triggered by cues in a secure environment that resemble those of the traumatic experience. Failure to discriminate between aversive and benign stimuli can lead to intrusive recollection of aversive memories. This type of reduced fear memory accuracy is an observed response to two stimuli that are not identical, and in humans may rely on mechanisms that allow rapid and efficient assessment of dangerous situations (Dunsmoor, Mitroff, and LaBar, 2009; Resnik, Sobel, and Paz, 2011).

Previous studies implicate prefrontal circuitry in the contextual fear memory specificity and generalization (Xu, Morishita, Buckmaster, Pang, Malenka, and Sudhof, 2012; Xu and Sudhof, 2013) or discrimination of more discrete multiple odor stimuli (DeVito, Lykken, Kanter, and Eichenbaum, 2010). We have recently demonstrated that prefrontal hypofunction of transcription regulators implicated in the mechanism underlying long-term memory consolidation results in a disruption of contextual and auditory discriminatory fear learning in mice (Vieira, Lovelace, Corches, Rashid, Josselyn, and Korzus, 2014). These data indicate that improvement of fear memory accuracy involves mPFC-dependent extinction of fear responses to benign stimuli (Vieira et al., 2014), which is consistent with studies showing that lesions in mPFC produce deficit in extinction of conditioned fear (Gewirtz, Falls, and Davis, 1997; Morgan, Romanski, and LeDoux, 1993; Orsini, Kim, Knapska, and Maren, 2011; Orsini and Maren, 2012; Quirk, Russo, Barron, and Lebron, 2000). The learning of fear extinction involves both increased neuronal activity and protein synthesis in the mPFC (Milad and Quirk, 2002; Orsini et al., 2011; Santini, Ge, Ren, Pena de Ortiz, and Quirk, 2004). In addition, there is strong evidence that extinction of conditioned fear depends on N-methyl-D-aspartate receptors (NMDARs)-mediated plasticity (Burgos-Robles, Vidal-Gonzalez, Santini, and Quirk, 2007; Santini, Muller, and Quirk, 2001).

Thus, discrimination between aversive and benign cues is likely to involve mPFC functional interactions. Still unknown are the neural mechanisms underlying the attainment of fear memory accuracy for appropriate discriminative responses to aversive and benign stimuli. To explore the potential impact of prefrontal NMDARs on discriminatory fear learning, we generated mutant mice with locally deleted obligatory subunit of the NMDAR in the mPFC and examined their capability to distinguish between aversive and safe stimuli in fear auditory conditioning. For behavioral evaluations, we used an auditory discriminatory task that depends on the ability to distinguish discrete auditory cues constructed of frequency modulated (FM) upward or downward tone sweeps. This auditory discriminatory task indicated that NMDAR-dependent neural signaling within mPFC circuitry is an important component of the mechanism for disambiguating the meaning of fear signals.

Results

Generation of mPFC-NR1 KO mice

In this study, we focused on the NMDAR, which is a known regulator of synaptic activity. This receptor has been implicated in forms of synaptic plasticity underlying memory (Bliss and Collingridge, 1993; Tsien, Huerta, and Tonegawa, 1996). Loss of NMDAR function is achieved by the conditional deletion of the *Grin1* gene, which encodes an obligatory NR1 subunit for functional NMDAR (Tsien et al., 1996). In most experiments in current studies, we injected Herpes Simplex Virus (HSV) expressing monomeric fluorescent protein mCherry (CamKIIα-mCherry) or mCherry and Cre recombinase under control of CamKIIα promoter (CamKIIα-mCherry/Cre) (Fig. 2.1A) into the mPFC of floxed-NR1 mice to generate Control or mPFC-NR1 KO mice, respectively (Fig. 2.1A, B). Cre recombinase is the second gene in our CamKIIα-mCherry/Cre bicistronic vector and linked to mCherry with the internal ribosome entry site (IRES) of encepahlomyocarditis virus (EMCV). We have found that more then 98% infected cell are neurons (data not shown, see (Vieira et al., 2014)). Immunostaining with antibodies directed against CamKIIα and mCherry revealed that Cre recombinase was targeted to CamKIIα positive cells (Fig. 2.1C). To evaluate of NR1 gene deletion in mPFC-NR1 KO, we employed the widely used anti-NR1 monoclonal antibody clone 54.1 (Brose, Gasic, Vetter, Sullivan, and Heinemann, 1993) to examine expression of NR1 protein levels in mCherry expressing cells. mPFC-NR1 KO mice show decreased levels of expression of NR1 gene product in cells infected with CamKIIα-mCherry/Cre virus within the mPFC (Fig. 2.1D, E). We found approximately 40% decrease of NR1 protein signal in neurons expressing mCherry protein in the mPFC-NR1 KO when compared to control (Figure 2.1E; Ctrl vs. mPFC-NR1 KO: t-test; $t_{(14)} = 3.415$, $p = 0.0042$, $r = 0.674$). This is consistent with previous studies indicating that IRES-dependent second gene expression is significantly less effective than cap-dependent first gene expression in a bicistronic vector (Mizuguchi, Xu, Ishii-Watabe, Uchida, and Hayakawa, 2000; Zhou, Aran, Gottesman, and Pastan, 1998).

Impairment of discriminatory fear learning in mPFC-NR1 KO mice

We tested mPFC-NR1 KO mice using an auditory sweep discrimination task, which examines the ability of mice to recognize the direction (upward or downward) of frequency modulated (FM)-sweeps (Fig. 2.2). This assay begins with 3 days of acquisition (single CS+ footshock pairing per day) followed by a 24hr test on day 4 and a generalization test on day 4-5. The mice are then run through discrimination training on days 7-12 in which they experience 3 sessions: first, they are tested for freezing to CS+ and CS- (in context C); second, they are exposed to CS+ (or CS-); third, they are exposed to CS- (or CS+).

We examined upward FM-sweep (CS+) fear conditioning acquisition in mPFC-NR1 KO mice and control mice, and both groups acquired this form of Pavlovian conditioning normally (Fig. 2.3A; RM-ANOVA of Day and Group: $F_{(2,38)} = 0.800$, $p = 0.457$). Both groups also showed similar retrieval on a 24-hr memory test (Fig. 2.3B; two way ANOVA of Group and Baseline/24 h-Test; Group: $F_{(1,38)} = 0.941$, $p = 0.338$; Baseline/24 h-Test: $F_{(1,38)} = 422.288$, $p = 3.509^{-22}$; Group x Baseline/24 h-Test: $F_{(1,38)} = 0.553$, $p = 0.462$). We next tested the amount of generalized fear expressed by mPFC-NR1 KO and control mice, in which we examined their freezing responses to a novel downward FM sweep (CS-), revealing no difference in the freezing responses to the CS- or CS+ between mPFC-NR1 KO and control mice (Fig. 2.3C; ANOVA of FM-sweep direction and group during day 4 and 5: Group: $F_{(1,38)} = 0.363$, $p = 0.550$; ANOVA of FM-sweep direction: $F_{(1,38)} = 0.385$, $p = 0.539$; Group x FM-Sweep Direction: $F_{(1,38)} = 0.002$, $p = 0.966$). Taken together, these data indicate that both groups of mice show normal conditioned fear acquisition, 24hr retrieval, and generalization.

We next ran control and mPFC-NR1 KO mice on auditory discrimination training (Fig. 2.3D-F). Initially, both groups of mice generalized their conditioned responses, exhibiting similar levels of freezing responses to both CS+ and CS- (days 7-8). However, following 2 days of training, control mice demonstrated the ability to consistently distinguish between similar yet different auditory patterns (days 10-12), exhibiting a higher number of freezing responses to CS+ and significantly fewer freezing responses to CS- compared to CS+ (Fig. 2.3D; RM-ANOVA of Day and FM-sweep direction: Day x FM-sweep direction: $F_{(5,45)} = 8.728$, $p = 0.000007$, $n = 10$). Post hoc analysis using Bonferroni correction (alpha = 0.0083) for multiple comparisons indicated that differences were present during days 10 ($t_{(9)} = 3.487$, $p = 0.007$, $r = 0.76$), 11 ($t_{(9)} = 7.209$, $p = 0.00005$, $r = 0.92$) and 12 ($t_{(9)} = 8.147$, $p = 0.00002$, $r = 0.94$) only.

mPFC-NR1 KO mice demonstrated deficient discrimination between CS+ and CS-, never showing a significant difference in freezing response between up and down auditory sweeps. (Fig. 2.3E, RM-ANOVA of Day and FM-sweep direction: Day x FM-sweep direction: $F_{(2.7,26.5)} = 2.787$, $p = 0.066$, $n = 11$). Post hoc analysis using Bonferroni correction for multiple comparisons indicated that no differences were present during days 9 (CS+ vs CS- t-test: $t_{(10)} = 2.515$, p = 0.031, r = 0.62), 10 ($t_{(10)} = 0.655$, p = 0.528, r = 0.20), 11 ($t_{(10)} = 3.131$, p = 0.011, r = 0.70) and 12 ($t_{(10)} = 1.728$, p = 0.115, r = 0.48). In contrast to the control animals, mPFC-NR1 KO mice continued to generalize their conditioned responses throughout the entire auditory discrimination training and failed to distinguish between CS+ and CS-.

There is a clear deficit in mPFC-NR1 KO mice in auditory memory specificity when compared to controls (Fig. 2.3D-E, RM-ANOVA of Group and FM-sweep direction and Day 7-12: Group x FM-sweep direction x Day: $F_{(5,95)} = 3.619$, p = 0.005; Group x FM-sweep direction: $F_{(1,19)} = 5.972$, p = 0.024; mPFC-NR1 KO, n=11; Ctrl, n = 10). Analysis of discrimination ratios supports the difference in performance between control and mPFC-NR1 KO animals on days 10-12 (Fig. 2.3F. Discrimination Index mPFC-NR1 KO vs. Ctrl t-test: Day 10: $t_{(19)} = 3.322$, p = 0.004, r = 0.61; Day 11: $t_{(19)} = 4.719$, p = 0.0001, r = 0.73, Day 12: $t_{(19)} = 3.850$, p = 0.001, r = 0.66; mPFC-NR1 KO , n=11; Ctrl, n = 10), but not during the initial 3 days of training (p>0.05). Control mice clearly show a better performance than mPFC-NR1 KO mice on auditory discrimination (Fig. 2.3D-F). In sum, these data suggest that knockout of NR1 in the mPFC results in abnormal auditory (FM-sweep direction) fear memory specificity.

We also analyzed fear responses to upsweep (CS+) and, separately, to downsweep (CS-) in control and mPFC-NR1 KO tested on FM-sweep direction fear discriminatory task (Fig. 2.3). There was no difference in responses to conditioned stimuli CS+ between mPFC-NR1 KO and control mice across the entire FM-sweep direction discrimination training (Fig. 2.3D-E; CS+/mPFC-NR1 KO vs Ctrl; RM-ANOVA of days 7-12 and group: Day X Group, $F_{(3.1, 59.1)} = 0.546$, $p = 0.659$). An analysis of learning curves (Fig. 2.3G) showed a positive slope to CS+ in control ($\alpha = 2.05 \pm 1.22$) and mPFC-NR1 KO ($\alpha = 1.91 \pm 1.06$) across the entire FM-sweep direction fear discriminatory task. In fact, there was no difference in the learning (slopes) of appropriate responses to CS+ between mPFC-NR1 KO and control groups (Fig. 2.3G; CS+ slope/Ctrl vs mPFC-NR1 KO t-test; $t_{(19)} = 0.088$, $p = 0.931$, $r = 0.02$). However, mPFC-NR1 KO mice responded differently to non-relevant stimuli CS- across training on the auditory discriminatory task when compared to control mice (Fig. 2.3D-E; CS-/mPFC-NR1 KO vs Ctrl; RM-ANOVA of days 1-5 and group: Day X Group, $F_{(3.4,63.7)} = 3.447$, $p = 0.018$). The marked improvement of discrimination observed on the FM-sweep direction fear discriminatory task in control mice (Figure 2.3D, F, G) coincides with the significant negative slope of the learning curve for CS- (Fig. 2.3G; $\alpha = -5.17 \pm 0.79$). The mPFC-NR1 KO group, which failed to improve fear memory accuracy across training (Fig. 2.3E-F), shows a slight positive slope for CS- across the training (Fig. 2.3G; $\alpha = 0.412 \pm 1.20$) and a marked difference when compared to the CS- slope observed in control animals (Fig. 2.3H; CS- slope/Ctrl vs mPFC-NR1 KO t-test; $t_{(19)} = -3.803$, $p = 0.001$, $r = 0.66$).

In summary, analysis of patterns of responses to CS+ and CS- in control animals tested on the FM-sweep direction fear discriminatory task revealed that the improvement of auditory fear memory accuracy was due to only slight incline in freezing to CS+ and rapid decline in freezing to CS-. NMDA receptor hypofunction in the mPFC altered the ability to learn auditory discriminatory responses to CS+ versus CS- by disrupting the pattern of learning for CS- only, while responses to CS+ remained similar to control mice. These data demonstrate that the mPFC supports the improvement of auditory fear memory accuracy by controlling acquisition of appropriate responses to non-relevant stimuli.

Impairment of auditory fear memory extinction in mPFC-NR1 KO mice

Previous work on fear memory and mPFC NMDA receptor function demonstrated that infusion of CPP, a potent NMDA receptor antagonist, impaired extinction memory recall (Burgos-Robles et al., 2007; Santini et al., 2001). We tested mPFC-NR1 KO on an auditory extinction task (Fig. 2.4A) in which mice are initially trained with 3 pairings of upward FM sweeps (CS+) and footshocks on day 1, followed by 7 days of extinction in which they are presented with non-reinforced upward (CS+) and downward (CS-) FM auditory sweeps in a novel context while measuring freezing responses. No further pairing of CS+ and footshocks was done after the initial training on day 1. Control mice extinguish freezing to CS+ and CS- differently across extinction training (Fig. 2.4B, RM-ANOVA of Day and FM-sweep direction: Day x FM-sweep direction = $F_{(6,60)} = 2.565$, $p = 0.028$), whereas mPFC-NR1 KO mice show a near significant difference (Fig. 2.4C RM-ANOVA of Day and FM-sweep direction: Day x FM-sweep direction = $F_{(6,66)} = 2.225$, $p = 0.051$). Analysis of learning curves (Fig. 2.4D, Control CS+ $\alpha = -9.51 \pm 0.72$, mPFC-NR1 KO CS+ $\alpha = -6.07 \pm 0.64$, Control CS- $\alpha = -9.11 \pm 1.10$, mPFC-NR1 KO CS- $\alpha = -6.34 \pm 0.74$) shows a significant difference in the rate of extinction of CS+ and CS- between control and mPFC-NR1 KO mice when comparing average slopes of both CS+ (Fig 2.4E, CS+ slope/Ctrl vs mPFC-NR1 KO t-test; $t_{(21)} = -3.573$, $p = 0.002$, $r = 0.61$)

and CS- (CS- slope/Ctrl vs mPFC-NR1 KO t-test; $t_{(21)} = -2.113$, $p = 0.047$, $r = 0.52$). Taken together, these data indicate that both control and mPFC-NR1 KO groups extinguish CS+ and CS- across extinction training, but that control mice extinguish fear responses to both FM-sweep directions more rapidly than mPFC-NR1 KO mice.

Discussion

In summary, this study shows that fear memory accuracy requires prefrontal NMDAR-dependent extinction of generalized fear responses to safe stimuli. Conditional deletion of the NMDAR in CamKIIα positive excitatory neurons within the mPFC resulted in abnormal discriminatory fear learning. Patterns of fear responses in control animals suggest that discriminatory learning involves extinction of freezing responses to safe stimuli. In fact, mPFC-NR1 KO mice show a deficiency in fear extinction training, which is consistent with prior studies demonstrating that infusion of NMDAR antagonist CPP into mPFC prevented consolidation of extinction learning (Burgos-Robles et al., 2007; Santini et al., 2001).

Evidence for the medial prefrontal cortex (mPFC) as a locus for gating fear discrimination and danger assessment includes animal studies in which a mPFC lesion impairs the ability to guide behavior, specifically when memory retrieval resolves conflicting aversive and benign contextual cues (Birrell and Brown, 2000; Dias, Robbins, and Roberts, 1996; Ragozzino, Kim, Hassert, Minniti, and Kiang, 2003; Rich and Shapiro, 2007). A fear decline is associated with elevated activity in the mPFC as determined by activation of immediate-early genes (Herry and Mons, 2004; Knapska and

Maren, 2009), increased blood oxygenation levels (Phelps, Delgado, Nearing, and LeDoux, 2004), cell firing (Burgos-Robles et al., 2007) and magnitude of local field potentials (Lesting, Narayanan, Kluge, Sangha, Seidenbecher, and Pape, 2011). The mPFC has dense reciprocal anatomical and functional connections with sensory cortices, thalamic sensory relays and memory systems including the hippocampus and basolateral amygdala (BLA), the critical locus for fear processing. Considerable evidence indicates that neurons in the mPFC, BLA and hippocampus are functionally coupled at the theta range (4-12 Hz oscillations) during fear conditioning (Popa, Duvarci, Popescu, Lena, and Pare, 2010; Seidenbecher, Laxmi, Stork, and Pape, 2003), conditioned extinction (Lesting et al., 2011) and discriminative fear learning (Likhtik, Stujenske, Topiwala, Harris, and Gordon, 2014). Moreover, memory retrieval elicits neuronal activity patterns in the mPFC that are reminiscent of neural representations of behavioral contexts that govern successful recollection. Different behavioral contexts evoke distinct firing of neuronal ensembles (Hyman, Ma, Balaguer-Ballester, Durstewitz, and Seamans, 2012), which can reset during uncertainty following environmental change (Karlsson, Tervo, and Karpova, 2012) or induce sudden transitions between neural ensemble states accompanied by behavioral transitions (Durstewitz, Vittoz, Floresco, and Seamans, 2010).

How can mPFC neurons control discriminatory learning? This study shows that discriminatory fear learning involves prefrontal NMDAR-dependent extinction of generalized fear responses to safe stimuli. Lesions in the mPFC have been linked to abnormal extinction of conditioned fear (Gewirtz et al., 1997; Morgan et al., 1993; Quirk et al., 2000). The PFC integrates convergent input from higher cortical association and sensory regions along with feedback information, including hippocampal, amygdala and thalamocortical pathways, and sends output signals to cortical and subcortical areas, including direct projections to the cholinergic and monoaminergic neurotransmitter systems to modulate the activity of cortical networks (Dalley, Cardinal, and Robbins, 2004; Heidbreder and Groenewegen, 2003; Ongur and Price, 2000). Prefrontal projections to the amygdala inhibit conditioned fear and it is postulated that the learning of fear extinction involves both increased neuronal activity (Milad and Quirk, 2002) and protein synthesis (Santini et al., 2004) in the mPFC. In addition, there is strong evidence that extinction of conditioned fear depends on N-methyl-D-aspartate receptors (NMDARs) -mediated plasticity (Burgos-Robles et al., 2007; Santini et al., 2001). NMDAR-dependent long-term potentiation is an experimental model of synaptic plasticity and is widely hypothesized to be the neural mechanism by which memory traces are encoded and stored in the brain (Martin, Grimwood, and Morris, 2000). Infusion of the NMDAR blocker 2-amino-5-phosphonovalerate (APV) into in the amygdala during extinction substantially interferes with extinction of conditioned fear to tone, light, and contextual stimuli (Falls, Miserendino, and Davis, 1992; Lee and Kim, 1998) while overexpression of NMDAR subunit NR2B in mice improves extinction learning (Tang, Shimizu, Dube, Rampon, Kerchner, Zhuo, Liu, and Tsien, 1999). Injections of antagonist of NMDA-type receptors 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) directly to the mPFC demonstrated that NMDA receptors are involved in long-term, but not short-term, memory of extinction (Santini et al., 2001) and consolidation of fear extinction requires NMDAR-dependent bursting in the mPFC

(Burgos-Robles et al., 2007) suggesting that fear extinction learning involves extinction memory through NMDAR-mediated plasticity in prefrontal–amygdala circuits.

It is generally believed that fear behavior is differentially regulated by the prelimbic (PL) and infralimbic (IL) divisions of the mPFC (Quirk and Mueller, 2008; Sotres-Bayon, Cain, and LeDoux, 2006) (Courtin, Bienvenu, Einarsson, and Herry, 2013; Sierra-Mercado, Padilla-Coreano, and Quirk, 2010), which may be due to differential connectivity with the amygdala (Gabbott, Warner, Jays, Salway, and Busby, 2005; Vertes, 2004). Electrophysiological findings suggest that the IL and PL cortices may have opposite effects on fear expression (Gilmartin and McEchron, 2005; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, and Quirk, 2006). It has been suggested that the PL promotes fear expression by activating neurons of the basolateral nucleus of the amygdala (BLA) projecting to the central nucleus of the amygdala (CeM) (Pape and Pare, 2010), a critical subregion for fear expression. Conversely, electrical stimulation of IL inhibits the CeM output through the amygdala intercalated neurons (ITC) relay (Royer, Martina, and Pare, 1999), providing an alternative mechanism for extinction (Likhtik, Popa, Apergis-Schoute, Fidacaro, and Pare, 2008). Since mPFC projections to amygdala inhibit conditioned fear, it is postulated that the learning of fear extinction in rat involves both increased neuronal activity (especially in IL) (Milad and Quirk, 2002) and protein synthesis (Santini et al., 2004) in the mPFC. In addition, it has been postulated that convergent (ventral) hippocampal and prefrontal (PL) projections to the amygdala mediate the control of fear after extinction in rat (Orsini et al., 2011). However, both the IL and PL exhibit increases in firing rates during extinction recall in mice (Holmes, Fitzgerald, MacPherson, DeBrouse, Colacicco, Flynn, Masneuf, Pleil, Li, Marcinkiewcz, Kash, Gunduz-Cinar, and Camp, 2012). Moreover, IL and PL projection patterns to principal neurons and interneurons in the amygdala are identical in mice (Cho, Deisseroth, and Bolshakov, 2013). Thus, the previously described difference between IL-amygdala and PL-amygdala projections in rat may not occur in mice.

Interruption of cAMP response element binding protein (CREB) function or inhibition of histone acetyltransferase CREB binding protein (CBP) activity in the mPFC leads to strong deficit in discriminatory fear learning (Vieira et al., 2014). Both CREB and CBP histone acetyltransferase (HAT) activity has been implicated in the putative molecular mechanism underlying memory consolidation and NMDAR-dependent synaptic plasticity. Thus, a context discriminatory task and an auditory discriminatory task indicated that NMDAR/CREB/CBP-dependent neural signaling underlying memory consolidation within mPFC circuitry is an important component of the mechanism for disambiguating the meaning of fear signals.

This study confirms previous evidence demonstrating the involvement of mPFC in discriminatory fear learning. In addition, current work shows that prefrontal NMDAR-dependent signaling in the CamKIIα positive excitatory cells is critical for disambiguating the meaning of aversive and safe stimuli. Previous study implicated also CREB and CBP HAT activity in the mPFC in discriminatory fear learning (Vieira et al., 2014). Considering that three components of molecular mechanisms underlying long-term plasticity and cellular memory in the mPFC are directly implicated in the fear memory accuracy, we conclude that discriminatory fear learning involves long-term encoding in the prefrontal excitatory circuitry. Further experiments are needed to reveal what type of information is encoded in the mPFC that is required for extinction of fear to safe stimuli and the improvement of fear memory accuracy.

Materials and Methods:

Subjects

All procedures were approved by the UC Riverside Institutional Animal Care and Use Committee in accordance with the NIH guidelines for the care and use of laboratory animals. We used C57BL/6J mice for all experiments. Mice were weaned at postnatal day 21, housed 4 animals to a cage with same sex littermates with ad libitum access to food and water and maintained on a 12 hr light/dark cycle. Old bedding was exchanged for fresh autoclaved bedding every week.

Surgery

We used the same rescue surgery protocol described in Vieira et al. (2014). Briefly, 2-3-month-old mice were separated into individual cages prior to surgery. Anesthesia was induced by placing individual mice in chamber filled with isoflurane. After induction, anesthesia was maintained by mounting the mouse in a heated stereotaxic apparatus and supplying a constant flow of isoflurane/oxygen mix. After adjusting the ear bars, bite bar, and nose clamp, the scalp was shaved, sanitized, and incised along the midline. A dental drill was used to thin the skull over the injection sites. The thinned bone was then removed with a needle tip. A 5-µl calibrated glass micropipette [8 mm taper, 8 µm internal tip diameter] was fitted with a plastic tube connected to a 10-ml syringe and lowered onto a square of Parafilm containing a 4-μl drop of virus. After filling the micropipette, it was lowered to the proper stereotaxic coordinates and pressure was applied to the syringe to inject 1 μl of solution at a rate of 50 nl/min. After completing the bilateral injection and removing the micropipette, the skin was sutured and antibiotic was applied to the scalp. The mouse was kept warm by placing its cage on a heated plate and injected with buprenorphine [0.05 mg/kg] for pain relief. The water bottle in the cage was mixed with meloxicam [1 mg/kg] to relieve pain during subsequent recovery days. Animals were monitored for any signs of distress or inflammation for 3 days after surgery. Behavioral experiments were initiated 3 days after surgery. The mPFC was targeted at the following stereotaxic coordinates: Bregma; AP 1.8, ML±0.4, DV 1.4.

Viruses

Surgical procedures were standardized to minimize the variability of HSV virus injections, using the same stereotaxic coordinates for the mPFC and the same amount of HSV injected into the mPFC for all mice. CRE and/or EGFP were cloned into the HSV amplicon and packaged using a replication-defective helper virus as previously described (Lim and Neve, 2001; Neve and Lim, 2001). The viruses were prepared by Dr. Rachael Neve (MIT, Viral Core Facility). The average titer of the recombinant virus stocks was typically 4.0 x 107 infectious units/ml. HSV viruses are effectively expressed in neurons in the PFC.

Behavioral Assays

All behavioral experiments were performed under blind conditions.

Fear conditioning. All fear conditioning was performed in a fear conditioning box (Coulburn Instruments Inc.) located in a sound attenuated chamber and analyzed automatically by a Video-based system (Freeze Frame software ActiMetrics Inc.). Freezing was expressed as "% Freezing", which was calculated as a percent of freezing time per total time spent in the testing chamber. The chamber was cleaned in between trials with Quatracide, 70% ethanol, and distilled water.

Auditory Discrimination. The FM-sweep direction discrimination task was performed according to a previously described protocol (Vieira et al. 2014). This task is divided into three phases: FM-sweep conditioning, generalization test and discrimination phase (Fig. 2.2). The conditioned stimuli (CS) were 20-s trains of frequency modulated (FM)-sweeps for a 400-ms duration, logarithmically modulated between 2 and 13 kHz (upsweep) or 13 and 2 kHz (downsweep) delivered at 1 Hz at 75 dB. The CS+ was paired with a foot shock (2 s, 0.75 mA). The onset of the US coincided with the onset of the last sweep for the CS. For fear conditioning acquisition (days 1-3; initial training phase), the animals were presented with a single US-CS pairing per day. The FM-sweep Fear Retrieval (day 4) and Generalization (day 4-5) were tested (freezing to 3x CS- for 30 s followed by 3x 30 s CS+ without US; 3 min baseline and 3 min ITI) in context C, which significantly differed from the training chamber (context A). The discrimination phase of FM sweep direction discrimination training was performed over three sessions a day for 6 days (days 7-12): Session 1 was the performance test, Session 2 was the presentation to 1x UC-CS+ pairing after 3 min baseline, and Session 3 was the presentation to the US-CS- pairing after a 3 min baseline.

Extinction. Auditory FM-sweep extinction followed a similar protocol to auditory discrimination (see Fig. 2.4A). On day 1, animals received 3 CS+ footshock pairings that were preceded and separated by a 180s delay. 24hr after acquisition, extinction training began with 3 randomized presentations (separated by 180s) each of CS- and CS+ (non-reinforced) in a novel context. This was repeated every day for 7 days while measuring freezing. We used upsweeps for CS+ and downsweeps for CS-, as described above.

Histology

Histology was performed as described before {Lovelace, 2014 #33424}. Briefly, anesthetized (Nembutal 200mg/kg, i.p. injection) mice were transcardially perfused first with PBS and then 4% PFA. Brains were extracted, soaked in 4% PFA overnight, soaked in 20% sucrose until they sank, and then flash frozen using embedding media, dry ice, and ethanol before being stored in a -80^{o}C freezer. The frozen brain was then mounted on cryostat for 50-µm-thick sectioning of the mPFC. Free-floating immunohistochemistry (IHC) was performed on sections according to a previously described protocol (Korzus, 2004). The sections are washed 3 times for 10 min in a wash buffer (PBS, 0.3% Triton x-100, 0.02% NaN2) followed by a 1-hr incubation in blocking buffer (5% normal goat serum in washing buffer), followed by a 10-min incubation in the wash buffer. The sections were incubated overnight at 4C° with primary antibodies (mouse anti-NeuN monoclonal antibody (Millipore, 1:2000) and rat anti-mCherry (Molecular Probes, 1:10,000) or mouse anti-CaMKII antibody (Fisher, clone: 6G9: 1:1,000) and rat anti-mCherry or mouse anti-NMDAR1 (Millipore, clone 54.1, 1: 1,000) and rat anti-mCherry

After three washes with the wash buffer, the sections were incubated with appropriate secondary antibodies (Alexa488 goat anti-mouse IgG or Biotin-anti-rat IgG or Alexa647 goat anti-mouse IgG; Molecular Probes, 1:1,000), in blocking buffer overnight at 4C°. The sections were washed again three times with the wash buffer, incubated with Streptavidin-Alexa 568 (Molecular Probes, 1:1,000) in blocking buffer for 4 hr at room temperature, and washed before mounting for viewing. Negative control slices were collected at the same time, undergoing the same IHC procedure in addition to receiving primary antibodies. After immunostaining, the tissue was mounted mounted with mounting medium (ProLong Antifade, LifeTechnologies) before imaging.

Imaging

Images were taken using an Olympus FV1000 laser scanning confocal microscope controlled using the FluoView software. Fluorescence was measured from mPFC slices using objective 40x/0.80 LUMPlanFL40x objective. Alexa488, Alexa568 and Alexa-647 were imaged using 473-nm, 559-nm and 647-nm lasers, respectively. Gain and offset of each channel were balanced manually using Fluoview saturation tools for maximal contrast. All settings were tested on multiple slices before data collection and brain slices were imaged using identical microscope settings once established. The fluorescence intensity was compared to the negative control slices, which did not receive any primary antibodies. Forty-micrometer z-stacks were obtained from the entire mPFC for assessing the site of injection. For other measurements, a single optical section was acquired and analyzed. The fluorescence intensity quantification was performed on original images by the use of Olympus Fluoview software without any non-linear image adjustments.

Data analysis

The experimenters were blind to the group conditions. Sample size is indicated by N and error bars use the standard error of the mean. Statistical analysis was performed using Excel (Microsoft Inc.) or SPSS (IBM Inc.). The Student's t-test or ANOVA was used for statistical comparisons. Pearson's correlation (r) was used as an effect size. In cases where the repeated measures ANOVA (RM-ANOVA) was utilized and assumptions of sphericity were violated (via Mauchly's Test), the analysis was performed using the Greenhouse-Geisser correction. Bonferroni corrected post hoc analysis was performed for multiple comparisons, which allows for substantially conservative control of the error rate. Significance values were set at $p<0.05$. The asterisks indicate statistical significance: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ and n.s. indicates not significant.

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Figure 2.1

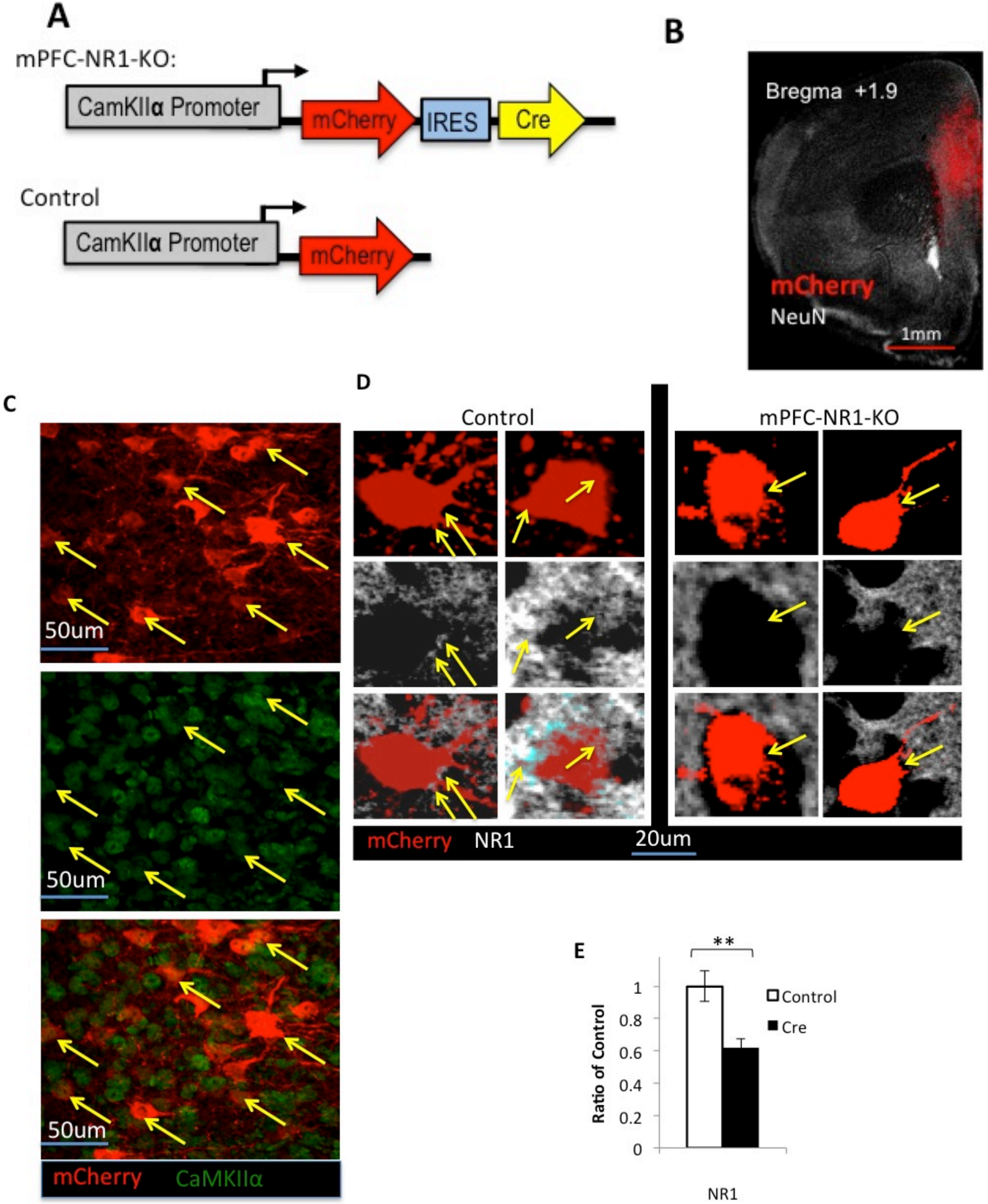

Figure 2.1 Generation of mPFC-NR1 KO mice. **(A)** Viral construct for generating mPFC-NR1 KO and control mice: HSV/CaMKIIα-mCherry-IRES-Cre and HSV/CaMKIIα-mCherry, respectively. **(B)** Representative image indicating mPFC infection of the virus. Long-term expression HSV-1 viruses carrying CRE

(HSV/CaMKIIα-mCherry-IRES-Cre) or mCherry as the control (HSV/CaMKIIα-mCherry) were injected into the mouse mPFC. To determine the pattern of mCherry-tagged virus expression, the imaged tissue was compared to the Paxinos and Franklin mouse atlas (Paxinos and Franklin, 2001) and areas of maximal mCherry expression were labeled as injection sites. Red, mCherry; white, NeuN neuronal marker. **(C)** Multiplex immunohistochemistry with anti-mCherry and anti-CamKIIα antibodies revealed that HSV/CaMKIIα-mCherry-IRES-Cre virus was targeted to CamKIIα positive cells in mPFC-NR1 KO mice. **(D)** Multiplex immunohistochemistry with anti-mCherry (red) and anti-NR1 (green) antibodies indicated that expression of Cre recombinase in NR1-floxed mice was effective to generate conditional NR1 gene K.O. in the mPFC. Control animals injected with HSV/CaMKIIα-mCherry show overlapping expression or NR1 (green) and mCherry (red) proteins in the mPFC of control mice, while NR1 expression is depleted in the mPFC-NR1 KO mice. **(E)** Cre recombinase decreases NR1 subunit expression in the mPFC of mPFC-NR1 KO mice. Cells co-expressing mCherry and Cre showed significantly lower levels of NR1 reactivity when compared to control animals expressing mCherry only (Ctrl vs mPFC-NR1 KO t-test; $t_{(14)} = 3.415$, $p = 0.0042$, $r = 0.674$). The asterisks indicate statistical significance: **, $p < 0.01$.

Figure 2.2

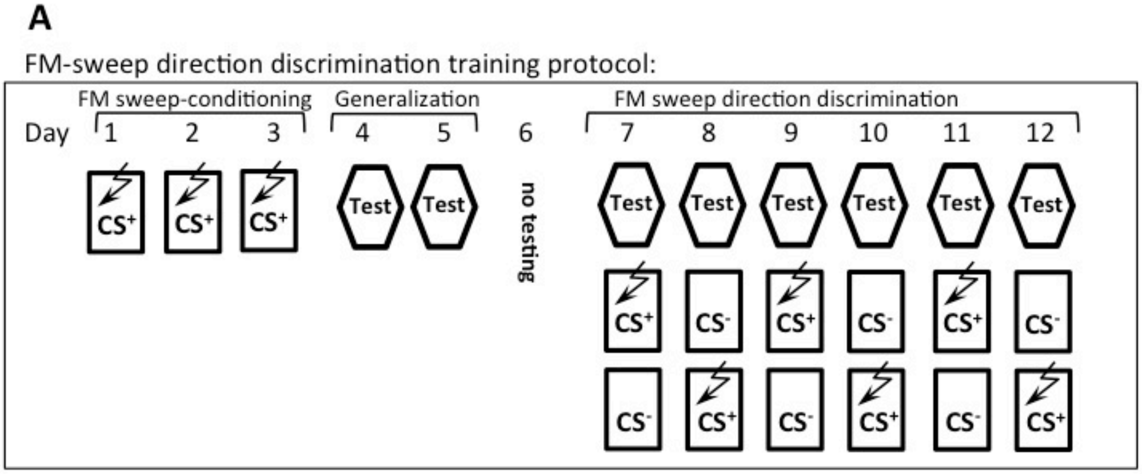

Figure 2.2 FM-sweep direction discrimination training protocol. (A) Schematic of the auditory discrimination protocol (see Methods). Following 3 days of paired sweep-footshock training, a generalization test assessed the levels of freezing in response to the non-conditioned stimulus (down sweep) compared to the conditioned stimulus (up sweep). Generalization was followed by 6 days of discrimination training in which each day consists of 3 sessions: a non-reinforced test of freezing to CS+ and CS-, a CS+ US (footshock) pairing, and a unpaired CS- session (counterbalanced). The conditioned stimuli (CS) for auditory fear conditioning were 20-s trains of FM-sweeps for a 400-ms duration, logarithmically modulated between 2 and 13 kHz (upsweep) or 13 and 2 kHz (downsweep) delivered at 1 Hz at 75 dB.

Figure 2.3

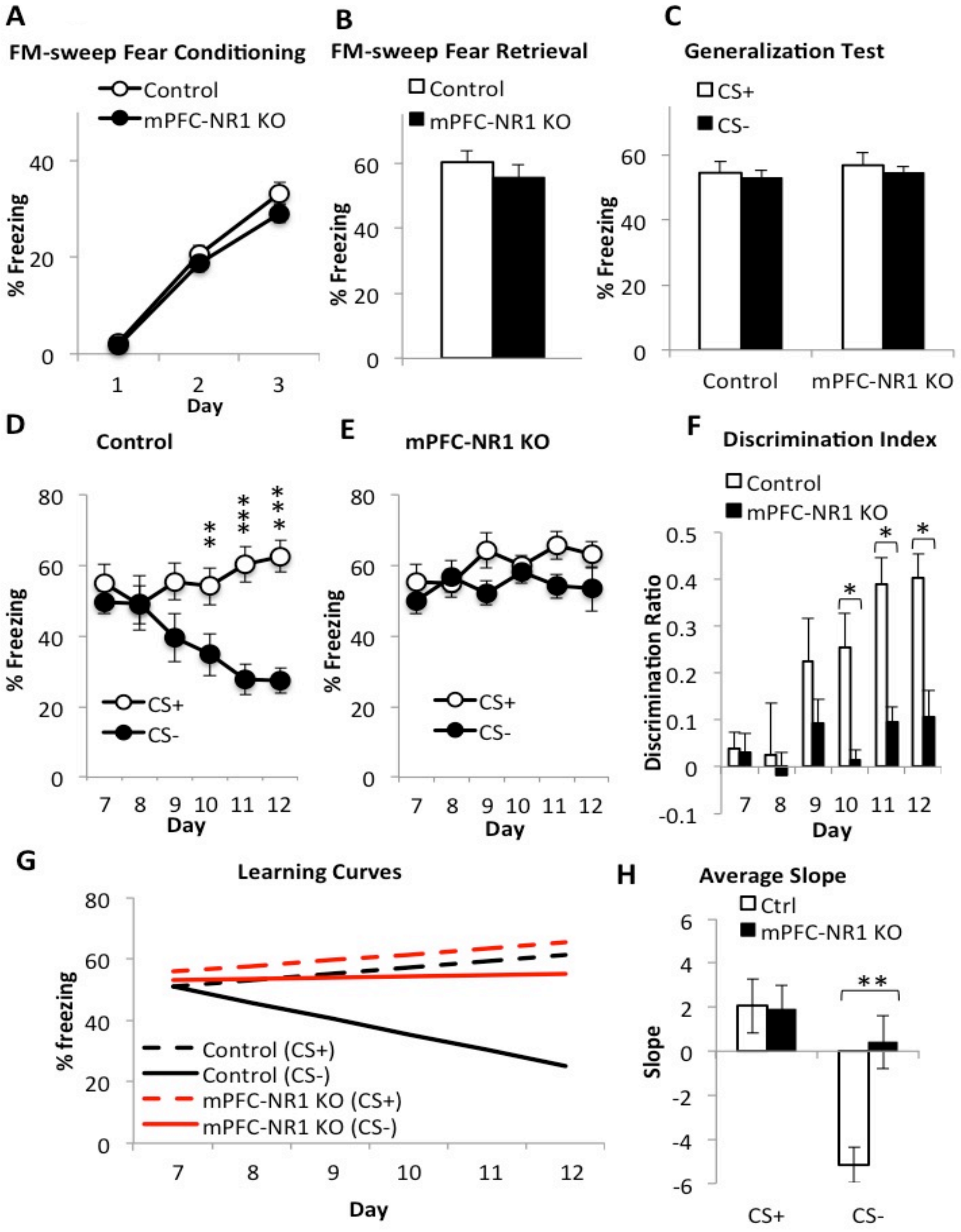
A
FM-sweep Fear Conditioning
Control
mPFC-NR1 KO
% Freezing
40
20
0
1
2
3
Day
B
FM-sweep Fear Retrieval
Control
mPFC-NR1 KO
% Freezing
60
40
20
0
C
Generalization Test
CS+
CS-
% Freezing
60
40
20
0
Control
mPFC-NR1 KO
D
Control
% Freezing
80
60
40
20
0
**
***
***
CS+
CS-
7
8
9
10
11
12
Day
E
mPFC-NR1 KO
% Freezing
80
60
40
20
0
CS+
CS-
7
8
9
10
11
12
Day
F
Discrimination Index
Control
mPFC-NR1 KO
Discrimination Ratio
0.5
0.4
0.3
0.2
0.1
0
-0.1
*
*
*
7
8
9
10
11
12
Day
G
Learning Curves
% freezing
80
60
40
20
0
Control (CS+)
Control (CS-)
mPFC-NR1 KO (CS+)
mPFC-NR1 KO (CS-)
7
8
9
10
11
12
Day
H
Average Slope
Ctrl
mPFC-NR1 KO
Slope
6
4
2
0
-2
-4
-6
**
CS+
CS-

Figure 2.3 FM-sweep direction fear memory specificity is deficient in mPFC-NR1 KO mice. (A-B). Pavlovian FM-sweep fear conditioning was normal in mPFC-NR1 KO, showing similar acquisition (RM-ANOVA of Day and Group: $F_{(2,38)} = 0.800$, $p = 0.457$) (A) and retention (two way ANOVA of Group and Baseline/24 h-Test; Group: $F_{(1,38)} = 0.941$, $p = 0.338$; Baseline/24h-Test: $F_{(1,38)} = 422.288$, $p = 3.509^{-22}$; Group x Baseline/24 h-Test: $F_{(1,38)} = 0.553$, $p = 0.462$) (B) of FM-sweep fear conditioning compared to control (Ctrl) mice. FM-sweep fear was tested in context C at 24 h after a three upsweeps-foot shock pairing. (C) Both groups (mPFC-NR1 KO and Ctrl) show no difference in the freezing responses to CS+ and CS- ($p>0.05$) during day 4 and 5 of training, indicating that initially, the mPFC-NR1 KO and control mice generalized responses and did not discriminate between upsweep and downsweep (ANOVA of FM-sweep direction and group during day 4 and 5: Group: $F_{(1,38)} = 0.363$, $p = 0.550$; ANOVA of FM-sweep direction: $F_{(1,38)} = 0.385$, $p = 0.539$; Group x FM-Sweep Direction: $F_{(1,38)} = 0.002$, $p = 0.966$). (D) After the initial generalization of fear conditioned responses, control mice exhibited robust fear memory specificity (RM-ANOVA of Day and FM-sweep direction: Day x FM-sweep direction: $F_{(5,45)} = 8.728$, $p = 0.000007$, $n = 10$). Bonferroni correction (alpha= 0.0083) showed differences on days 10 ($t_{(9)} = 3.487$, $p = 0.007$, $r = 0.76$), 11 ($t_{(9)} = 7.209$, $p = 0.00005$, $r = 0.92$) and 12 ($t_{(9)} = 8.147$, $p = 0.00002$, $r = 0.94$) only. (E) mPFC-NR1 KO mice demonstrated a deficit in auditory memory specificity when compared to controls (RM-ANOVA of Day and FM-sweep direction: Day x FM-sweep direction: $F_{(2.7,26.5)} = 2.787$, $p = 0.066$, $n = 11$). (F) The FM-sweep direction discrimination ratios (DI) were calculated using the freezing responses to CS+ and CS-according to the formula DI = ((Upsweep - Downsweep) / (Upsweep + Downsweep)). Analyses revealed differences between mPFC-NR1 KO and control mice in the performance during Days 10 – 12 (mPFC-NR1 KO vs. Ctrl t-test: Day 10: $t_{(19)} = 3.322$, $p = 0.004$, $r = 0.61$; Day 11: $t(19) = 4.719$, $p = 0.0001$, $r = 0.73$, Day 12: $t_{(19)} = 3.850$, $p = 0.001$, $r = 0.66$; MPFC-NR1 KO , n=11; Ctrl, n = 10). (G) Average learning curves for learning of appropriate responses to CS+ and CS- were calculated based on the performance of control and mPFC-NR1 KO group across the entire training (Fig. 2.3D-E; Days 7 to 12) followed by fitting the regression line and t-test analysis on the mean of those slopes (α). The analysis of patterns of responses to CS+ and CS- in control animals tested on the FM-sweep direction fear discriminatory task revealed that the improvement of auditory fear memory accuracy was due to slight incline in freezing to CS+ and rapid decline in freezing to CS- (CS+/Ctrl: $\alpha = 2.05 \pm 1.22$; CS-/Ctrl: $\alpha = -5.17 \pm 0.79$). There was no difference in the learning (slopes) of appropriate responses to CS+ between mPFC-NR1 KO and control groups (CS+ slope/Ctrl vs mPFC-NR1 KO t-test; $t_{(19)} = 0.088$, $p = 0.931$, $r = 0.02$). The mPFC-NR1 KO group, which failed to improve fear memory accuracy, showed a positive slope for CS-, a marked difference from control responses to the CS- (CS- slope/Ctrl vs mPFC-NR1 KO t-test; $t_{(19)} = -3.803$, $p = 0.001$, $r = 0.66$). (H) Graph showing average slopes on the same analysis. The asterisks indicate statistical significance: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Figure 2.4

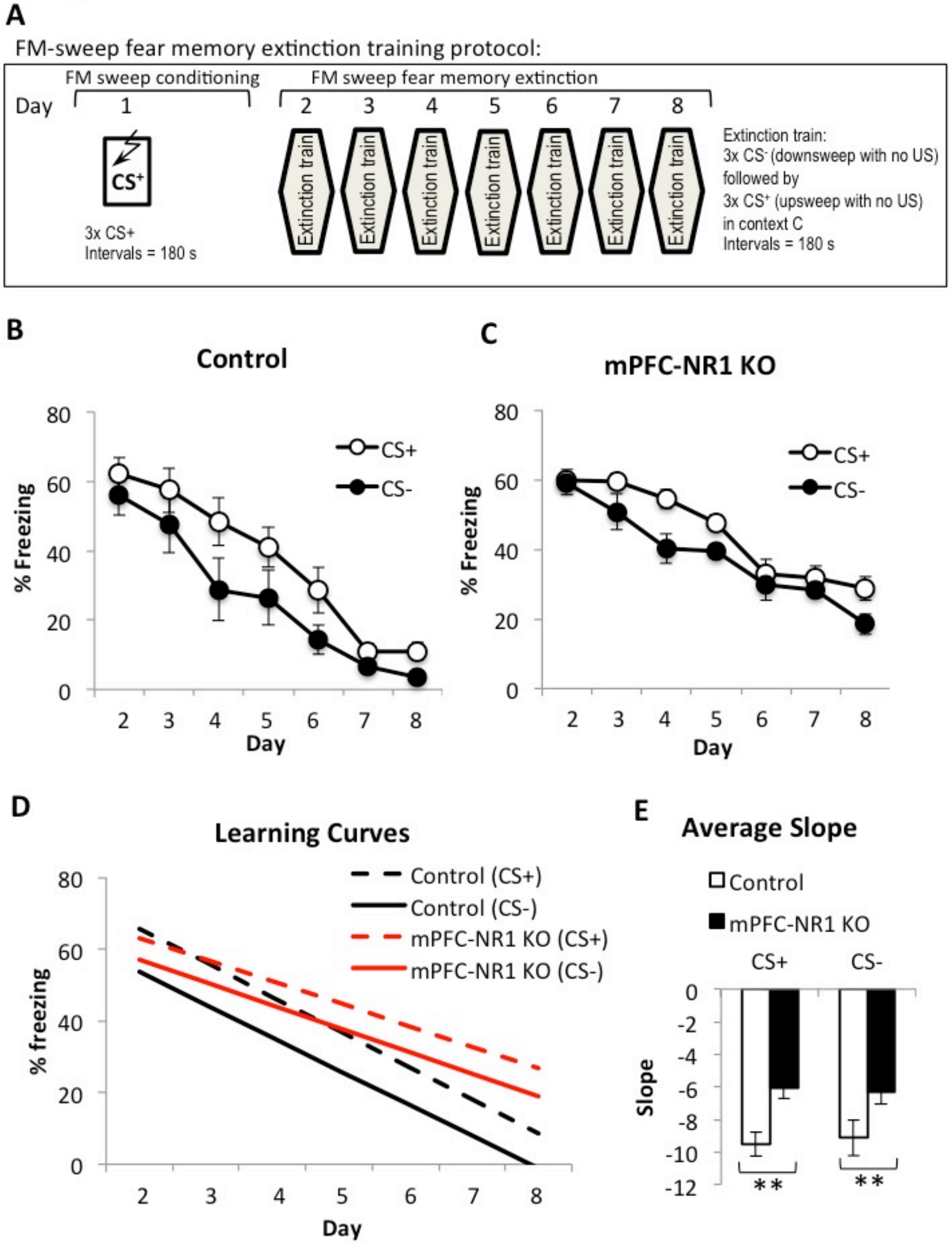

Figure 2.4 mPFC-NR1 KO mice show deficient fear memory extinction. (A) Experimental design for the extinction protocol. A single day of training in which the

animal receives 3 pairings of upward sweeping FM tones and footshock is followed by 7 days of extinction in which freezing is measured during the presentation of 3x CS- (downward sweeping FM tones) followed by 3x CS+ (unpaired with footshock) in a separate context from training (Context C). (B) Percent of total time spent freezing during the sweep presentation plotted across days. Control animals extinguish freezing to both CS+ and CS- significantly different (RM-ANOVA of Day and FM-sweep direction: Day x FM-sweep direction = $F_{(6,60)} = 2.565$, $p = 0.028$). (C) mPFC-NR1 KO mice extinguish CS+ and CS- nearly significantly different (RM-ANOVA of Day and FM-sweep direction: Day x FM-sweep direction = $F_{(6,66)} = 2.225$, $p = 0.051$). However, there was no significant difference when comparing groups in a 3-way RM-ANOVA (RM-ANOVA of Day and FM-sweep direction and group: $F_{(6,126)} = 0.927$, $p = 0.478$). (D) Learning curves comparing slopes of extinction to CS+ and CS- between control (CS+ slope(α)= -9.51±0.72, CS- slope(α)= -9.11±1.10) and mPFC-NR1 KO mice (CS+ slope(α)= -6.07±0.64, CS- slope(α)= -6.34±0.74. (E) Average slope comparison of extinction curves to CS+ and CS- between control and mPFC-NR1 KO mice shows a significant difference in rate of extinction to CS+ (CS+ slope/Ctrl vs MPFC-NR1 KO t-test; $t_{(21)} = -3.573$, $p = 0.002$, $r = 0.61$) and CS- (CS- slope/Ctrl vs MPFC-NR1 KO t-test; $t_{(21)} = -2.113$, $p = 0.047$, $r = 0.52$). The asterisks indicate statistical significance: **, $p < 0.01$.

Conclusion

We have established that the mPFC plays a critical role in fear memory accuracy by guiding appropriate responses to aversive and non-aversive stimuli, which contributes to our understanding of memory specificity and generalization. First, we demonstrated a requirement for CBP HAT activity and CREB functionality in discriminating similar but different auditory sweeps. Mice with disrupted CBP HAT or CREB activity were indistinguishable from controls on fear acquisition, long-term retrieval, and initial generalization to similar non-aversive stimuli. Based on learning curve analysis, however, the requirement for functional CBP HAT and CREB activity was predominantly pronounced in the extinction of freezing to the neutral CS- stimulus, not in the attainment of appropriate freezing to the aversive CS+ stimulus. This suggest that the mPFC plays a strong role in discriminating fear memory by acquiring appropriate responses to non-aversive stimuli that closely resemble aversive stimuli. Though more detailed research is needed to tease apart the specific molecular pathway involved in these processes, it likely includes long-term consolidation and synaptic plasticity comprising CREB, CBP HAT, gene translation/transcription, and protein synthesis. Future studies should look more closely at constitutive and inducible transcription factors involved in the consolidation of long-term memory, the most studied group of inducible transcription factors being the immediate early genes (IEGs), which show rapid and transient expression following various treatments that is independent of protein synthesis (Herdegen and Leah 1998). Protein product of many IEGs was shown to act as transcriptional regulators that mediate genes involved in stable neuronal changes associated with learning and memory, including c-fos (Tischmeyer and Grimm 1999), arc (Guzowski, Lyford et al. 2000), and BDNF (Lubin, Roth et al. 2008).

The subsequent study confirmed previous evidence demonstrating the involvement of the mPFC in discriminatory fear learning by showing that prefrontal NMDAR-dependent signaling in CamKIIα-positive excitatory cells within the mPFC is critical for disambiguating the meaning of aversive and safe stimuli. Considering that three components of molecular mechanisms underlying long-term plasticity and cellular memory (i.e., CBP HAT, CREB, and NMDAR) within the mPFC are directly implicated in the accuracy of fear memory, we conclude that discriminatory fear learning involves long-term encoding in the prefrontal excitatory circuitry. Further experiments are needed to reveal what type of information is encoded in the mPFC that is required for extinction of fear to safe stimuli and the improvement of fear memory accuracy.

The described data are consistent with previous reports implicating the mPFC in extinction of fear memory (Quirk, Garcia et al. 2006). Lesions to the mPFC (Quirk, Russo et al. 2000), infusion of anisomycin, a potent inhibitor of protein synthesis, (Santini, Ge et al. 2004), or infusion of CPP, a competitive NMDA receptor antagonist (Burgos-Robles, Vidal-Gonzalez et al. 2007), block long-term recall of fear extinction memory without affecting initial acquisition, and single unit recordings from the mPFC show correlated activity in response to long-term recall of extinction memory but not during acquisition (Milad and Quirk 2002).

These data fit well into the complementary learning systems theory, in which systems consolidation transfers specific memories from the hippocampus to the cortex where they become more generalized (McClelland, McNaughton et al. 1995, Kumaran and McClelland 2012). Discrimination of specific memories is maintained by “pattern separation,” whereas generalization occurs through “pattern completion,” both processes borrowed from computational literature (Marr 1971, Rolls and Treves 1994, Rolls 1996) to describe observations of animal behavior when manipulating the hippocampus (Kesner, Lee et al. 2004, Rolls and Kesner 2006, Leutgeb, Leutgeb et al. 2007, Leutgeb and Leutgeb 2007, McHugh, Jones et al. 2007, Aimone, Deng et al. 2011, Motley and Kirwan 2012, Nakashiba, Cushman et al. 2012) or, more recently, the olfactory bulb (Sahay, Wilson et al. 2011). There is even support in human literature for neurophysiological functions of pattern separation using fMRI (Kirwan, Jones et al. 2007, Kirwan and Stark 2007, Bakker, Kirwan et al. 2008, Lacy, Yassa et al. 2011). However, little research has looked at the role of the mPFC in functional pattern separation and completion, that is, discriminatory processes as a part of memory specificity and generalization that guide eventual behavioral outcomes (Preston and Eichenbaum 2013, Xu and Sudhof 2013). Our studies, therefore, contribute to the young but growing body of literature that implicates the mPFC in a more active role in memory specificity/generalization. Future work should look to isolate specific parts of the physiological pathway (e.g. synaptic plasticity, protein synthesis) as well as neural circuitry (e.g. prefrontal-hippocampal reciprocity) that are necessary for discrimination memory to be acquired, consolidated, and retrieved for appropriate responses to relevant and non-relevant stimuli.

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